BLOOD GROUPING

G S Williams, Northern Illinois University, DeKalb, IL, USA

Introduction

Standard blood grouping involves typing for antigens found on the surface of blood cells using serology. These blood cell antigens are also found on other body tissues and in body fluids of secretors. A broad range of antigen systems on red blood cells (RBCs) and white blood cells (WBCs) are stable, polymorphic markers for a wide variety of uses. Blood grouping has been performed for medicolegal purposes since the 1920s. Although it is not used at all in many forensic laboratories, due to the availability and convenience of automation of DNA profiling methods, it is still used for parentage testing in some areas in order to decrease cost. Parentage testing by blood grouping can be applied to inheritance issues, immigration of relatives, kidnapping cases, baby mix-ups, and traditional paternity cases. In addition, blood grouping is critical for transfusion medicine and transplantation medicine.

Blood grouping is performed by directly typing fresh blood samples or fresh stains collected in normal saline (0.85% NaCl). Methods for typing dried blood stains include absorption–inhibition, absorption–elution, or microagglutination methods. Other body fluids of secretors are typed for ABH substances and Lewis antigens. Although most tissues of the body also have ABH and Duffy antigens on them, most RBC antigens are only on RBCs.

WBCs may also be typed for human leukocyte antigens (HLAs). These antigens are on all nucleated cells in large amounts, and reduced amounts on nonnucleated cells such as RBCs and platelets. The traditional microlymphocytotoxicity method is cumbersome and technically challenging. Newer nucleic acid testing has replaced much of the serological HLA testing. Platelets have polymorphic antigens as well, but the typing of them is challenging and is not routinely performed in medicolegal laboratories.

The purpose of this article is to cover key blood groups useful for identity and parentage testing, and the methods used to detect them in fresh and dried samples.

Major RBC Systems

ABO, Secretor, and Lewis

The ABO system has been used the longest and is very useful for excluding a wrongfully accused person. The reagents are readily available and the testing is easy to perform and read. Gene frequencies have been established for almost every regional and ethnic population and the frequencies have been stable over time. Refer to **Table 1** for a sample of phenotype

frequencies in diverse populations. Mutations resulting in a new viable phenotype are rare. The full expression of the ABO antigens encoded on chromosome 9 is dependent upon genes at the *Hh* and *Se* loci closely linked on chromosome 19 as well. Lewis antigen expression is also related to *Hh* and *Se* loci.

ABO, H, and Lewis antigens are carbohydrates. They are added to type 1 or type 2 precursor molecules composed of chains of sugars with different linkages. The H glycosyl-transferase prefers type 2 chains present on cell membranes. The precursor substance attached to type 2 chains is H (fucose) and a person must inherit *Hh* or *HH* to express H substance. Glycosyl-transferases add H to soluble glycoproteins, sphingolipids, lipids, or to glycoproteins or glycopingolipids embedded in the RBC membrane and other tissue cell membranes. H is then enzymatically changed to A (terminal *N*-acetyl-glucosamine) or B (terminal β-D-galactose) or both by glycosyl-transferases. These are the gene products of group A, B, or AB blood types. The O allele codes for a nonfunctional gene product. H is expressed for nearly 100% of group Os. When individuals are *hh*, they do not express H substance. Even though they may inherit A or B glycosyl-transferases, they will not express it on cells; although a few people with *hh* genotypes inherit Se and have converted type 2 chains to A, B, and/or H. The RBCs will type like an O by standard typing methods. This is called the Bombay phenotype and is extremely rare. All Bombays produce anti-H that reacts with all normal O cells strongly, thus they are easy to differentiate from a true O. Bombay and rare subgroups must be considered when typing discrepancies occur.

For ABH to be present in secretions another locus is required. *Se* is the secretor gene. People who inherit *SeSe* or *Sese* express ABH antigens in their secretions and people who inherit *sese* do not. Type 1 chains present in secretory glands are preferred. The ABH antigens in secretions match those on RBCs. Rare exceptions exist and will be discussed later. ABH is found in easily measurable amounts on all tissues except eye lens, cartilage, hair, and nails. For secretors it is found in saliva, sputum, sweat, semen, tears, pus, nasal and bronchial secretions, gastric juice, urine, and other tissue fluids except cerebrospinal fluid.

Common blood types for the ABO system include: O, A_1, A_2, B, A_1B, and A_2B. The alleles A_1, A_2, B, and O are codominant alleles; each person has only two, one from each parent. A group O is genetically OO. A group A could be A_1A_2, A_1O, A_1A_1, A_2A_2, or A_2O. A group B genotype could be BB or BO. People with blood type AB are either A_1B or A_2B.

Secretors are Le(a–b+) and nonsecretors are Le(a+). *Le* encodes for a glycosyl-transferase also but only acts on type 1 chains in secretory glands. The resulting substance is a glycosphingolipid. Lewis is, therefore, adsorbed on to RBCs, not an integral part of the membrane. Lewis typing is not commonly done for parentage testing due to complex inheritance patterns and delayed full expression until about age 6. It can be useful for determining secretor status of older children and adults by testing blood, saliva, or stains. Le^a will be found in the saliva of an Le(a–b+) person because Lewis substance is not completely converted. An extremely rare phenotype, Le(a+b+), found in Asians and Australian Aborigines, has been described; Le(a+b+) may be secretors or nonsecretors. Le(a–b–) is also a less common phenotype; most secretors have ABH in their secretions but, rarely, someone who secretes B and no H, or A and no H is found. The antigens that would be expected on RBCs and in secretions depending upon expression of Hh, Se, and Le phenotypes are listed in Table 2.

RH System

The RH system was first recognized in 1939 and defined as D in 1940. RH is short for Rhesus. The antibody produced by guinea pigs and rabbits in response to Rhesus monkey cells agglutinated 85% of human RBCs. This was called anti-Rhesus and the corresponding human antigen was called Rhesus. It is now known that RH is not identical to D. The

Table 1 ABO phenotype percentages in selected populations

Population	*O*	*A1*	*A2*	*B*	*A1B*	*A2B*
Vietnamese	45	21	0	29	5	0
Bengalese	22	22	2	38	15	1
South American Indians	100	0	0	0	0	0
Australian Aborigines	44	56	0	0	0	0
Lapps	18	36	19	5	6	6

Data from Mourant AE, Kopec AC, and Domaniewska-Sobczak K (1976) *The Distribution of the Human Blood Groups and Other Biochemical Polymorphisms*, 2nd edn. Oxford, UK: Oxford University Press.

Table 2 ABH and Lewis antigens in secretions and on blood cells based on phenotype

Phenotype	*Antigens on red cells*	*Antigens in secretions*
Le, Se, H, ABO	Le^b, ABH	Le^a, Le^b, ABH
le, Se, H, ABO	ABH	ABH
le, se, H, ABO	ABH	None
Le, Se, h, ABO	Le^a	Le^a
Le, se, h, ABO	Le^a	Le^a
le, Se, h, ABO	None	None

"RH" antigen on human RBCs is actually LW. Now that the molecular biology of the Rh system has been worked out, two types of nomenclature are accepted for current use in documents: numerical and CDE. The Weiner nomenclature Rh-Hr based on a one-locus hypothesis is no longer used except in verbal communication about phenotypes. The Rh-Hr nomenclature is an easy shorthand method for the most common phenotypes and haplotypes. It is easier to say R^1 rather than *DCe* (verbally: big D, big C, little e). Similarly, the numerical system works well for computerization, but is too cumbersome for verbal communication. There are 46 alleles associated with the Rh system but only a few have readily available monospecific antisera and useful polymorphism. The RH system has its greatest usefulness in parentage testing. RH antigens are well developed at birth and are inherited as autosomal dominant genes. Testing for DCEce does not provide genotype information that is critical to excluding alleged fathers unless a family study can be done with his parents, siblings, or multiple children. However, when race is known, an educated guess of probable genotypes from the phenotypes may be made. Additional testing for combination antigens Ce and ce helps to establish genotypes even when only the alleged father and child are available. Blood stains that are fresh and intact RBCs can be RH-typed; older stains are tested in only a few laboratories. Table 3 shows frequencies of Rh haplotypes.

RH genes are on chromosome 1 and are closely linked. RHD and RHCE are closely linked loci. The alleles for RHD are either *RHD* (D+ or Rh+ or RH:1) or deleted (D– or Rh– or RH:–1) for Caucasians, and RHD (D+ or Rh+ or RH:1) or inactive (D– or Rh– or RH:–1) for Blacks, Japanese, or Chinese. The alleles for RHCE are *RHCe*, *RHcE*, *RHCE*, and *RHce*.

RHD and *RHCE* genes code for proteins with no carbohydrates. These proteins are integral membrane proteins that are also required for full expression of other gene products: LW, Duffy (Fy5), and SsU. Another gene product, RHAG, a glycoprotein encoded on chromosome 6, is also required for RH expression. Most RH null RBCs do not express any RH even though they have the genes for RHD and RHCE because they lack RHAG. Other RH null RBCs result from a deletion of RHCE and already have the deleted RHD. Complete or partial deletion of RHCE and increased expression of RHD is also a rare finding.

Table 3 Rh system phenotype incidence using DCcEe nomenclature

Haplotypes	*Blacks*	*Whites*	*Asians*	*Native American*
DCe	17	42	70	44
DCE	<1	<1	1	6
DcE	11	14	21	34
Dce	44	4	3	2
Ce	2	2	2	2
CE	<1	<1	<1	<1
cE	<1	1	<1	6
ce	26	37	3	11

MNS

Glycophorin A and B contain MNSs antigens and are well developed at birth. Now over 40 antigens have been found in the MNS system. Of these, only MNSs and U are routinely typed. In laboratories doing DNA testing, the results for glycophorin A and B closely linked on chromosome 4 would overlap the RBC antigen studies for this system. Glycophorin A has M and N antigens and glycophorin B has S, s, and U. These genes are closely linked and viewed as haplotypes *MS*, *NS*, *Ms*, and *Ns* for parentage studies. One precaution: anti-N commercial antisera may cross-react with M+ cells.

Duffy

Duffy antigens that are routinely typed are well developed at birth. Duffy antigens are glycoproteins encoded on chromosome 1 but not linked to the RH system. This glycoprotein is the receptor for entry of *Plasmodium vivax* into RBCs; therefore, the distribution of the antigens is profoundly different for countries with endemic malaria. It is a selective advantage to be Fy(a–b–) where exposure to malaria is high. There are six possible alleles: Fy^a, Fy^b, *Fy*, *Fy3*, *Fy4*, and *Fy5*. Fy^a and Fy^b are the most frequently tested antigens in the system.

Duffy is present in other tissues besides RBCs. Brain, kidney, spleen, heart, and lung tissue cells also express Duffy antigens. Blacks who are Fy(a–b–) negative on RBCs may express Duffy antigens on solid tissue cells. Fy(a–b–) is rare in whites and common in African-Americans (68%).

Kidd

Kidd antigens are only on RBCs as urea transport molecules. Jk^a, Jk^b, *Jk3*, and *Jk* are alleles. *Jk*, an amorph, is extremely rare and found in Polynesians and other Pacific Islanders of Chinese descent with an even rarer occurrence in North Europeans by a different mechanism (suppressed by the Lutheran antigen inhibitor). Kidd antigens are enhanced by enzymes. Antisera to Jk^a is scarce and antisera to Jk^b is even scarcer, thus typing for this antigen is

not routine. In addition, Kidd antibodies are fragile. Kidd antibodies often disappear rapidly from people who produce them. In addition, a few days after putting a clotted specimen in the refrigerator, the antibodies lose potency. Freeze-dried antisera appear to retain their activity. This system shows useful polymorphism and adequate expression on fresh samples; thus, Kidd system typing can give extra information in parentage testing where antisera are available.

Kell

Kell antigens are proteins bearing homology with neutral endopeptidases. These proteins contain a large number of cysteine residues forming disulfide bonds required for tertiary structure and thus intact antigenic expression. Kell antigens are destroyed by acid or reducing agents such as dithiothreitol or 2-mercapto-ethanol and structure disruptor ethylenediaminetetraacetic acid-glycine. There are 11 alleles, all rare except K (K1) and k (K2). Other allelic antigens in the Kell system include Kp^a, Kp^b, Js^a, Js^b, and Ko. A person homozygous for *Ko* expresses no Kell antigens.

Lutheran

Lutheran (Lu) antigens are found on a RBC glycoprotein with adhesion properties. The genes encoding *Lu* are linked to *Hh* and *Se* genes found on chromosome 19. These antigens are poorly expressed at birth, in low density in adults, and the antisera scarce; therefore, these are not routinely used for parentage studies.

The common antigens are Lu^a (Lu1) and Lu^b (Lu2). In whites, the frequency of Lu(a+b−) is 0.15%, Lu(a+b+) is 7.5% and Lu(a−b+) is 92.35%. Lu(a–b–) is extremely rare. Twenty other antigens are in the Lu system, but only Au^a (Lu18; 80% of whites) and Au^b (Lu19; 50% of whites) might be of use for parentage studies. The other antigens are high-incidence or low-incidence antigens. The inheritance of one dose of the inhibitor of Lu antigen (In(Lu)) gene suppresses the expression of Lu on RBCs and must be considered when analyzing data.

Xg

Xg^a is an antigen found with higher frequency in females than males. It is located on the X chromosome. The frequency in whites of Xg^a in females is 88.7% and 65.6% in males. It is most useful for parentage studies involving alleged fathers who are Xg(a+) and mothers who are Xg(a–). All sons would be Xg(a–) and all daughters would be Xg(a+) if he is the biological father.

Standard Methods with Intact Blood

Blood group antigens are detected with antibodies to each specific antigen. Each antibody–antigen reaction is optimized by modifying the ratio of antigen to antibody, time, temperature, and additives. Many of the blood group antigens can be tested by slide, tube, or microplate methods and require no incubation. Agglutination (clumping) or hemolysis (breakdown of the RBCs) is traditionally observed for positive reactions. Certain antibody–antigen reactions require incubation at room temperature or 37 °C for 20 min to 2 h. For reactions at 37 °C, an antiglobulin test (Coomb's test) involves washing excess unbound antibody from the sample, antihuman globulin is mixed with the RBCs to detect bound antibody, and the tube or microplate is centrifuged and read for agglutination. Additives that enhance or modify reactivity include low-ionic-strength solutions, polyethylene glycol, enzymes (ficin, bromelain, papain, or trypsin), albumin, and positively charged polymers (hexadimethrine bromide, protamine sulfate, poly-L-lysine). Newer methods use enzyme immunoassays or solid-phase RBC adherence to view positive reactions. In addition, fluorescent and ferritin-bound antibodies can be used to distinguish homozygosity from heterozygosity. Currently, DNA sequencing is available for detecting heterozygotes and blood group DNA microarrays are available to determine complete genotypes.

Systems that react rapidly and best at room temperature or cooler temperatures include ABO, MN, P, Lewis, and Lu(a+). The P system is not useful for forensics because the antisera are unreliable and the expression quite variable. Antigens that are best typed at body temperature (37 °C) and incubated include most of the CcEe and some weak expressions of D, Kell, Duffy, Kidd, Ss, and Lu(b+). RH system antigens react best with antisera containing extra protein or enzymes. Proper controls of antisera to rule out nonspecific binding must be done whenever enhancement materials are present. These controls contain the same contents as the antisera but lack the antibody.

Standard ABO slide testing involves mixing a drop of antisera (anti-A, anti-B) with a drop of whole blood and viewing for agglutination (clumping of RBCs and antisera). The reverse typing for ABO involves taking RBCs of known type A_1 and B and sometimes O and reacting these against the plasma or serum of the test case. For ABO tube or microplate grouping, the RBCs of the individual are diluted to 2–4% in normal saline (0.85% NaCl) and mixed with antibodies to A and B (anti-A,B, or anti-H may be used to clear up discrepancies). These are

centrifuged briefly and then read for agglutination. A reverse type would also be set up. For detection of the two common subgroups of A, *Dolichos biflorus* lectin is used. A_1 cells are positive and A_2 cells are negative.

RH typing for parentage testing is usually limited to mixing RBCs with anti-D, C, c, and E. Antisera are not always monospecific and care must be used when dual specificity is found, such as with anti-Ce; e-negative RBCs would not react well with this antisera. It is so rare to be e-negative in most populations that anti-e is not used routinely. Exceptions include Mexicans, Native Americans, and Asians (Table 3).

Testing for M and N is performed by mixing RBCs with anti-M and anti-N, centrifuging, and observing for agglutination. Testing for S and s, Duffy (Fy^a, Fy^b), Kidd (Jk^a, Jk^b), and Kell (K, k) involves incubation at 37 °C and antihuman globulin. Special controls of test RBCs without reagent antibody must be included to ensure that they are not already coated with antibody. These Coomb's-positive cells would test positive for every marker requiring antihuman globulin as part of the procedure.

Not all antigens are expressed well at birth; system antigens with nil to moderate expression include Lewis, P, Lutheran, and Kx. In addition, reverse typing for ABO will not be reliable in newborns up to 6 months of age because they have circulating maternal antibodies and do not produce their own ABO antibodies until their immune system is mature. They need effective exposure to the environmental antigens that are ubiquitous in nature to stimulate production of anti-A, anti-B, and/or anti-A,B. Elderly individuals also have waning expression of the ABO antibodies. Other considerations include transfusion history, chimeras, transplantation, dispermy, and cancer (especially leukemia and colon cancer with known alterations in ABO expression).

Testing Blood Stains

In selecting antigens for testing blood stains, stability of the antigen in diverse conditions is an important consideration. For instance, ABO is very stable whereas Kidd antigens are not. The availability of highly specific antisera is also an important consideration. Anti-C without anti-G is hard to find; anti-Jk^b is extremely rare; anti-N that does not cross-react with M is uncommon. Anti-Lutheran antibodies are also not easily obtained. Testing RH, Fy, K, MNSs, and Jk^a (in fresher stains) are plausible choices. ABO, RH, and MN are most likely to be successful on blood stains, and are the most widely used by crime laboratories.

Methods to test for RBC antigens in stains include absorption–inhibition, absorption–elution (100 times more sensitive than absorption–inhibition), mixed agglutination, fluorescent and ferritin-labeled antibody, formalin-treated RBCs (not widely used), and reversible agglomeration (used to retrieve RBCs from putrefied blood clots, not for typing itself).

The absorption–inhibition test has been used since the early 1920s and was the only one until 1960. It requires large amounts of sample compared to the newer methods. Basically, biological fluids, RBCs, or stroma from a stain are tested or scraped into a tube with saline (0.85%), anti-A, anti-B, and *Ulex europaeus* anti-H lectin are incubated with the sample. The serum is removed and tested for its ability to agglutinate A cells, B cells, or O cells compared to a saline (no RBC control). A significant reduction in the ability of the test sera to agglutinate cells indicates that the original sample was that blood type. For instance, anti-A after incubation with group A sample will not agglutinate group A RBCs well because the anti-A was absorbed by the sample.

The absorption–elution technique is widely used and requires 100 times less RBC sample. First, an optional RBC fixation can be achieved by heating blood-stained samples to near boiling for 30 s in a buffer. Then anti-A, anti-B, and/or anti-H are absorbed on to the fixed or unfixed extracted sample; excess antisera are washed off at cool temperatures. The absorbed antibody is then eluted off the sample. Test the eluates from the sample as follows: anti-A eluate with A_1 cells and A_2 cells, anti-B eluate with B cells, and anti-H eluate with O cells. All main blood types have been successfully typed by this method.

Microagglutination also requires only minute samples. The sample is treated with antisera, washed, and mixed with the same blood type as the antisera used. It is viewed under a microscope for RBCs attached to the fibers of blood-stained cloth or materials.

Testing Other Body Fluid Stains for Blood Groups

Some antigens are only on RBCs (RH, Kidd, Kell, MNS, Lutheran). Testing of body fluid stains requires careful collection of the stain and an adjacent area with everything but the stain to be sure the typing is not from the environmental source rather than the specimen of human body fluid. ABH antigens are found on bacteria, wood, soil, other mammals, dust, and other places. Therefore, careful collection is a must. In addition, organisms in the environment may enzymatically alter blood group antigens. *Clostridium* spp., *Bacillus* spp., and *Aspergillus niger* as

well as coffee beans can change or eliminate certain blood group substances. In addition, it is advised to test for A or B when the test for H is negative. Testing for anti-A and anti-B in stains or fresh body fluids besides blood is also possible; awareness of false negatives and positives is important.

Testing for Secretor Status

Testing for secretor status may be accomplished by two different methods: testing blood for Lewis antigens or secretions for ABH substances. The concentration of ABH is high in saliva, semen, gastric juice, breast milk, and amniotic fluid and low in tears and urine. When testing body fluids, special precautions and procedures must be followed. For instance, saliva has enzymes that degrade the ABH so it must be either dried immediately or heat-treated to inactivate the enzymes. Urine requires concentration; semen is fragile.

Testing blood for adhered Lewis antigens is one way to predict secretor status. Le(b+) people are secretors and Le(a+) are nonsecretors of ABH substance. Alternatively, and more specifically, test saliva or other body fluids for H substance using *Ulex europaeus* anti-H lectin to absorb on to the H in the sample. The supernatant will not be able to agglutinate O cells if there is H substance in the sample. If the individual is a nonsecretor, no anti-H will be absorbed, and the O RBCs will agglutinate with equal strength compared to the saline control. If the test is negative for H, test for A or B to detect secretions that express A or B, and not H.

Blood Groups on White Cells and Platelets

HLA is on all nucleated cells of the body in large amounts and reduced amounts on nonnucleated cells. Until DNA testing became widely used for typing purposes, HLA testing was done by microlymphocytotoxicity assays. Trays with multiple wells are filled with antisera from multiparous women and overlayed with oil, then frozen until needed. Each antigen is represented by at least three different sources of antisera as a control check. Typed cells are T lymphocytes or B lymphocytes from fresh ACD anticoagulated blood. The T lymphocytes are used for the HLA-A, B, C antigen plates and the B lymphocytes are used for the HLA-DR, DP, DQ antigen typing. The cells are added to an entire plate of antisera, incubated, and then rabbit complement is added. An indicator dye helps differentiate dead from living cells using phase contrast. Brightly refractile cells are alive. The dark cells are dead and therefore had antibody plus complement to kill them. The grading system is from 0 to 8 where 0 = no dead cells and 8 = 100% dead cells. Most procedures resulted in lots of scores of 6 or 4 and require very experienced people to interpret results.

HLA typing using DNA probes is also cumbersome and time-consuming, requiring experienced personnel for interpretation. Systems are now available using DNA testing with allele-specific primers for HLA-A, B, and DR and are interpreted by the computer.

Conclusion

Blood grouping has been replaced by DNA methods in most forensic laboratories throughout the world. Classic blood grouping is still performed for parentage testing and for transfusion and transplantation medicine.

See Also

Crime-scene Investigation and Examination: Collection and Chain of Evidence; **Immunoassays, Forensic Applications**; **Mass Disasters:** Principles of Identification; **Postmortem Changes:** Overview; **Serology:** Overview; Blood Identification

Further Reading

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