TECHNICAL PROGRESS IN PARENTAGE ANALYSIS

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There are no illegitimate children—only illegitimate parents.119

EARLY PARENTAGE ANALYSIS: ABO

In 1921, Ottenberg proposed the use of blood grouping in a genetic analysis of paternity.74 His proposal was based on three earlier discoveries: first, Landsteiner's ABO blood groups and naturally occurring antibodies to A and B antigens permitted universal human bloodgroup classification. Second, von Dungern and Hirschfeld reported that ABO groups followed Mendel's first law of inheritance (segregation of alleles at one locus) and suggested studying them in paternity disputes. (Curiously, there were a number of anomalies in their pedigree data, caused by either technical error or nonpaternity!) These workers believed that A and B groups were determined by separate loci, but Bernstein refuted that hypothesis and demonstrated that the ABO system was in Hardy-Weinberg equilibrium (i.e., that genotype frequencies are constant from one generation to the next). Third, Ottenberg found that all genetically unexpected results could be attributed to either common technical errors or rare recombination events. Notably, Ottenberg was the first to carry out routine ABO grouping and pretransfusion compatibility tests of blood donors and recipients. Thus, parentage testing and blood banking had common origins. The ABO system, however, could only exonerate 13% (0.13) of falsely accused men.

MNS, INFORMATIVENESS, AND PATERNITY EXCLUSION

A man is excluded from parentage of a child when an allele observed in the child could not have been maternally inherited (i.e., it is a paternal obligatory allele,

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Table 1. THE EFFECT OF EQUIVALENCE OF ALLELE FREQUENCIES ON PATERNITY EXCLUSION*

<table>
<thead>
<tr>
<th>Phenotypes (Genotypes) That Demonstrate Exclusion of the Man</th>
<th>Proportion of Men Excluded, Given:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal Child Accused Man</td>
<td>q = 0.5  q = 0.6  q = 0.7  q = 0.8  q = 0.9</td>
</tr>
<tr>
<td>p = 0.5  p = 0.4  p = 0.3  p = 0.2  p = 0.1</td>
<td></td>
</tr>
<tr>
<td>P(PP) P(PP) Q(QQ)</td>
<td>0.0625  0.0576  0.0441  0.0256  0.0081</td>
</tr>
<tr>
<td>Q(QQ) Q(QQ) p(pp)</td>
<td>&quot;     &quot;     &quot;     &quot;     &quot;</td>
</tr>
<tr>
<td>p(pp) p(qq) p(pp)</td>
<td>&quot;     &quot;     &quot;     &quot;     &quot;</td>
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<tr>
<td>Q(qq) Q(qq) P(PP)</td>
<td>&quot;     &quot;     &quot;     &quot;     &quot;</td>
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Total probability of exclusion (PE) = 0.2500  0.2304  0.1764  0.1024  0.0324 |
Heterozygosity of the system = 2 pq = 0.50  0.48  0.43  0.32  0.18

* Maternity assured, two codominant alleles P and Q. Frequency of P = p, frequency of Q = q, p² + 2pq + q² = 1.

or POA) and the allele is lacking in the man. When characteristics of the polymorphic MNS blood group systems were shown to be inherited independently of the alleles of the ABO system (Mendel’s second law: independent assortment of alleles at different loci), parentage tests became more valuable in excluding men who were falsely accused of paternity. A polymorphic genetic system may be defined as one in which there are two or more alternative characteristics (alleles), no one of which has a frequency in the population of greater than 99%. If the alleles in a system have population frequencies that are more or less equal, then their value is increased in demonstrating exclusions of falsely accused men. Both the number of alleles in a system and the equivalence of their population frequencies contribute to the overall ability of a system to exclude falsely accused men. Polymorphisms of alleles with similar frequencies show greater “heterozygosity” or “information content.” The related specific measure of the ability to demonstrate paternity exclusion is termed the probability of exclusion, or PE. It is defined as the probability that tests for alleles of one genetic system will exclude a falsely accused man (or the percentage of falsely accused men who will be excluded by the defined extent of testing). The effects of an equivalence of frequencies of two alleles on heterozygosity and PE in a simple genetic system can be seen in Table 1. The addition of the MNS system to the ABO system, by Landsteiner and Levine in 1927, markedly increased the ability to demonstrate nonpaternity. (The linked S/s locus alleles of the haplotypic MNS system were discovered later.)

The combined PE (CPE) is the cumulative probability of exclusion using two or more genetic systems (see reference 49). Using ABO alone, the PE for caucasian men is about 0.13, but when combined with the MNS system (PE = 0.24), CPE is about 0.34. The CPE is not the simple sum of PEs of independent systems. If ABO can show exclusion of 0.13 of falsely accused men, then 0.87 of these men remain unexcluded. Of these 0.87, an additional 0.24 can be excluded by MNS: 0.87 * 0.24 = 0.21; 0.13 + 0.21 = 0.34. A simple general formula is as follows:

\[ CPE = 1.0 - (1 - P_1)(1 - P_2)(1 - P_3) \ldots (1 - P_n) \]

where \( P \) is the PE of each independent locus.113

It is of interest to consider why MNS has a much higher PE than ABO. First, it should be noted that subgroups of A are often problematic and are not usually used in parentage studies.14 Second, although there are three usable and common alleles
in the ABO system, the most frequent one is silent (O); its presence cannot be observed directly by any antiserum. The genotypes of AA and AO persons (e.g., men) cannot be differentiated (nor can BB and BO). When a paternal obligatory allele (POA) is O, men homozygous for A or B cannot be ascertained and cannot be excluded. Third, the last allele is infrequent: The B gene’s frequency (among whites) is only about 6%. On the other hand, the common alleles of the MNS system are codominant. Without considering the Ss locus alleles (the MN and Ss loci are closely linked), the M allele is found in 55% and the N allele is found in 45%. Thus, there is a relative equivalence of the two alleles in the population and relatively “high heterozygosity” for the diallelic system (see Table 1). Phenotypes of heterozygotes generally denote genotypes (except for a few rare variants of M that may be silent under routine test conditions because identifying antisera are unavailable or too expensive for routine use). Therefore, men lacking a child’s paternal obligatory allele are easily identified and excluded from paternity.

Theoretically, if the AO heterozygote could be easily distinguished from the homozygous AA individual (or genotypes BO from BB), then a man homozygous for either A or B could be excluded from paternity whenever the paternal obligatory allele was O. Actually, it may be possible to determine zygosity by immune determination of the presence of the nonfunctional enzyme O-transferase, by inference studies of an extended family, by antigen density using flow cytometry, by immunocytochemistry of spermatozoa, and by DNA analysis of the ABO locus. None of these methods, however, are readily available for parentage analyses.

MORE IMMUNOCHEMATOLOGY AND IMMUNOGENETICS: RH, KEL, FY, JK

In the United States, Wiener may be considered the father of parentage analysis for several reasons. First, Wiener introduced a major blood-group system (RH) to parentage studies in 1947. The RH system was much more polymorphic than those described earlier, and it had considerable value in demonstrating exclusions. (RH phenotypes, however, do not denote genotypes, and the population frequencies of various alleles are not close to equivalence.) Second, Wiener’s expertise in the immunology of RH and other blood-group marker systems was important in securing immunogenetic information as legally admissible. Third, Wiener advocated probability calculations, initially to describe PE and subsequently to present and quantify the genetic evidence in favor of paternity. Calculations for paternity inclusion are discussed in a later section.

The CPE of ABO, MNS, and RH systems is about 0.50; that is, on average, about half of falsely accused men can be excluded by study of these three systems. In addition, POAs may be ascertained in three independent systems. If a child happened to inherit three POAs that were infrequent in the population, and the accused man possessed them, it intuitively strengthens the suspicion that he, in fact, is the child’s biologic father.

Three additional red cell marker systems have been recommended in parentage analysis. The Kell, Duffy, and Kidd systems raise CPE of erythrocyte surface antigen studies to over 60%. Although these systems are neither very polymorphic nor highly heterozygous, blood banks generally possess reagent antisera that are carefully controlled for avidity, titer, and specificity, and the sera meet requirements of the Food and Drug Administration because they are used in blood transfusion.
Blood bank personnel use standardized methods and have knowledge of the technical and interpretive problems that arise in using these systems.

ADVANTAGES AND LIMITATIONS OF RED CELL MARKERS

Red cell immunogenetic systems are regarded as very well defined: Virtually all alleles and their frequencies in various populations are known, most genetic oddities have already been described, and the mutation and recombination rates are very low. It became evident, however, that there was a diminishing rate of return of information about parentage as additional, but less powerful blood groups were applied.

There are several limitations to phenotyping erythrocytes. Reagents for red cell antigens are mostly of human sources. (Some monoclonal antibodies, lectins, and animal sera are used.) Human sera are expensive, sometimes contain unexpected antibodies, and are occasionally unavailable. At times, manual serologic methods produce subjective errors, and procedures are labor intensive and expensive. Rare alleles can cause misinterpretation, especially those that are unobservable (silent) either because they require rare antisera to induce agglutination or because they are immunologically unexpressed. For example, a heterozygous biologic father with one undetected antigen may be considered homozygous for his detected antigen. If a child inherits an expressed maternal obligatory allele (MOA) and the silent allele from its father, the child will appear to be homozygous for the allele inherited from the mother, but is actually heterozygous: MOA/paternal silent allele. A false exclusion can be inferred from the misinterpretation of homozygosity of the biologic father and child. This false "indirect" ("second order") exclusionary finding is a well-known pitfall in red cell systems. An indirect exclusion is defined as one that requires deducing genotypes before inferring nonpaternity. A "direct" (first-order) exclusion, by contrast, is based on finding an allele in the child that is absent from both the alleged father and mother or finding a heterozygous alleged father, but finding neither paternal allele in the child. The possibility of error, caused by inference of genotype, has produced a widely held laboratory policy that paternity should not be ruled out on the basis of a single indirect exclusionary result. (Direct exclusionary findings are rarely erroneous because of faulty genetic inferences.)

A frequently encountered problem is that ambiguous red cell phenotypes can represent several genotypes (Fig. 1). A systematic way of accounting for all the possible genotypes that may produce a given phenotype is to construct a two-dimensional table of all known alleles at a locus. The first vertical column and first horizontal row list the possible alleles. The cells of the matrix are then filled in with possible genotypes. Each genotype can be assigned a probability of representing the phenotype that is based on the frequencies of the alleles in a population and the

**Figure 1.** A phenotype may represent several genotypes. The boldfaced symbol E represents a phenotype that can be produced by superimposing the two letters in each genotype combination.
Hardy-Weinberg law. The sum of probabilities of genotypes should equal the frequency of the phenotype in the population.

An infrequent problem of red cell immunogenetic analysis involves alteration of an inherited antigen. Some antigens can undergo posttranslational change to another antigen. A striking example is conversion of A1 to B in the ABO system. Other antigens can be suppressed or enhanced by disease, pregnancy, and very young age. Very uncommonly, antigens may be affected by genetic events, including presence of a specific allele at the same locus of a homologous chromosome (e.g., in trans-position), presence of specific alleles at other loci (epistasis), by recombination, and rarely by mutation, chimerism, despermy, and so on.

Despite the best efforts, misclassification of blood groups used to occur in up to 2% to 3% of red cell determinations. The usual error, misclassification of uncommon alleles in the alleged father as common ones, caused false inclusions of paternity. In a highly informative polymorphism, misclassification has a trivial effect on the PE of the system, but obviously it can be disastrous in individual cases. It is of interest that in the 1940s, although some prosecutors were overly skeptical of bloodgroup evidence of exclusions and denied their validity, others regarded them as infallible. (Notably, both these legal positions were taken again in the 1980s when DNA technology was introduced.) Technical and clerical errors in red cell pheno
typing are now largely avoided by adopting a standard of testing that demands two separate analyses using separate reagents and interpretation of data by two independent observers.

**BLOOD PROTEIN POLYMORPHISMS:**
**IMMUNOGLOBULIN ALLOTYPING**

Extensive immunogenetic polymorphisms were described and characterized after their discovery, in 1956, at two independent loci encoding the constant domains of gamma immunoglobulin heavy (IGH or Gm) and kappa light (IGK or Km) peptide chains.

The phenotypes comprising the alleles (allotypes) are determined by red cell agglutination inhibition. One reagent is prepared by sensitizing Rh-positive red cells with reagent anti-Rh IgG of known allotype. The antigens (haplotypes) are detected by determining if an unknown serum contains the same allotypic antibody that absorbs a specific second reagent antiallotype antibody. If absorption occurs, there is lack of agglutination of the sensitized red cells. Inhibition of agglutination indicates a positive test for a specific allotype (Fig. 2). The combined PE of IGH and IGK is 0.27 in whites (and 0.39 in blacks). The tests require very little serum, and antigens are stable, but sources of specific human antisera reagents are limited, and accurate results require expert technique. Children of less than 6 months of age present difficulties because their serum contains placentally transferred maternal antibodies. The system’s use in parentage analysis appears to be waning.

**BLOOD PROTEIN POLYMORPHISMS:**
**ELECTRICAL CHARGE VARIANTS**

The first electrophoretic protein variant (S hemoglobin) was described in 1949. Electrical charge of a protein molecule depends on its amino acid content; variant alleles possess different amino acids and different charges. Electrophoresis
Figure 2. Agglutination inhibition test for immunoglobulin allotyping. Positive test (A), negative test (B).

involves separation of proteins according to their rate of migration in an electrical field; migration is primarily a function of each protein’s net charge at a fixed pH. In 1955, Smithies described the electrophoretic separation of allelic variants of haptoglobin, a protein which binds free hemoglobin in serum. Many other polymorphisms of serum proteins and red cell enzymes were described subsequently (in genetic forensic and anthropologic literature) as suitable for parentage analyses. The application of these genetic polymorphisms to parentage analysis increased CPE to above 0.90. Electrophoretic methods were crude initially, but they underwent dramatic changes by the 1970s when they were commonly applied by forensic laboratories and by a few parentage testing laboratories. Currently, in addition to high-resolution electrophoretic methods, laboratories can identify additional allelic
protein variants by isofocusing, by reaction with specific antibody, and by their enzymatic activity.

Isofocusing requires a gel containing ampholytes that establish a pH gradient. (An ampholyte is a polyamine of known pK.) Proteins are added and migrate only until they encounter a region of the gel in which the pH is the isoelectric point of a protein; migration stops at that point. The proteins with enzymatic activity are observed by their in situ catalytic action on a specific substrate that is converted to a visible product directly on the gel or on a blot of the gel. Some proteins are immunoprecipitated after electrical separation by specific antibodies, and the fixed immune complex can be visualized on the support matrix by simply washing to remove unfixed proteins and staining the precipitated complex. Others proteins are so concentrated in the sample that they are simply detected by a general protein stain.

Protein-separation analyses (electrophoresis, isofocusing) have become inexpensive and rapid. Many protein variants are stable under conditions of transport, storage, and analysis. The alleles are codominant and readily observable. Systems that are used commonly in parentage studies include PGM1, ACP1, ESD, GC, HP, TF, PLG, G6PD, GALT, GSR, HBB, and many others. The PE's of individual systems range up to about 0.35. Advantages of electrical separations of protein variants for parentage analysis are small sample volume requirements, possible simultaneous separation of alleles at different loci, simultaneous testing of many individuals, commercial availability of reagents, and detection of all reactive alleles using common reagents (unlike immunogenetic analyses, in which a single anti-serum identifies single alleles or only epitopes of alleles). Procedures can be technically demanding and may occasionally produce poor resolution of similarly migrating variants (and their secondary products) with misinterpretation (Fig. 3). The relative instability of some enzymatic proteins can also pose problems. The presence or quantity of total expressed proteins at a few loci are affected by age and disease. (For example, haptoglobin is bound by free hemoglobin, and the complex may be removed from serum following intravascular hemolysis.) There are known silent alleles and variants that are difficult to differentiate because they have similar migration characteristics.
MAJOR HISTOCOMPATIBILITY ANTIGENS: HLA CLASS I

Immunogenetics resurfaced in the field of parentage analysis when, in the 1970s, Terasaki\textsuperscript{95} showed that the very highly polymorphic and heterozygous HLA system was applicable.\textsuperscript{37} The haplotypes of serologic loci (class I HLA-A,B,C) alone demonstrated a PE of about 0.92. Test results could be obtained in 1 day without the use of special equipment. Assays required microscopic, semiquantitative observations of lymphocytotoxicity as demonstrated by vital stains. Unlike some red cell systems, the class 1 locus antigens of HLA are expressed in very young children, including newborns. Mutations are infrequent, although the recombination rate between the A and B loci is 0.7%. Recombination can be problematic when analysis involves more than one child.

In Europe, the extensive and early use of a variety of genetic systems had suggested calculations that estimated likelihood of paternity of an unexcluded, accused man.\textsuperscript{32,93} (See section on paternity-inclusion calculations.) In the United States, use of HLA, a single but "extensive" polymorphism, helped to change the bias that parentage tests should be simply exclusionary.\textsuperscript{96} (In general, cumulative PE and inclusion calculations are related.\textsuperscript{69}) A single locus, consisting of a polymorphism whose alleles have similar population frequencies, yields great information content with respect to both PE and calculations of odds or probabilities of paternity. Inclusion calculations derived from HLA phenotypes are sometimes higher than those of combined red cell and protein markers by several orders of magnitude.

Using the markers of the HLA system, if a child possesses an obligatory paternal allele (e.g., A,B haplotype), and that allele is carried by a man accused of paternity but is rare in the population of men, then, intuitively, the chance seems great that the man is truly the biologic father. The large number of HLA haplotypes and their even distribution in the population ("balance") cause each haplotype to be relatively rare. For example, the most frequent HLA-A,B haplotype’s frequency is about 0.07 in whites.

Powerful as the HLA system may be, any single-system parentage test has shortcomings.\textsuperscript{97} A single system will not suffice when several related men have been accused of paternity because of allele sharing among close relatives.\textsuperscript{35} Large volumes of fresh blood are required to harvest sufficient viable lymphocytes for HLA analysis. Lymphocytopenia generates the same problem, even if the blood sample volume and preservation appear adequate. Sometimes, as can be seen in large series, the single HLA system is not revealing, but a number of other systems, each of limited power, accumulate diagnostic information regarding paternity.\textsuperscript{23,41,91} It may also be difficult to obtain specific antisera. Reagent scarcity increases cost, as does technical labor. Unlicensed reagent antisera, derived from multitransfused patients or multigravida, may contain transmittable viruses. Multiple antibodies with different specificities, strengths of reaction, and cross reactions may be present in HLA antisera. (HLA antigens belong to cross reactive groups: CREGS.) Each of three situations give rise to silent alleles or "blanks": (1) rare variants may not be detectable with the antisera available; (2) there is homozygosity; and (3) there may be unexpressed alleles. Blanks are more frequent in blacks than whites, although PEs are similar in these two groups, suggesting that undefined specificities remain unidentified.\textsuperscript{109} Phenotypic suppression has been described.\textsuperscript{109} Haplotypes may be ambiguous, and antigen expression can vary with disease activity and inversely with age. Test variables include the potency of complement reagent, the dyes used, cell and reagent storage conditions, and technical expertise in typing cells. The cytotoxicity assay itself is somewhat subjective.

It is probable that HLA will continue to serve as an important test of parentage because it is now widely accepted, its power to exclude or include paternity is
equivalent to the newer DNA systems, and it is available in many clinical laboratories because it has medical utility (in diagnosis, in study of disease associations, in transfusion and transplantation, and so forth.)

**PATERNITY-INCLUSION CALCULATIONS**

The growing power of genetic tests, both in the number and the heterozygosity of polymorphic loci, allowed for meaningful probability estimates of paternity. By the 1970s, many laboratories could achieve exclusion of 95% to 99% of falsely accused men. Use of tests with a high CPE yields an actual exclusion rate of about 25% to 30% among US cases. Of the 70% to 75% of men who are not excluded by tests, an estimate of the likelihood of their paternity is helpful in describing the relative chances of paternity of an accused man (or men) as compared to the possibility that another man actually sired the child in question. One commonly used calculation is an odds (likelihood) ratio comparing the chance that a man of the phenotype of the accused man fathered the child+ the chance that another man (from the same population as the alleged father) sired the child. The ratio is a simple comparison of frequencies of two conditional probabilities. One condition (possibility) supposes that the alleged father (whose phenotype is genotypically consistent with paternity) mated with the child’s mother (of known phenotype) and that a child (of known phenotype) was born to the couple. The other condition supposes that the child was born to the mother, but was sired by a man in the population whose genotype is consistent with paternity of the child. The likelihood ratio (paternity index, or PRI) assigns no prior probabilities to the nongenetic evidence of the alternative hypotheses: (1) that the alleged father (AF) is the biologic father (BF), or (2) some other man is the BF.

The second calculation, based on Bayes theorem, does assign prior probabilities to the nongenetic evidence. Because the prior probability of paternity and the conditional probability (based on the genetic evidence) are independent events, the probability that an unexcluded, accused man is the BF is given by the product of the prior and conditional probabilities. Traditionally, equally weighted prior probabilities have been assigned to the alternate hypotheses under consideration. Thus, when a mother accuses one man of paternity, he is arbitrarily assigned a prior probability (P1) of paternity of 50% and an unnamed “random man” (who is the alternate possible BF) is assigned a prior probability (P2) of 50%. In a two-man case, each accused man may be assigned a prior probability (P1a and P1b) of 33.3%, and the random man may also be assigned 33.3% (P2). Many different sets of alternative hypotheses arise in actual cases, and prior probabilities may be assigned accordingly.

The product of a prior P multiplied by a conditional P is a joint probability. There is a joint probability (j1) that, a priori, an AF is the BF (P1) and he has the observed phenotype that is consistent with paternity (Cj). (Prior P is supposedly based on nongenetic evidence, and conditional P is based on the phenotypes of mother, child, and AF if he actually sired the child.) There is a second joint probability (j2) that, a priori, some other man (random man, or RM) is the BF (P2) and, if so, he has contributed obligatory paternal alleles that are consistent with paternity (C2).

Two posterior probabilities (W, for the German word wahrsecheinlichkeiten) may be calculated from the two joint probabilities (in a case involving one accused man). The two posterior probabilities represent the relative chance that alleged father is the biologic father (W1) and that random man is the biologic father of the child (W2). The sum of the posterior probabilities must equal 1.0 (100%), just as the sum of the prior probabilities (P1 and P2) must equal 1.0 because the two alternative
hypotheses are mutually exclusive events (i.e., there are no other hypotheses to consider). Thus, in a simple case in which one man is accused of paternity, there are two alternative hypotheses: $H_1: AF = BF$ and $H_2: RM = BF$. The following probability statements apply (where $o$ = phenotype):

$$P_1 = P(AF = BF) \text{ and } P_2 = P(RM = BF), P_1 + P_2 = 1.0$$

$$C_1 = P(o \text{ of child given maternal } o \text{, if } AF = BF)$$

$$C_2 = P(o \text{ of child given maternal } o \text{, if } RM = BF)$$

$$C_1 + C_2 = \text{ Paternity index (PI) = likelihood ratio (independent of } P_1 \text{ and } P_2)$$

Multiplying the independent probabilities $P$ and $C$ produces joint probabilities:

$$P_1 \times C_1 = J_1 \text{ and } P_2 \times C_2 = J_2$$

$$J_1/(J_1 + J_2) = W_1 \text{ and } J_2/(J_1 + J_2) = W_2 \text{. } W_1 + W_2 = 1.0$$

$W_2$, the relative probability that $AF \neq BF$ and that $RM = BF$, is usually not reported. $W_1$ (or simply $W$), a posterior probability of paternity, is sometimes termed the *plausibility of paternity* and is often expressed as a percentage instead of a decimal fraction: $W(\%) = 100 \times J_1/(J_1 + J_2).$  

The meanings of the calculations for determining paternity inclusion have been debated.  

For example, there has been argument about the arbitrariness of prior probabilities that seem ethically fair but are not based on any real nongenetic evidence (calculations or frequency data). Some workers have advocated that the court (judge or jurors) assign their own (subjective) value to the prior probabilities, based on the nongenetic evidence presented. A receiver-operator characteristic (ROC) curve can be presented that graphically depicts posterior probabilities as functions of the assigned value of the prior probabilities. In view of some state laws that now mandate an inference of paternity based on calculated posterior $P$, the use of arbitrary and generic (not case specific) prior $P$'s remains disturbing.

Aside from the controversy about prior $P$, there is a growing consensus that both $PI$ and $W$ descriptively convey the relative paternity likelihood. There is also empirical evidence that calculated $PI$ values are valid. Some point out that the percentage descriptor ($W$) is valuable because it information appears to be understood better by nonscientists (judges and juries), especially in paternity cases involving more than one accused man. Typically, in the United States, both $PI$ and $W$ values are determined and reported. The prior $P$'s, assigned to alternative hypotheses and used to calculate $W$, are usually reported too.

**VARIATIONS IN NUCLEOTIDE SEQUENCES: DNA RESTRICTION FRAGMENT LENGTH POLYMORPHISMS**

The nucleotide sequences of DNA can be "cut" using a purified endonuclease, an enzyme that recognizes specific sequences of 4 to 10 nucleotide bases and cleaves bonds within the chain of nucleotides at specific restriction sites, to yield many restriction fragments of various lengths. Genetic variations in the sequences of nucleotide units in DNA include, but are not limited to, deletions or restriction sites of endonucleases. The restriction fragments of DNA can be separated by gel electrophoresis, denatured into single-stranded DNA, and blotted from gel onto solid phase (Southern blotting). Electrophoresis of DNA fragments separates them primarily on the basis of size rather than charge (in contrast to protein electrophoresis). Specific fragments of DNA can be detected by labeled sequences of single-stranded DNA (probes) that are complementary to, and bound by, target sequences of nucleotides within one or more fragments. Some probes bind (hybridize) to a specific
sequence within only one fragment derived from a single chromosomal locus. (Hybridization is hydrogen bond-driven interaction of complementary strands.) Probe-hybridized fragments, differentiated by length, are the equivalent of alleles, which are identified as label-signaled visible bands that migrate specific distances on electrophoresis. (The labels that permit visualization may be radiisotopes, fluorophores, enzymes, and so on.) Migration distance of DNA fragments on gel electrophoresis is inversely related to the length of the DNA fragment (usually stated in thousands of nucleotide bases or kilobases).

Diallelic loci of unexpressed DNA have been termed restriction fragment length polymorphisms, or RFLPs. (Only infrequently are more than two alleles detected at an RFLP locus.) The two alleles at a given locus are defined by presence or absence of an endonuclease recognition site. The RFLPs (which are plentiful in the genome) became candidate parentage tests and, when combined, showed promise of achieving CPES equivalent to HLA as early as 1985 (Fig. 4A). The number of RFLPs required to achieve utility in parentage studies, however, was a limitation because of the work and expense involved; that is, individual RFLP loci are not very informative but can be empowered by using a number of enzymes and probes in combination.

VARIATIONS IN NUCLEOTIDE SEQUENCES: VARIABLE NUMBER OF TANDEM REPEATS

The first highly polymorphic DNA locus was described in 1980. At about the same time, another highly polymorphic DNA locus was identified adjacent to the human insulin gene. The restriction fragments detected by Southern blotting at these loci were inherited in a mendelian fashion, and displayed high heterozygosity and a wide range of different lengths in unrelated individuals. Analysis of these two DNA regions revealed that each allele was composed of a tandemly repeated sequence of variable copy number (variable number of tandem repeats, or VNTRs); that is, the alleles of this hypervariable region of DNA were produced by insertions or deletions of multiples of a "core" (consensus) sequence between unaltered restriction enzyme recognition sites. The repeated sequence of the two different loci described by Wyman and Bell were not related. Although cross-hybridization to other unlinked loci had been detected at low stringency in some experiments, the observations were initially dismissed because no simple explanation was apparent. Subsequently, other highly polymorphic loci were identified at the Harvey-ras oncogene, the zeta-globin pseudogene, and elsewhere.

In 1985, a "family" of tandemly repeated sequences was detected at multiple loci in humans. The term minisatellite sequences (MSS) was used to characterize these types of repeats to relate them to satellite sequences described in genomic structural organization. (Satellite DNA refers to DNA of buoyant density different from the main fraction of DNA because it has different proportions of purine and pyrimidine bases. MSS repeats possess a lower copy number than the highly repetitive satellite sequences.) Members of MSS families each contain specific derivations of a consensus repeat sequence that can be used at low stringency to detect most family members, or at high stringency to detect an individual locus. Many MSS families have now been identified after screening genomic libraries with previously cloned VNTRs.

Probes complementary to a portion of a repeat sequence stringently hybridize to single target loci. If an allele contains many repeats, its length inhibits migration on an electrophoretic gel, and many copies of probe will hybridize to it. If an allele contains few repeats, it migrates further and binds fewer probe molecules. Single-
locus probes detect two target alleles in heterozygotes and one allele in homozygous individuals. Each allele appears as a single band on a Southern blot (see Fig. 4B).

Actually, the core sequence itself shows variability caused by substitutions, insertions, and deletions. Core sequence variants form longer sequences that are also repeated tandemly so that each minisatellite DNA locus can be a hierarchy of short, intermediate, and long repeat sequences. Minisatellites are generally distributed
telomerically (and centromerically) on hundreds of sites on many different chromosomes. They are sites of greater recombination frequency and possible lateral (saltatory) amplification of sequences. These activities produce many allelic mutations but contribute to a potentially problematical mutation rate. Similar sequences possess different mutation rates at different locations; location appears to determine mutation rate in some way to produce genomic recombination “hotspots.” (See the section on technical issues.)

Probes that hybridize to consensus sequences at many loci are termed multilocus probes. On Southern blots, many bands are evident and present a complex, barcode-like appearance that is highly informative if not unique to an individual. These patterns constitute human “DNA fingerprints.” They have been used as forensic identifiers in numerous criminal investigations and in parentage analysis. DNA may be retrieved from semen, bloodstains, exfoliated somatic cells of various body sites, and hair follicles. Multilocus minisatellite probes can be used successfully in parentage analysis, but DNA fingerprint systems require skill in pattern interpretation and do not lend themselves to the traditional logic and mathematics used in parentage analysis. Currently, most laboratories in the United States utilize single-locus probes to identify allelic restriction fragments on Southern analyses. Empirically, however, multilocus probes show clear separation of the proportions of bands shared by related and unrelated individuals and continue to show promise in parentage analysis.

OTHER TECHNIQUES USING DNA

Amplification of DNA sequences by the polymerase chain reaction (PCR) permits detection of extremely small quantities of DNA. The advantage of PCR in parentage studies is that blood specimens are unnecessary: hair bulbs, buccal cells, urine cells, and the like can provide sufficient material without use of “invasive” methods of sampling. The PCR methods are susceptible to contamination by extraordinarily small quantities of contaminant DNA of various sources. This can be controlled by meticulous technique, including physically separating, in different rooms, the DNA that has already been amplified from material to be tested. It is also possible to experience loss of larger fragments of DNA, if it has been partly degraded, preferential amplification of small fragments, and artificial bands caused by a frame-shifted enzyme or annealing error.

HLA haplotypes are of great biologic interest (in immune responsiveness, immunologic self-recognition, immune targeting, organ transplantation, disease susceptibility and association, and so forth) and are alleles of the most extensive protein polymorphism known. Thus, it is not surprising that methods for examination of the DNA of the HLA region were developed rapidly. The Class 2 major histocompatibility (HLA D-region) loci have been investigated and suggested for parentage analysis. Allele frequencies have been determined, but polymorphism is limited as compared to other investigated DNA loci. Of interest, the proprietary technology for examining HLA-DQα alleles does not involve electrophoresis or Southern blotting: rather, it involves the use of the PCR to amplify the DNA of interest. Oligonucleotide probes (allele specific oligonucleotides, or ASOs) with biotin-avidin-peroxidase labels detect six specific alleles on solid-phase dot blots. Advantages include the use of very small DNA samples, possible collection of specimens other than blood, rapid acquisition of results, and clear-cut identification of qualitatively discrete alleles.

It is possible to combine PCR with gel separation of amplified target DNA fragments. Electrophoresis can be followed by silver staining of the DNA in the gel for direct visualization (without Southern blotting), and the observed bands require
no hybridization with labeled probe. The amplified fragment length polymorphisms (AmpFLPs, or “amplips”) can be assigned to allele classes that are determined by a set of controls. The controls, which are pools of DNA from a number of individuals, serve as calibrators that can be visualized along with the unknown DNA. The calibrator AmpFLPs constitute an observable allele “ladder.” This system requires the least quantity of material, yet it is rapid (2 days) and qualitative (i.e., alleles are discrete and clearly differentiated from one another, unlike the quasi-continuous distribution of quantitatively sized alleles in the usual electrophoretic method). Because polymorphisms with many alleles present difficulties because alleles of similar size cannot be differentiated (a problem in any Southern analysis), it is important to select limited polymorphisms. One must also take care to avoid contamination with very small amounts of DNA from other sources.

Other methods using DNA are under development but have not been applied to casework. Oligonucleotide ligase amplification (OLA) is a method, like PCR, that specifically amplifies particular regions of DNA for which sequence data are available. Oligonucleotide ligase amplification uses DNA ligase to link two abutting oligonucleotides that can accurately pair to a complementary target sequence in tandem. The method is more sensitive for detecting single-base mismatches at the oligonucleotide junction than any other method currently available. A thorough knowledge of all possible allelic types is necessary to apply the procedure.

Aside from VNTRs, there are DNA polymorphisms composed of dimeric (microsatellite) short tandem repeats (STRs), Alu sequences, and trimeric and tetrameric STRs, which may be useful in parentage analysis. PCS amplification may be carried out on a number of loci simultaneously (multiplex). Use of fluorescent labeled probes and fluorescence detectors may allow automation in the future.

Another method, made easier by PCR, is the direct sequencing of DNA. This method, currently used in molecular archeology to determine hierarchical evolutionary relationships among species, involves comparison of base sequence at highly variable regions of DNA. This method has the potential to produce the most information possible about the relatedness of two samples. A major constraint is the difficulty in interpreting the sheer mass of information. A region of DNA that undergoes enough mutation to be variable could create problematical sequence variation in family studies. Extended measurements of mutation rate for any sequenced region, along with a study to show that mutation is consistent in most species members, will require a concerted effort and much hard data. No studies of this type are currently underway. Direct sequencing has been suggested as a technical method for differentiating two qualitatively different fragments of identical length.

Finally, a recent PCR-based method takes advantage of minor differences in core nucleotide sequences of VNTR alleles (i.e., two alleles of even identical length may be differentiated). First, amplification of minisatellite variant repeats (MVR ± PCR) is carried out using a “tagged” primer to a repeat sequence and a second primer to DNA flanking the minisatellite region. The tag sequence does not hybridize to target DNA but serves as an end to a novel secondary DNA strand. Each secondary strand is then amplified and Southern analysis is performed. The resultant multibanded patterns demonstrate extremely high heterozygosities and probabilities of exclusion (> 0.99) for single systems. Shortcomings include high mutation rates at some loci and interpretive difficulties related to the need to evaluate band intensity.

DNA AND PARENTAGE: TECHNICAL ISSUES

Technical and ethical problems of DNA analysis for testing parentage have already been identified. The usual restriction fragment length alleles of DNA
polymorphisms are identified in a way that is different from the qualitative observations used with polymorphisms of expressed red cell and HLA systems, or DNA systems that identify specific alleles with oligonucleotide probes of complementary sequences. Seemingly, the expressed alleles of the earlier systems could be *qualitatively* and discretely characterized, but on Southern blots, a DNA allele is characterized only by (1) its fragment length and (2) the presence of target sequences that bind a probe. Allele identification by *size* is *quantitative*, and the measurement is imprecise. (Protein polymorphisms should be subject to this criticism, but the errors of misclassification are less frequent. Indeed, in practice, measurement error of migration distance of protein variants is a problem only infrequently.) A DNA restriction fragment phenotype can represent several genotypes because (1) variable lengths of sequences of DNA flanking the VNTR sequence contribute to the fragment length so that two restriction fragments of identical size may have compensatory differences in VNTRs and in flanks;19,26 (2) small deletions, insertions, and substitutions within the VNTR may qualitatively alter the restriction fragment without changing its length;19 and (3) restriction fragments of slightly different lengths may appear to be of identical size at the limits of resolution.

The uncertainty of potential analytic error, however, is not a reason to reject DNA tests. As has been stated throughout this article, there has always been error associated with parentage tests: false indirect red cell exclusions, misclassification of protein alleles, mutations, epistasis, and so on. Uncertainty can be managed probabilistically. For example, a number of mathematical treatments to correct for errors in size measurement have been proposed.7,15,34 To some extent, one can also deal with uncertainty about the results of DNA tests by technical means.

Just as additional tests often resolve the problem of finding one indirect red cell exclusion of parentage, additional tests can demonstrate additional evidence of exclusion or inclusion. There are already sufficient combinations of highly heterozygous DNA probes and enzymes to deal with problems of questionable homozygosity, alleles of similar length in two individuals, and possible mutation or recombination within a test system (mutation rates are in the range of 0.001 for individual VNTRs). In fact, with the variety of tests available, our and other laboratories already require more than one exclusion, regardless of the test system, and regardless of whether the finding is indirect or direct.79 (See the section on advantages and limitations of red cell systems.)

There are also technical means of enhancing resolution in a DNA marker test. A comigration experiment can be carried out in which a mixed sample is electrophoresed. The mixture is composed of the DNA of two persons who carry an apparently size-identical restriction fragment (e.g., AF and child). If two different restriction fragments are mistaken for the same one on Southern analysis of individuals, then a two-band pattern can appear in the lane containing the mixture. This approach is based on the fact that intralane resolution is greater than interlane or intergel resolutions. If the apparently size-identical bands are truly identical, then they will merge into one "fat band," similar to the double-dose band that is characteristic of homozygous individuals. (Unfortunately, nonidentical but size-similar fragments can also produce this pattern because the variables that affect band resolution are resolving power of the gel, diffusion or interaction of alleles, diffusion of the probe label's signal over time, and so forth.) An alternative to a comigration experiment involves the spiking of internal sizing standards into each unknown sample. In this way, each electrophoretic lane is accurately calibrated, and alleles of apparently identical length can be distinguished.9,56,57

The error of mistaking two closely migrating bands as one band would be a cause of false inclusions of high probability in paternity studies. It would also produce "fat bands" that might result in an apparent excess of homozygotes in a population study. The excess would invalidate the assumption of the Hardy-Weinberg equilibrium, a necessary assumption in calculations of parentage. (See the
sections on early parentage analysis and advantages and limitations of red cell systems.) Recently, however, it has been demonstrated that there is "no consistent evidence of violation" of the equilibrium in the American caucasion, black, or hispanic populations commonly recognized by parentage testing laboratories.15,25

The alleles of VNTR loci, like HLA alleles, are numerous, and some of their individual population frequencies are relatively low.2,5 As expected, the PE of single-locus probe systems is great and the magnitude of their PIs is equivalent to the HLA-A,B,C system.5,8 Because it was initially difficult to obtain sufficient data from different populations and subpopulations, there was criticism of sampling estimates of allele frequencies used to determine PIs and paternity probability statements. The issues of population genetics that are relevant to determining probability statements have been extensively debated.17,6,5,14 Furthermore, there had been limited evidence to support the conclusion that there is independence of loci, inheritance of alleles, or inheritance of alleles within subpopulations.22 Lack of independence is obviously a problem when calculations reflect combining loci that may demonstrate linkage disequilibrium.66 Most laboratories select loci known to reside on different chromosomes (e.g., VNTR loci with different repeat sequences).

A consensus has been developed by a committee of the National Academy of Sciences and published.71a DNA is considered generally reliable in forensic work, but investigations are required to establish better allele frequency information among various population subgroups, and the field is in need of quality assurance oversight and accreditation by government and scientific organizations. Proficiency testing of laboratories should be carried out. Until allele frequencies are known, the upper boundary of empirically-determined frequencies of each locus' alleles should be identified among 100 unrelated individuals within three defined populations (black, white, hispanic). A laboratory should use the highest frequency found (in any of the studied populations) or 10%, whichever is higher. This "ceiling principle" assures a conservative and acceptable estimate of probabilities.

With time and experience, laboratories will develop allele frequency data, measurement precision, and standardization of methods.92 Several studies already indicate that population substructuring is less important than was initially believed. In Canada, studies have attempted to compare the VNTR allele frequencies found in traditionally defined subpopulations involving persons of English, French, and Eskimo ancestry. The variation found between English and French populations was below the level of statistical significance. In the Eskimo-derived populations, only those tribes that were complete isolates showed significantly different proportions, and there was as much deviation between different Eskimo tribes as there was between Eskimo and French or English groups.33 The lowest level of heterozygosity detected was 75% using a method that generally yields 90% heterozygosity in other populations. In Finland, no statistically significant variation was found between Finnish groups and mainland European groups except for isolates on remote Baltic islands.80 A study of three geographically distinct populations (New Guinea Highlanders, Canadian Dogrib Indians, and the Kachari of India) has shown that genotype distributions at six VNTR loci conform to Hardy-Weinberg expectations and that there is no apparent association of VNTR alleles at loci that are physically separated in the genome.24

ATYPICAL LABORATORY INVESTIGATIONS

Special circumstances arise in parentage analyses in which usual methods are inappropriate or insufficient. A typical parentage analysis involves a mother, her child, and a man accused of paternity. Other evaluations of filiation or pedigree are
carried out in the following situations: (1) immigrants, who as beneficiaries of federal immigration laws wish to enter the United States, claim to be first-degree relatives of US citizens (petitioners); (2) persons claim to be the heirs to an inheritance left by a decedent relative; (3) criminal investigators need to know if a pregnancy is the result of rape or incestuous union; (4) kidnapped children or foundlings need to be placed with their rightful parent or nearest of kin; (5) biologic parentage (maternal and paternal) may need to be established in cases involving an infertile couple and a gamete donor or surrogate mother; (6) changlings need to be matched to their rightful parents; and (7) several men (including brothers) are accused as possible fathers.

In some of these situations, the usual technical methods may need modification or may not be useful at all. For example, there are times when either or neither parent is available for study. Study of relatives may indicate the parental obligatory alleles. At times, the suspected father is deceased and HLA typing or DNA may be possible from tissues.

On other occasions, the child is unborn. Although chromosomal heteromorphisms, determined from cultured amniotic cells, have been used to determine paternity, this method is technically difficult, subjective, and may not meet the "general acceptance" standard (Frye rule) in court. It is possible, however, to serologically phenotype the expressed HLA-A antigens (and the weak B antigens) from cells retrieved and cultured from amniotic fluid. Antigenic expression of HLA-B may be increased, and expression of loci HLA-C, -DR and -DQ may be induced by incubation of the cells with gamma interferon. A more direct method of study involves analysis of DNA polymorphisms using amniotic cells, fetal blood, or chorionic villus samples. Maternal blood is used as a source of material to phenotype the mother. Paternal obligatory alleles are then readily identified in the fetal sample, which may be contaminated with maternal cells introduced at the time of fetal or placental sampling. In fact, it may be possible to obtain trophoblastic cells directly from the maternal blood by using solid phase-associated monoclonal antibodies directed against human syncytiotrophoblast cells. The small number of cells are a sufficient source of DNA if amplification of target DNA is carried out.

CONCLUSIONS

Parentage analysis arose as a subspecialty of bloodbanking at the beginning of the twentieth century when tests only permitted exclusion of about 1 in 10 falsely accused men and reliability of results was justifiably questioned. Currently, by using immunologic, biochemical, and molecular methods, greater than 99% of falsely accused men can easily be exonerated with virtual certainty. Unexcluded men are frequently assigned paternity indices that often exceed 100 to 1 \((W > 99\%\), given a prior \(P = 50\%\). The next decade should produce the data to resolve most of the present scientific and legal controversies. Predictably, by the twenty-first century, there will be technical resolution of almost all questions of biologic parentage.

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