Performance evaluation study of ID CORE XT, a high throughput blood group genotyping platform

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Background. Traditionally, red blood cell antigens have been identified using serological methods, but recent advances in molecular biology have made the implementation of methods for genetic testing of most blood group antigens possible. The goal of this study was to validate the performance of the ID CORE XT blood group typing assay.

Materials and methods. One thousand independent samples from donors, patients and neonates were collected from three research institutes in Spain and the Netherlands. DNA was extracted from EDTA-anticoagulated blood. The data were processed with the ID CORE XT to obtain the genotypes and the predicted blood group phenotypes, and results were compared to those obtained with well-established serological and molecular methods. All 1,000 samples were typed for major blood group antigens (C, c, E, e, K) and 371-830 samples were typed for other antigens depending on the rarity and availability of serology comparators.

Results. The incorrect call rate was 0%. Four "no calls" (rate: 0.014%) were resolved after repetition. The sensitivity of ID CORE XT for all phenotypes was 100% regarding serology. There was one discrepancy in E– antigen and 33 discrepancies in Fy^b– antigen. After bidirectional sequencing, all discrepancies were resolved in favour of ID CORE XT (100% specificity). ID CORE XT detected infrequent antigens of Caucasians in the sample as well as rare allelic variants.

Discussion. In this evaluation performed in an extensive sample following the European Directive, the ID CORE XT blood genotyping assay performed as a reliable and accurate method for correctly predicting the genotype and phenotype of clinically relevant blood group antigens.

Keywords: blood group genotyping, sensitivity, specificity, ID CORE XT.

Introduction

Human blood can be classified into different groups based on the presence or absence of specific antigens (proteins, carbohydrates, glycoproteins or glycolipids) on the surface of red blood cells[1]. To date, the International Society of Blood Transfusion has recognised 346 antigens, 308 of which are involved in 36 discrete blood group systems, while 38 antigens have not yet been assigned to a system[1,2]. The vast majority of the antigenic differences that give rise to blood groups have a well-known genetic basis[3].

Blood group antigens are clinically important in blood transfusion and during the progression of pregnancy[4,5]. A transfusion between incompatible blood groups can cause an immune response with severe consequences, i.e. a transfusion reaction whereby the immune system of the recipient attacks the donated blood cells[6]. Similarly, haemolytic disease of the foetus and newborn is a related alloimmune condition in which antibodies from the mother pass through the placenta and attack the red blood cells in the foetal circulation. Blood group compatibility is also very important in tissue/organ transplantation. For instance, transplantation of an ABO-incompatible organ will generally result in rejection, unless the recipient is subjected to prior plasmapheresis[7].

Traditionally, red blood cell antigens have been identified using serological methods based on haemagglutination reactions, which are the reference tool for blood group testing. However, serology has potential limitations, such as weak antibody reactivity (e.g. for the detection of the Fy^b antigen), weak antigen expression (e.g. DEL and "weak D"), scarcity of some reagents (e.g. Do^a) and the presence of unwanted autoantibodies or alloantibodies (e.g. after transfusion) in the plasma[8].

In recent years, there has been a significant increase in molecular blood group typing methods[9,10]. Fortunately, the advancements and declining costs of molecular techniques have revolutionised the way in

which we can test genetically for the presence/absence of a blood group antigen in circumstances such as multi-transfused recipients, weakly expressed antigens[11] or lack of serology reagents for specific antigens[12]. Moreover, it is now possible to efficiently screen large numbers of blood donors for the benefit of patients in need of a blood transfusion[13,14].

In the light of the above information, this study focused on ID CORE XT, a polymerase chain reaction (PCR) and hybridisation-based genotyping assay for the simultaneous identification of multiple alleles, encoding 37 human red blood cell antigens of ten blood group systems (Rh, Kell, Kidd, Duffy, MNS, Diego, Dombrock, Colton, Cartwright, Lutheran) by analysing 29 polymorphisms in genomic DNA extracted from whole blood. The ultimate goal of this study was to validate the overall performance claimed for erythrocyte antigen genotype and phenotype prediction of ID CORE XT as an *in vitro* diagnostic kit.

Materials and methods

Study design

This study was based on the molecular and serological analysis of 1,000 independent human samples from three collaborating centres in Spain and the Netherlands (Table I). An ethics committee at each of the three participating centres approved the protocols used in this study and all participants gave their informed consent in compliance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans.

The performance of the ID CORE XT genotyping assay (Progenika Biopharma, a Grifols Company. Derio, Spain) was compared to that of well-established serological and/or molecular platforms as reference methods (BLOODchip®, Progenika Biopharma, a Grifols Company, Derio, Spain) and bidirectional DNA sequencing in subsets of individuals.

Sampling procedures

Samples were obtained from donors, patients and neonates in the proportion required by the European Directive for validation studies (10% patients, 2% neonates and 10% weak D; Table I)[15]. Samples were randomly collected and anonymously coded with no prior knowledge of their ID CORE XT molecular genetic basis. The 10% weak D samples were selected by serological typing.

DNA was extracted from EDTA-anticoagulated whole blood samples using QIAgen mini or Flexigen kits (Qiagen, Hilden, Germany)[16], according to the ID CORE XT package instructions. The DNA in the samples was within a concentration range of 8-80 ng/µL and DNA purity (A260/A280 ratio) 1.63-2.1 as quantified by NanoDrop spectrophotometry (ThermoFisher Scientific, Wilmington, DE, USA). The DNA samples prepared by the blood centres were kept frozen at –20 ºC and sent to Progenika until the ID CORE XT assay.

Most of the samples (n=800) were analysed at the Progenika Biopharma laboratories in Derio, Spain, while the remaining 200 were analysed at Progenika Inc. in Boston, USA. A process control sample from the National Institute of General Medical Sciences (NIGMS) collection, courtesy of the Coriell Institute for Medical Research (Camden, NJ, USA), was also included in each analysis run.

Blood group typing procedures

For the ID CORE XT genotyping analysis, targeted DNA regions from the entire sample (n=1,000) were amplified through a multiplex PCR using biotinylated dCTP. The PCR products obtained were subsequently hybridised onto oligonucleotide probes, attached to microspheres and labelled with streptavidin-conjugated phycoerythrin. Finally, the beads were analysed with a Luminex® 200 system (Millipore Corporation, Billerica, MA, USA). The raw data were processed with the ID CORE XT Analysis Software to obtain the genotypes, as well as the predicted blood group phenotypes. The possible predicted phenotype results of each of the antigens determined by ID CORE XT were as follows: positive (normal antigen expression), negative (undetectable antigen expression), "no call" (inability of the genotyping platform to assign a phenotype) and

Table I - Type and number of samples provided by each of the three participating centres.

Sample type	Institution			Total (%)
	CTZ (blood)	*CVTTH (DNA)*	*SBS (DNA)*	
Donors	52	550	180	782 (78.2%)
Clinical samples	0	53	47	100 (10%)
"Weak D" donors	0	97	0	97 (9.7%)
Neonatal specimens	0	0	21	21 (2.1%)
Total	52	700	248	1,000 (100%)

CTZ: Zaragoza Blood Transfusion Centre; CVTTH: Basque Human Transfusion and Tissue Centre; SBS: Sanquin Blood Supply.

"unknown" (that particular combination has not been previously described as associated with a phenotype).

The participating centres evaluated the majority of the ID CORE XT antigen phenotypes under standard serological assays using commercially available kits from Immucor-Gamma (Immucor, Peachtree Corners, GA, USA); CLB-Pelikloon (Sanquin, Amsterdam, The Netherlands) and DiaMed cards (Bio-Rad, Hercules, CA, USA). All one thousand samples were typed for the major antigens of the Rh (C, c, E, e) and Kell (K) systems. For other antigens (C^w, k, Kp^a, Kp^b, Jk^a, Jk^b, Fy^a, Fy^b, M, N, S, s, Di^a, Di^b, Co^a, Co^b, Lu^a and Lu^b), the number of samples typed depended on the availability of serology comparators associated with their rarity. For the remaining antigens in which no antibodies were available for serology testing, the comparison technique was a molecular method (n=100): BLOODchip® assay (VS, Js^a, Js^b, U, Mi^a, Do^a and Do^b) and bidirectional DNA sequencing (V, hr^B, hr^S, Hy, Jo^a, Yt^a and Yt^b). The BLOODchip® assay was also used as an additional comparator (n=100) for the samples already tested for serology.

Data analysis

The performance of the ID CORE XT assay was evaluated based on the following parameters: system failure rate (valid tests; acceptance criterion: ≤5% invalid), call rate (genotype assignment; acceptance criterion: ≤1% for incorrect calls, ≤5% for "no calls"), sensitivity (or true positive rate; acceptance criterion: ≥99%), and specificity (or true negative rate; acceptance criterion: ≥99%). All discrepancies between the genotype result obtained with ID CORE XT and the serology reference method were analysed by bidirectional DNA sequencing of the region tested.

Additionally, the frequencies of the antigens tested by ID CORE XT were calculated and compared with the frequencies reported in the literature for the predominant local population (Caucasian)[1]. Apparent deviations (no statistical test applied) were interpreted.

The rare blood group genotype variants (≤1%) detected by ID CORE XT in the entire sample (n=1,000) were also listed. These rare variants were not a part of the comparison study with reference methods.

Results

ID CORE XT failure and call rates

A total of 29,000 ID CORE XT calls (29 polymorphisms × 1,000 samples tested) were obtained. No samples showed invalid analysis (0% system failure rate).

Initially, ten samples yielded an apparently erroneous result with ID CORE XT; five of these were due to sample misidentification, one due to no available serology data, and four due to a low signal or indeterminate genotype message ("no call"). Three of the "no calls" were observed for the Lutheran blood group (low signal or indeterminate genotype in LU:c.230A>G) and one for the Rh blood group (indeterminate genotype in RHCE:c.712A>G). All ten cases were resolved after retesting and were not, therefore, considered discrepancies [0% incorrect call rate and 0.4% / 0.014% "no call" rate (4/1,000 samples / 4/29,000 calls)].

ID CORE XT sensitivity and specificity

All 1,000 samples were typed for C, c, E, e and K. The number of samples typed for the other antigens with available serology comparators ranged from 31/32 for Co^a/Co^b and 2/2 for Di^a/Di^b, to between 371 and 830 for the rest of the antigens. Based on valid results, the phenotypes predicted by ID CORE XT and those obtained from the serological and molecular methods for the same samples are shown in Table II.

Sensitivity and specificity were not calculated for antigens with no positive/negative phenotype expression by reference methods (serology: Kp^b–, Di^a+, Di^b–, Co^a–, Lu^b–; molecular: C^w+, hr^S–, Kp^b–, Js^a+, Js^b–, U–, Mi^a+, Di^a+, Di^b–, Hy–, Jo^a–, Co^a–, Yt^a–, Lu^b–) (see Table II).

For all the phenotypes with expression, there was 100% agreement between the positive results predicted by ID CORE XT and those of serology (100% sensitivity). The agreement regarding negative results was also 100% for all phenotypes except for one discrepancy for E antigen (99.9% agreement) and 33 discrepancies for Fy^b antigen (95.5% agreement). After retesting with bidirectional sequencing, all discrepancies were resolved in favour of ID CORE XT (34 false negatives for serology, 100% specificity for ID CORE XT). Agreement of both positive and negative results predicted by ID CORE XT with those of molecular methods was 100% for all phenotypes (100% sensitivity and 100% specificity).

Analysis of the discrepancies

The only discrepancy in E antigen was found in a patient for whom serology showed expression of both E and e antigens (E+e+) whereas ID CORE XT found expression only in e (E–e+). The discrepancy was resolved with bidirectional sequencing of the 676G>C polymorphism in *RHCE* exon 5 amplicon which determines the expression of the E and e antigens. The sequence obtained showed G homozygosis in *RHCE**676 GG, indicating that the sample was homozygote for e antigen, which confirmed the E–e+ phenotype prediction of ID CORE XT. It was further verified that the sample was obtained from a recently transfused patient.

The presence of the 125A/G and 265C/T polymorphisms of the *FY* gene was detected with

Table II - Phenotypes predicted by ID CORE XT compared to serological and/or alternative molecular reference methods (BLOODchip®/bidirectional sequencing).

Blood group	Antigen (ISBT)	Phenotype (n)					
		Serology			*Molecular method*		
		(+)	*(–)*	*Total*	*(+)*	*(–)*	*Total*
Rh	C (RH2)	619	381	1,000	55	45	100
	E (RH3)	240	760*	1,000	20	80	100
	c (RH4)	848	152	1,000	85	15	100
	e (RH5)	977	23	1,000	98	2	100
	C^w (RH8)	32	484	516	0	100	100
	V (RH10)	N/A	N/A	N/A	1	99	100
	hr^S (RH19)	N/A	N/A	N/A	100	0	100
	VS (RH20)	N/A	N/A	N/A	1	99	100
	hr^B (RH31)	N/A	N/A	N/A	98	2	100
Kell	K (KEL1)	84	916	1,000	9	91	100
	k (KEL2)	816	14	830	95	5	100
	Kp^a (KEL3)	46	564	610	6	94	100
	Kp^b (KEL4)	610	0	610	100	0	100
	Js^a (KEL6)	N/A	N/A	N/A	0	100	100
	Js^b (KEL6)	N/A	N/A	N/A	100	0	100
Kidd	Jk^a (JK1)	560	175	735	73	27	100
	Jk^b (JK2)	543	191	734	73	27	100
Duffy	Fy^a (FY1)	516	222	738	77	23	100
	Fy^b (FY2)	457	281*	738	48	52	100
MNS	M (MNS1)	445	147	592	75	25	100
	N (MNS2)	421	170	591	69	31	100
	S (MNS3)	412	314	726	64	36	100
	s (MNS4)	629	96	725	82	18	100
	U (MNS5)	N/A	N/A	N/A	100	0	100
	Mi^a (MNS7)	N/A	N/A	N/A	0	100	100
Diego	Di^a (DI1)	0	2	2	0	100	100
	Di^b (DI2)	2	0	2	100	0	100
Dombrock	Do^a (DO1)	N/A	N/A	N/A	79	21	100
	Do^b (DO2)	N/A	N/A	N/A	80	20	100
	Hy (DO4)	N/A	N/A	N/A	100	0	100
	Jo^a (DO5)	N/A	N/A	N/A	100	0	100
Colton	Co^a (CO1)	31	0	31	100	0	100
	Co^b (CO2)	2	30	32	4	96	100
Cartwright	Yt^a (YT1)	N/A	N/A	N/A	100	0	100
	Yt^b (YT2)	N/A	N/A	N/A	9	91	100
Lutheran	Lu^a (LU1)	32	342	374	6	94	100
	Lu^b (LU2)	371	0	371	100	0	100

ISBT: International Society of Blood Transfusion; N/A: not available; 33 discrepancies in Fy^b and one in E in serology were resolved in favour of ID CORE XT by bidirectional sequencing.

sequencing in the samples of all 33 donors (32 of them from the same centre) with a discrepancy for Fy^b antigen. This discrepancy was due to the lack of sensitivity of the commercially available serological kits used in both centres (Anti-Fy[b] micro Immucor-Gamma) for the determination of the Fy^b antigen, which renders the detection of "weak Fy^b" expressers impossible. In this study, five more samples were genotyped by ID CORE XT as FY*A, FY*B[265T]_FY*X, but there were no Fy^b serology results available for comparison.

Antigen frequency and rare genotype variants

The frequency of each of the antigens as determined by ID CORE XT compared to the frequency in the Caucasian population as described in the available literature is shown in Table III. Most antigen frequencies were apparently similar for both populations, except for an unusually higher frequency of VS (>100 times), Js^a (>30 times) and Kp^a (>4 times), as well as a lower frequency of Fy^b and Co^b (almost 15 percent points lower) observed in the tested samples.

The rare blood group genotype variants detected by ID CORE XT in the present study are summarised in Table IV. These rare variants had to be sequenced in order to confirm the ID CORE XT genotype results.

Discussion

In this study, a complete evaluation of the ID CORE XT blood genotyping test was performed in an extensive sample of non-selected donors, patients and neonates in comparison to serological and molecular reference methods. Results showed that ID CORE XT was a robust blood group typing assay which successfully genotyped and predicted phenotype for all tested samples. Following the European Directive[15], 1,000 typing tests were performed for the major antigens (C, c, E, e, K), while for minor antigens an effort was made to type as many as possible. All 37 antigens interrogated by ID CORE XT were represented, with the exception of Di^a and Mi^a.

The system failure rate was 0%, which met the acceptance criterion of ≤5%. There were very few "no calls" (0.014%) which also met the acceptance criterion of ≤5%. Since all "no calls" were resolved after retesting, the rate of incorrect calls was 0%, again meeting the acceptance criterion of ≤1%.

Both the sensitivity and specificity of ID CORE XT for blood group antigen prediction were 100% in all samples with detected positive or negative phenotype expression, using serology, BLOODchip® Reference and bidirectional sequencing as reference methods. This successfully met the acceptance criterion of ≥99% correct identification rate. The phenotypes to which sensitivity and specificity calculations were not applicable are rare in the Caucasian population[1].

There were a number of discrepancies with serology that involved the E– and Fy^b– antigens. All of these were resolved in favour of ID CORE XT, which meant 100% of true negatives for the tested genotyping system, adding to the 100% true positives. In accordance with our results, in a recent study that compared the performance of blood-MLPA (a multiplex ligation-dependent probe amplification method) and ID CORE+ (the precursor of ID CORE XT) to that of standard serological typing methods, the molecular methods provided more accurate and reliable blood type information than did classical serological typing[17].

The discrepancy regarding E (E–e+ in ID CORE XT and E+e+ in serology) was considered a false positive serological result in a previously transfused patient. Limitations of serological typing after a recent

Table III - Frequency of antigens typed with the ID CORE XT compared to existing data from a Caucasian reference population[1].

Blood group	Antigen (ISBT)	Frequency (%)	
		Observed	*Reported in Caucasians*
Rh	C (RH2)	62	68
	E (RH3)	24	29
	c (RH4)	85	80
	e (RH5)	98	98
	C^w (RH8)	3.3	2
	V (RH10)	1.6	1
	hr^S (RH19)	100	98
	VS (RH20)	1.6	<0.01
	hr^B (RH31)	97.5	98
Kell	K (KEL1)	8.5	9
	k (KEL2)	98.6	99.8
	Kp^a (KEL3)	8.1	2
	Kp^b (KEL4)	100	100
	Js^a (KEL6)	0.3	<0.01
	Js^b (KEL6)	100	100
Kidd	Jk^a (JK1)	75.3	77
	Jk^b (JK2)	75.5	74
Duffy	Fy^a (FY1)	66.8	66
	Fy^b (FY2)	68.1	83
MNS	M (MNS1)	76.8	78
	N (MNS2)	71.6	72
	S (MNS3)	56.1	55
	s (MNS4)	87.3	89
	U (MNS5)	100	99.9
	Mi^a (MNS7)	0	<0.01
Diego	Di^a (DI1)	0	<0.01
	Di^b (DI2)	100	100
Dombrock	Do^a (DO1)	68.1	67
	Do^b (DO2)	81	82
	Hy (DO4)	100	100
	Jo^a (DO5)	100	100
Colton	Co^a (CO1)	99.9	99.9
	Co^b (CO2)	6.9	10
Cartwright	Yt^a (YT1)	99.8	99.8
	Yt^b (YT2)	9	8
Lutheran	Lu^a (LU1)	7.8	8
	Lu^b (LU2)	99.5	99

ISBT: International Society of Blood Transfusion.

Table IV - Summary of rare variants detected by ID CORE XT in the study.

Blood group	Genotype	Samples (n)
Rh	*RHCE*Ce, RHCE*CeCW*	16
	*RHCE*ce, RHCE*CeCW*	12
	*RHCE*cE, RHCE*CeCW*	5
	*RHCE*ce, RHCE*ce[733G]*	15
	*RHCE*ce[733G]* homozygous	1
Kell	*KEL*k_KPB_JSB, KEL*k_KPB_JSA*	3
Kidd	*JK*B, JK*B_null(IVS5-1a)*	1
Duffy	*FY*A, FY*B[265T]_FY*X*	38
	*FY*B, FY*B[265T]_FY*X*	9
	*FY*B_GATA, FY*B[265T]_FY*X*	1
	*FY*A, FY*B_GATA*	18
	*FY*B, FY*B_GATA*	14
	*FY*B_GATA* homozygous	4
Dombrock	*DO*A, DO*B_HY-*	1
	*DO*B, DO*B_HY-*	1
	*DO*B, DO*A_JOA-*	2
Colton	*CO*B* homozygous	1
Cartwright	*YT*B* homozygous	2
Lutheran	*LU*A* homozygous	2

transfusion are well known[9], which supports the implementation of DNA-based typing methods.

Interestingly, the Fy^b antigen was involved in the remaining 33 discrepancies. The weak expression of Fy^b is determined by the presence of the *FY*A*, *FY*B[265T]_FY*X* or less common *FY*B_GATA*, *FY*B[265T]_FY*X* genotypes and, therefore, weak expression of Fy^b antigen[18]. Lack of serological detection of weak Fy^b is due to the antigen being expressed below the limits of detection. In fact, not all serological reagents detect weak Fy^b reliably. The Fy^b typing was not repeated in the test-tube with a different antiserum because there is no CE mark- approved, commercially available serology reagent that detects the weak expression of Fy^b associated with *FY*B[265T]_FY*X* allele. However, the blood centre in which most of the Fy^b discrepancies were detected did test and confirm the samples with an"in house" absorption/elution technique using the same antibody with PEG to detect Fy^b expression (*data not shown*).

The results regarding antigen frequency were mostly comparable to those previously reported in the literature for samples of the Caucasian population[1]. There were, however, some variations. The higher frequencies of the antigens VS and Js^a, antigens with low occurrence in Caucasians (<0.01%)[19-21] in the tested samples suggest the presence of genes of African origin in the population (demographic information was not collected from the donors or patients). The observed higher frequency of Kp^a is more difficult to explain. Although the major participating centre was from the Basque region in Spain (700 out of the 1,000 samples), some red blood cell phenotype singularities that have been reported in the literature in the Basque population (such as low K, Do^a and Do^b frequencies)[22] were not observed in our samples.

The rare genotype variants found in the Rh, Kell, Duffy, Colton, Cartwright and Lutheran blood groups could be expected in a European population study[20]. In addition, ID CORE XT detected one *JK*B, JK*B_null(IVS5-1a)* sample in the Kidd blood group, although the *JK*B_null(IVS5-1a)* allele has been reported only in Finnish and some Asian populations[23]. ID CORE XT also detected rare variants in the Dombrock blood group: one *DO*A, DO*B_HY-* sample; one *DO*B, DO*B_HY* sample and two *DO*B, DO*A_JOA-* samples. The Dombrock alleles *DO*B_HY-* and *DO*A_JOA-* have been reported in the Black population[24]. Again, this suggests an African origin or genetic ancestry for the samples.

Conclusions

In conclusion, the results of this study showed that the ID CORE XT assay performed as a reliable and accurate method for correctly predicting red blood cell phenotypes of clinically relevant blood group antigens.

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Authorship contributions

MR, MP, MA, BV, MAV, EVDS and FP collected the samples and generated the serology data; ML, IA and DT designed the work, generated the genotyping data and analysed and interpreted the data. All Authors critically revised the work for intellectual content and approved the final version of the manuscript to be published.

Disclosure of conflicts of interest

ML, IA and DT are employees of Progenika Biopharma, a Grifols Company. The other Authors declare that they have no competing interests in relation to this manuscript.

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