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Paternity testing: a review of theoretical and practical issues

by

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Abstract

Seen the legal, psychological and social implications, errors cannot be tolerated in paternity tests. Correct identification of the individuals as well as the blood samples drawn is of the utmost importance. Present molecular biological techniques, the foremost of which is the polymerase chain reaction (PCR), do not only permit the exclusion of paternity, but also the demonstration of paternity with a probability close to absolute certainty.

Key-words

Paternity – DNA fingerprinting – Forensic anthropolgy

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Introduction

The indications to demand a paternity test are various. First, there are the discussions concerning the attribution of alimentation in a divorce case or with a natural child. In a divorce case, the mother may claim that the child should not receive the name of the legal father, with whom the mother was still officially married during the period of conception. When a man who is not married to the mother of their child wants to recognise the child as his, the judge may also require biological evidence. Additionally, an increasing number of immigration cases is examined. Here, the prospective immigrant must prove that he is the father of the child of a Belgian citizen or of another immigrant who has permission to reside in Belgium.

In principle, a paternity test should be orderded by the Court of Justice, by means of a sentence.

This paper gives a general review of parentage testing. Sample collection, technical aspects and interpretation of the results are discussed.

Sample collection

The most important aspect of the sampling procedure is the meticulous documentation of the individual's identity. One can imagine that a man who wants to prove he is not the father, might send another man in his place for blood collection, when these persons are not known personally by the physician drawing the blood.

The identity is thus documented by means of 1° a photocopy of the identity card, passport or birth certificate, 2° a photograph, 3° the signature and 4° a fingerprint with ink. On the blood collection form, the parties declare 1° to agree with the blood sampling in view of a paternity test, 2° to have verified that his/her name is correctly noted on the tube containing his/her blood and 3° that he/she has never received a bone marrow or stem cell transplantation. The latter certification is needed because a transplantation with hematopoietic cells induces a complete change of the cells in the blood circulation to the genetic profile of the donor. Moreover, if blood group antigens are applied as genetic markers, one should not have received a blood transfusion during the last three months. When only extracted DNA is examined, that condition does not

have to be met, as was proven in a study on DNA-profiles of recently transfused trauma patients (1). The blood tube itself must be punctually identified with the donor's surname, first name and date of birth. He or she then declares to have verified that the name is correctly stated on the tube.

In contrast to criminal investigations, where all kinds of biological traces are examined, a paternity test is carried out on a freshly drawn blood sample. The molecular techniques applied in our laboratory require 2 mL of EDTA anticoagulated blood. Ideally, the blood is collected in the laboratory which will perform the test and write the report. If a sample is mailed to the laboratory, the responsibility for the identification of individual and sample rests with the phlebotomist and sender of the blood. The Central Laboratory of the Blood transfusion service (CLB) of Amsterdam, the only laboratory performing paternity tests in the Netherlands, requires samples to arrive within 24 hours after they are mailed. This is not only to preserve optimal quality of the blood sample, but also to reduce the possibility of tampering with the samples.

Buccal swabbing forms a non invasive alternative for blood sampling (2). Here, cells from the mouth mucosa are collected by swabbing the inside of the cheek with a cytology brush or cotton swab. The condition for applying this technique is that only DNA-markers are used for the paternity analysis.

A conclusive result is obtained through comparison of the genetic markers found in the mother, the child and the alleged father. Without the mother's blood, a result is possible, but not with the same degree of reliability. Furthermore, the mother's permission is required for blood sampling of her child in view of a paternity test. As a principle, we never comply with the request of a man for "a DNA test" without the knowledge and the collaboration of the child's mother.

Paternity analysis

Principle

Parentage testing is based on the fact that each genetic marker of a child is inherited from one of both parents. In practice, a number of genetic markers are determined in the blood of the mother, the child and the alleged father. A man is excluded from paternity if he does not

possess at least two genetic markers present in the child but not in the mother. The opposite situation, when all genetic markers which the child did not inherit from its mother are found in the alleged father, constitutes so-called "inclusion of paternity". The probability that such a man is the true biological father is subsequently calculated (see further).

Genetic markers

Biological characteristics detectable in blood can be applied for parentage analysis on the following conditions: 1° they must show Mendelian inheritance; 2° the allelic distribution in the population must be in accordance with the Hardy-Weinberg law (3); 3° the different genetic systems are inherited independently from each other, i.e. they are localised on different chromosomes and 4° their mutation frequency should be low.

Among the used genetic markers, there are on the one hand gene products such as membrane and serum proteins and on the other hand DNA fragments. This division coincides with the chronological and technical evolution of paternity analysis.

Conventional markers, still typed for at this time, include the blood group systems ABO, Rhesus, MNS, P, Kell, Lutheran, Duffy, Kidd, Wright. To the polymorphic serum proteins belong haptoglobin, IgG and IgA heavy chains, kappa and lambda immunoglobulin light chains and the enzymes phosphoglucomutase and acid phosphatase.

A next step in the evolution of parentage testing was set by HLA typing by serology. Here, leukocyte membrane antigens are identified using specific antibodies and a complement mediated cytotoxicity assay.

Developments in molecular biology during the last 15 years have permitted direct typing of DNA. The polymorphism of the HLA system was thus found to be much more comprehensive at the DNA than at the membrane antigen level. A following step was the discovery of the polymorphic system of variable restriction sites, the Restriction Fragment Length Polymorphism (RFLP). Genomic DNA is cut into fragments by means of a restriction endonuclease. After separation of the restriction fragments by electrophoresis, they are transferred onto a membrane. Then, a labelled (radioactive, fluorescent or enzyme) DNA probe is added which binds to the complementary nucleotide sequences of the restriction fragments, thus revealing them. Because restriction enzymes

cut the DNA at specific nucleotide sequences, which vary between individuals and are inherited, fragments of variable length are created. A child inherits DNA from both parents which can be seen in the length of the restriction fragments. This implies that if a DNA fragment, present in the child, cannot be detected in the mother, it must be found in the biological father. An RFLP assay is named by the restriction enzyme (cf. HinfI) and by the hybridisation probe (cf. 33.15). A milestone in forensic DNA analysis was the discovery by Jeffreys (4) in 1985 of a "multilocus probe". This simultaneously detects alleles of several loci. Here, the term "genetic fingerprint" was coined. Since restriction enzymes produce relatively large DNA fragments, the original DNA should be of good quality. It should not be degraded by age or poor storage conditions into short, aspecific fragments.

In 1988 Mullis (5) developed the polymerase chain reaction (PCR), an enzymatic nucleic acid amplification method for which its inventor 5 years later received the Nobel Prize for Chemistry. This technique has found countless biomedical applications, like in forensic research and paternity testing. The human genome contains repeats of definite sequences of 2 to 5 nucleotides. The coding function of these Short Tandem Repeats (STRs) (6) is unclear; they are sometimes called "junk DNA". The number of repeats displays an inherited polymorphism where each allele determines the number of repeats and thus the total length of the DNA fragment. These DNA fragments can be amplified by PCR and then separated according to length (figure 1). Visualisation of the band pattern is realised by fluorescent labelling and automated reading or by silver staining and "manual" inspection. The band pattern is interpreted as in RFLP; each non maternally inherited band of the child must be present in the biological father. The amplification products of alleged father, child and mother are flanked by allelic ladders. The latter consist of a mixture of most alleles of the STR marker in question and permit denomination of the alleles of the paternity "trio".

Forensic science and paternity analysis require only a limited number of the over 2000 markers that have been developed for genetic mapping and the "Human Genome Project" (7). Selected STRs should fulfil the following conditions in order to be used for human identification. First, the STR locus should be highly polymorphic, i.e. display a large number of alleles. Secondly, PCR products should be clearly discernible by electrophoresis. One should avoid markers with frequent microvariants, i.e. alleles differing less than one full repeat in length. Amplification artefacts one repeat above or below the true allele, called stutter bands, have led to the discontinuation of the use of dinucleotide STRs. While in criminal

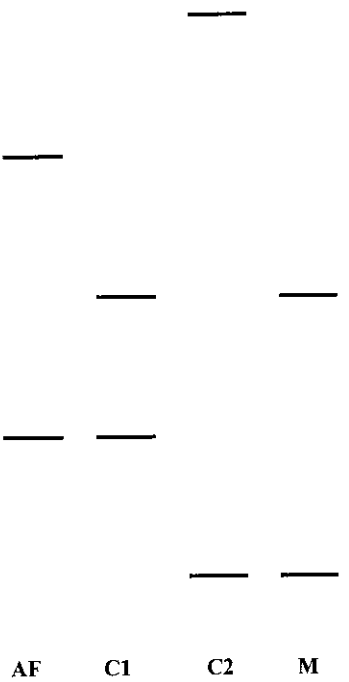

Fig. 1: Electrophoretic pattern of a family with two children. AF is the biological father of child C1 but not of C2. M is mother of both children.

investigations it is essential to be able to detect minute (1 ng) amounts of DNA, in paternity analysis it is imperative that the mutation frequency of the genetic marker is low in order to prevent the false exclusion of the biological father.

By accurate selection of STR-loci according to the length of their alleles, it is possible to simultaneously amplify several STRs and to visualise them in the same lane of one gel. Thus, a STR multiplex system is created, increasing efficiency and laboratory throughput.

PCR offers advantages over conventional but also over RFLP typing methods. In PCR, a thermostable DNA polymerase generates millions of copies of a DNA sequence, requiring only a minute quantity of DNA as starting material. We indeed perform PCR of twelve STR loci with the DNA extracted from 2 mL of blood. The CLB on the other hand requires 25 mL of blood for their standard set of conventional markers and DNA RFLP. Since with PCR of STRs very short DNA fragments are amplified, there is no interference due to DNA degradation during storage. This advantage proves its importance during investigation of biological traces of crimes. Identification of allelic bands by the number of repeats of a

STR is better standardised than with RFLP, where the bands are named by their migration distance in cm during electrophoresis. The latter way of identification hinders interlaboratory comparison of results.

As a first approach, we amplify a set of nine STRs in three triplex PCRs. The used loci are called D18S849, D3S1744, D12S1090, CSF1PO, THO1, PLA2A1, F13A01, CYAR04 and LIPOL (8,9). When paternity is excluded with at least two of the nine loci, no further assays are performed. Otherwise, analysis is pursued with a fourth triplex PCR of the STR loci HPRTB, FABP and CD4 (10). Typing of these twelve loci should permit differentiation between inclusion of paternity with a mutation in one of the nine "first line" loci and exclusion with accidentally only one excluding marker of the first series. In a case of inclusion based on these twelve loci, a probability of paternity greater than 99.9% is always obtained (see further).

Calculations and statistical aspects

When paternity is excluded, calculations are not necessary. With at least two excluding loci, the conclusion is fixed. The chance of a genetic system to show that a random man is excluded from paternity, is called the "Power of exclusion" of that system (11). It depends on the polymorphism, i.e. the number of alleles of the system and the distribution of these alleles in the population. The ABO blood group system has a power of exclusion of 16%. This implies that the chance that a random man can be excluded from paternity when only the ABO type of child, mother and this random man are determined, amounts to only 16%! The Rhesus and MNS blood group systems have a power of exclusion of respectively 25 and 35%. These three conventional markers together have a combined power of exclusion = 1 – [(1-0.16) x (1-0.25) x (1-0.35)] = 0.59 or 59%. With our standard combination of nine "first line" STRs, a power of exclusion of 99.98% is reached.

When paternity cannot be excluded, several indices are calculated to measure the probability of paternity. The paternity index I is defined as the ratio of the probability of a gamete of the non excluded man possessing all obligatory paternal alleles versus the frequency of this combination of alleles in a random man of the same racial background. Although exact and objective, the paternity index proves difficult to grasp for outsiders. To meet this objection, it has become customary to derive from the I-value the W-value (from the German *Warscheinlichkeit*).

Therefore a new numerical value p is introduced, the a priori probability of paternity. If the a priori probability of paternity equals p, then the chance of non paternity is (1-p), and W = (p x I) / [(p x I) + (1-p)]. Since in reality it is impossible to make a fair estimate of p, a value of 0.50 is arbitrarily chosen for p. Consequently, one derives that W = I / (I+1).

To calculate the above mentioned indices, the allelic frequencies of the population to which the father belongs, are required. For the local population we developed an database of genotypes and determined allelic frequencies (12,13). For foreign populations, published frequencies are used.

Standards / quality control

To demonstrate the reliability of its results, a laboratory should proceed according to international standards. For paternity testing based on HLA typing, the standards of the European Federation for Immunogenetics (EFI) can be followed. More comprehensive, and including all categories of genetic markers, are the Standards for Parentage Testing of the American Association of Blood Banks (AABB). Participation to an external quality control scheme forms a crucial chapter of a quality system. Together with 30 other European laboratories, we participate to the proficiency testing of the English Speaking Working Group of the International Society of Forensic Haemogenetics (ISFH).

Problem cases

Since mutations occur, paternity shall never be excluded based on only one locus. Preferably, genetic systems with low mutation frequency are used. Through merging of data from all laboratories from the English Speaking Working Group of the ISFH (14), mutation frequencies based on significant numbers of meioses are obtained. For most STRs applied in paternity testing, mutation frequencies vary between 0.1 and 0.5%. Among the 602 paternity cases we have examined with STRs, we observed 2 mutations (14,15). The other type of pitfall is encountered when the alleged and the biological father are close relatives (cf. brothers), especially when they have numerous alleles in common. As many genetic markers as possible should then be typed, to be able to exclude paternity.

Conclusion

New molecular techniques have permitted the direct study of the polymorphism of human DNA. One application of this fundamental research is found in paternity testing. While during the first half of the century a blood test could only exclude paternity, it can now be demonstrated with a statistically high degree of certainty. It must be stressed that there is no sense in performing the most sophisticated biological assays if the blood tubes are not correctly identified.

Samenvatting

Bij vaderschapsonderzoek worden, gelet op de juridische, psychologische en sociale implicaties, vergissingen niet geduld. Nergens is de juiste identificatie van de bemonsterde personen en van het afgenomen bloed van groter belang. De huidige moleculair-biologische technieken, waaronder op de eerste plaats de polymerase kettingreactie (PCR), laten niet alleen toe vaderschap uit te sluiten, maar ook met een aan zekerheid grenzende waarschijnlijkheid aan te tonen.

Résumé

Vu les conséquences juridiques, psychologiques et sociales, aucune erreur ne peut être tolérée dans un examen de paternité. L'identification correcte des individus autant que des échantillons, tient une importance impérative. Les techniques actuelles de biologie moléculaire, parmi lesquelles en premier lieu la réaction de polymerase en chaîne (PCR), permettent non seulement d'exclure la paternité, mais aussi de la démontrer avec une probabilité près de la certitude absolue.

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