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Simultaneous Phenotyping of Genetic Markers for Paternity Testing

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Summary. Time- and cost-saving methods for paternity testing are described. Seventeen genetic systems were divided into six groups: (1) transferrin (Tf), factor B (Bf), and phosphoglucomutase 1 (PGM1); (2) group-specific component (Gc) or α1-antitrypsin (PI) and α2HS-glycoprotein (HSGA); (3) complement components C6 and C7, factor 13B (F13B), and plasminogen (PLG); (4) haptoglobin (Hp), C8 α-γ chain (C81), and factor I (IF); (5) red cell acid phosphatase (ACP), esterase D (ESD), and glutamic-pyruvic transaminase (GPT); and (6) 6-phosphogluconate dehydrogenase (PGD) and glyoxalase I (GLO). Each group of systems was typed simultaneously by electrophoresis or isoelectric focusing (IEF) followed by staining or immunoblotting. These methods are very practical because they afford a considerable saving of time, work and expense, and facilitate semipermanent preservation of electrophoretic patterns.

Key words: Simultaneous phenotyping, genetic markers – Isoelectric focusing, electrophoresis – Immunoblotting, paternity testing

Zusammenfassung. Es werden zeit- und kostensparende Methoden für die Vaterschaftsbegutachtung beschrieben. Siebzehn genetische Systeme werden in sechs Gruppen unterteilt. 1. Gruppe: Transferrin (Tf), Faktor B (BF) und Phosphoglucomutase 1 (PGM1); 2. Gruppe: Gc-System (Gc) oder α1-Antitrypsin (PI) und α-2HS-Glycoprotein (HSGA); 3. Gruppe: Komplement-Komponenten C6 und C7, Faktor 13B (F13B) und Plasminogen (PLG); 4. Gruppe: Haptoglobin (Hp), C8 α-γ Kette (C81) und Faktor I (IF); 5. Gruppe: saure Erythrozyten-Phosphatase (ACP), Esterase D (ESD), Glutamat-Pyruvat-Transaminase (GPT); 6. Gruppe: 6-Phosphogluconat Dehydrogenase (PGD) und Glyoxalase I (GLO). Jede Gruppe wurde gleichzeitig mittels Elektrophorese oder isoelektrische Fokussierung (IEF) mit Färbung

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oder Immunoblotting untersucht. Diese Methoden erwiesen sich in der Praxis als zeit- und kostensparend und erleichtern die vorübergehende Aufbewahrung und Dokumentation der Elektrophorese-Bilder.

Schlüsselwörter: Phänotypisierung genetischer Marker – Vaterschaftsbegutachtung, kostensparende Methoden und Dokumentation

Introduction

Recent advances in hematogenetics have made it necessary to use a large number of genetic marker systems in paternity testing, which in turn has become more and more time-consuming, laborious, and expensive over the years. Thus, there has been an increasing need for time-, labor-, and preferably cost-saving methods for paternity testing. This is reflected in the current upward trend in the number of papers on the simultaneous typing of genetic markers. Burdett and Whitehead (1977) and Dorrill and Sutton (1983) described simultaneous IEF of ACP and PGM1; Randall et al. (1980) that of ACP and adenosine deaminase (ADA); Adamo et al. (1984) simultaneous electrophoresis of ESD and PGM1; Hoste and Suys (1984) that of C3 and Tf and of Gc and BF; Finney et al. (1985) simultaneous IEF of ACP and ESD; Tamaki et al. (1985) that of C6 and C7; Berghaus and Staak (1986) that of Gc and Tf; Komatsu et al. (1987) that of ACP, ADA, and PGM1; Murch et al. (1986), simultaneous electrophoresis of ADA, adenylate kinase, and carbonic anhydrase II; and Wraxall et al. (1986) that of GLO, ESD, and PGM1.

Since the time needed for red cell antigen and HLA typing cannot be shortened, the present study focuses on the portion of paternity testing that involves electrophoretic separation of genetic markers. Combining previous methods and our own, we have devised a paternity testing protocol that saves much time, work, and expense as well as facilitates semipermanent recording of electrophoretic patterns.

Materials and Methods

Sample Collection and Pretreatment

Paternity casework material was used. Red cell suspensions, hemolysates, and sera were prepared by the routine procedure and subjected to genetic typing on the day of collection or stored at −80°C overnight. Sera were pretreated, where necessary, with *C. perfringens* neuraminidase (CPN), dithiothreitol (DTT), or ferric chloride ($FeCl_3$) as follows.

Five units of CPN was dissolved in 100 µl of a 0.5 *M* potassium phosphate solution (pH 7.0). Forty-five microliters of serum was treated with 5 µl of the CPN solution overnight at room temperature (RT).

Ten microliters of a 0.05 *M* DTT solution in distilled water was mixed with 40 µl of serum, and the mixture was incubated at RT for 15 min. To this mixture was added 25 µl of a 0.03 *M* iodoacetamide solution, and the resulting mixture was incubated at 4°C for 1 h.

Twenty-five microliters each of serum and a 0.01 *M* $FeCl_3$ solution in 5 m*M* hydrochloric acid were mixed, and the mixture was kept at 0°C for 15 min and diluted with 100 µl of 0.15 *M* saline.

For ACP, ESD, GPT, GLO, and PGD typing, hemolysates were treated with DTT. Forty-five microliters of hemolysate and 15 µl of a 0.1 *M* DTT solution were mixed, and the mixture was incubated at RT for 15 min.

Reagents and Equipment

Anti-Gc, anti-Hp, and anti-PLG sera were obtained from DAKO-immunoglobulins; antisera to HSGA, BF, IF, and F13B, from Behringwerke; anti-Tf, anti-C6, anti-C7, anti-C8, and horseradish peroxidase-labeled anti-rabbit (or goat) IgG, from Cappel Laboratories. The other chemicals used were of analytical grade.

Two units of Flat Bed Electrophoresis Apparatus FBE-3000 (Pharmacia), a Power Supply 2103 (LKB), and a Power Supply PS-1515 (ADVANTEC, Tokyo, Japan) were used.

Polyacrylamide Gel IEF (PAGIF)

Polyacrylamide gels (5% *T*, 3% *C*, 140 × 0.5 mm) containing 2.5% carrier amphyolytes were prepared by chemical polymerization. The distance between the electrode wicks was 9 cm except in ACP/ESD/GPT typing (12 cm). Unless stated otherwise, the gel was prefocused at 15 mA for 30 min, samples were applied with 3 × 5 mm paper applicators (Whatman no. 3), and focusing was performed at 4–10°C for 2–3 h depending on the Vmax used and the content of urea in the gel.

Simultaneous PAGIF of Tf, BF, and PGM1

A PAG of pH 4–6.5 (Ampholine) was used. Undiluted serum samples (for BF) and hemolysates (for PGM1) were applied at the anodal side, and $FeCl_3$-treated samples (for Tf) were applied at the cathodal side. The electrode solutions were 1 *M* phosphoric acid and 1 *M* sodium hydroxide. Focusing was performed at 1500 Vmax and 9 Wmax. The portion of the gel carrying the Tf lanes were cut off and stained with Coomassie Brilliant Blue R-250 (CBB). Alternatively, Tf as well as BF was detected by immunoblotting as described below. PGM was visualized with a cellulose-acetate membrane overlay moistened with a reaction mixture similar to that described by Spencer et al. (1964) except for its content of 3 m*M* Meldola Blue instead of phenazine methosulfate. The membrane was filed for semipermanent record.

Simultaneous PAGIF of Gc and HSGA

A pH gradient of 3.5–5.4 was generated by 1:3 mixture of Ampholine 3.5–5 and Pharmalyte 4.5–5.4. The electrode solutions were 1 *M* phosphoric acid and 0.2 *M* sodium hydroxide. Sera were applied at the cathodal side, and electrophoresed for 2 h at 2000 Vmax and 12 Wmax. The focused proteins were detected by immunoblotting.

Simultaneous PAGIF of PI and HSGA

PI was typed, singly or concurrently with HSGA, by a slight modification of the separator IEF procedure of Frants and Eriksson (1978) on a PAG containing a 2:1 mixture of Servalyt 4–5 and Servalyt 4–4.5. The anolyte and the catholyte were 0.5% phosphoric acid and 0.5% ethylenediamine. After prefocusing for 2 h, DTT-treated (for PI) and undiluted serum (for HSGA) samples were applied at the cathodal side, and the gel was run for 3 h at 1000 Vmax and 6 Wmax. PI was stained with CBB, and HSGA was detected by immunoblotting.

Simultaneous PAGIF of ACP, ESD, and GPT

The simultaneous PAGIF of ACP and ESD of Finney et al. (1985) was used with slight modification. Prefocusing was omitted. The PAG contained a 1:1 mixture of Ampholine 4–6 and Ampholine 6–8 but no chemical spacer MOPS. ACP and ESD samples shared applicators (2 × 3 mm) which were placed in the middle of the gel, while GPT applicators (3 × 5 mm) were

placed at the cathodal side. The ACP/ESD applicators were removed 15 min later, and the GPT applicators after a further 30 min. The running conditions were 1300 Vmax, 2.8 Wmax, and unlimited mA for a total of 115 min. ACP and ESD were detected according to Finney et al. (1985). GPT was visualized as described by Kishi et al. (1985).

Simultaneous PAGIF of Hp, C81, and IF

A PAG containing 2.4 g urea and a 1:1 mixture of Ampholine 3.5–9.5 and Ampholine 6–8 (or 5–8) was used. The electrode solutions were 0.5 *M* phosphoric acid and 0.5 *M* sodium hydroxide. CPN-DTT-treated sera (for Hp) on 3 × 3 mm applicators were placed at the cathodal side. Undiluted sera (for C81) on 3 × 10 mm applicators and CPN-treated sera (for IF) on 3 × 3 mm applicators were placed at the anodal side. Hemoglobin was focused together as a pI marker. After focusing for 3 h at 1500 Vmax and 9 Wmax, a 4 cm-wide Hp zone and a 3 cm-wide C81 zone, both anodal to the hemoglobin, and a cathodal 3 cm-wide IF zone were overlaid with nitrocellulose membranes (NCM) for immunoblotting.

Simultaneous Agarose Gel IEF (AGIF) of C6, C7, F13B, and PLG

A 245 × 125 × 1 mm agarose gel (pH 5–8) was made according to the instruction book of Pharmacia Biotechnology. CPN-treated C7 samples and untreated C6 samples, both diluted twice with distilled water, were applied with Whatman No. 1 filter paper (2.5 × 3 mm) at the anodal side. CPN-treated F13B samples were placed with 3 × 5 mm applicators at the cathodal side. If PLG typing is to be included, CPN-treated sera may be applied at the anodal side. The electrode solutions were 0.05 *M* sulfuric acid and 1 *M* sodium hydroxide. The running conditions were 300 V for 15 min, 400 V for 15 min, 600 V for 10 min, 850 V for 5 min, and 1250 V for 75 min. The paper applicators were removed after 1 h of focusing. The focused proteins were detected by immunoblotting.

Simultaneous Agarose-Starch Gel Electrophoresis of GLO and PGD

A 1% agarose-0.5% starch gel (150 × 110 × 1 mm) was used. The tank buffer consisted of 0.1 *M* Tris, 0.1 *M* maleic acid, 0.0087 *M* EDTA, and 0.01 magnesium chloride (pH 7.4). The gel buffer was a 1:14 dilution of the tank buffer. Paper applicators (1 × 5 mm) wetted with DTT-treated hemolysates were stood on edge on the gel surface 3 cm from the cathodal edge of the gel and removed after 10 min. The gel was run at 4°C at 350 V (35–40 mA) for 45 min. A 2 cm-wide zone immeditely anodal to the points of origin was overlaid with a strip of cellulose acetate membrane moistened with the usual PGD substrate mixture. The rest of the anodal area was stained for GLO according to the method of Paar et al. (1977).

Immunoblotting Procedure

Focused serum proteins were transferred to NCM by diffusion. The blotting buffer was 0.02 *M* Tris-buffered saline (pH 7.5). The protein blots were detected as described previously (Tamaki et al. 1985).

Results and Discussion

Devised after a number of trials, the methods described here have rendered satisfactory results in the paternity studies conducted in our laboratory for the past year. Figure 1 shows some of the phenotypes revealed by simultaneous IEF or electrophoresis. Since the conditions of PAGIF for typing of PI, Tf, or PGM1 in isolation and in combination with other genetic systems are essentially the same, their IEF patterns are not shown. AGIF patterns of C6 (Nishimukai

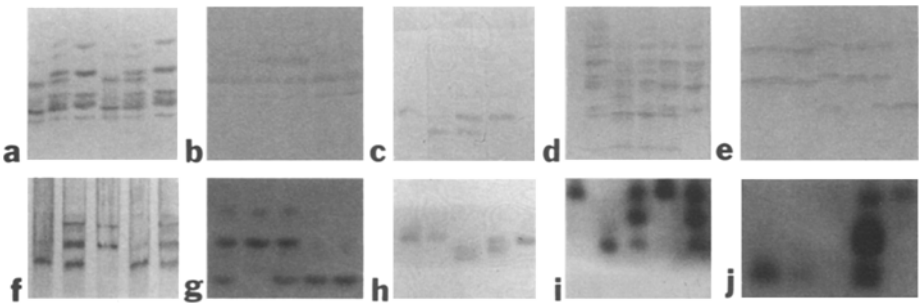

Fig. 1a–j. Phenotype patterns revealed by simultaneous IEF or electrophoresis. Anode *at the top*. From *left* to *right:* **a** Hp 2, 2–1, 1, 2, 2–1, 1; **b** IF B, B, AB, AB, B, B; **c** C81 A, B, AB, A; **d** HSGA 2–1, 5–2, 2, 2–1, 1; **e** Gc 1F, 1F–1S, 1S, 2–1F, 1F–1S, 2–1S, 2; **f** BF S, FS, F, S, FS; **g** F13B 3–1, 1, 3–1, 3, 3; **h** PGD A, A, C, CA, A; **i** GLO 2, 1, 2–1, 2, 2–1; **j** GPT 1, 1 (*GPT*1/GPT*QO*), 2 (*GPT*2/GPT*QO*), 2–1, 2 (This family with a GPT silent allele will be described elsewhere)

et al. 1985), C7 (Nishimukai and Tamaki 1986a), IF (Nishimukai and Tamaki 1986b), and PLG (Nishimukai et al. 1986) were described elsewhere. For phenotype patterns of ACP and ESD, reference should be made to the paper of Finney et al. (1985).

Combination methods for genetic typing pose no problem unless they involve changes in the usual conditions of isolated typing. If they do, however, care should be taken not to sacrifice resolution. Important considerations include selection of the genetic systems to be combined, pH range of carrier ampholytes, interelectrode distance, type of sample pretreatment (the resulting pI shift taken into account), and running conditions. A prime consideration is the choice of a pH range of carrier ampholytes that covers the pI's of the genetic systems combined.

Excessive widening of the pH range of ampholytes may bring the bands of a protein too close to each other to distinguish one phenotype from another, although this effect can be partially counteracted by generation of a nonlinear pH gradient and/or adoption of a longer interelectrode distance.

The selection of genetic systems for paternity testing should depend upon the ethnic group to which the persons concerned belong. The number of systems to be included in a typing program should be as large as possible to give adequate information but, at the same time, as small as possible from the practical point of time, cost, and technical skill. Otherwise, only a limited number of forensic institutions could carry out the typing program. Taking into consideration the possible implication of a silent allele in a case of false exclusion by one system only, we believe that non-fathers should be excluded from paternity by more than one system, and that the probability of paternity of non-excluded putative fathers should exceed 0.99, preferably 0.9975. Experience tells us that when the 27 systems listed in Table 1 are tested, 91% of non-excluded men show values over 0.99, and that mostly over 0.9975. For this reason, we are using these 27 systems, but we do not intend to argue that HLA typing is not necessary. Indeed it is the most informative system for paternity testing. However, on the one hand its inclusion considerably raises the cost of the testing,

Table 1. Twenty-seven systems used in a routine typing program with reference to the chance of paternity exclusion of the single system (P_0), the combined chance of exclusion (P), and the typing method

System	P_0	P	Method
Tf	0.162	0.162	
BF	0.124	0.266	PAGIF
PGM1	0.264	0.460	
Gc	0.382	0.666	PAGIF
HSGA	0.160	0.720	
PI	0.218	0.781	PAGIF
C6	0.174	0.819	
C7	0.077	0.833	AGIF
F13B	0.173	0.862	
PLG	0.038	0.867	
C81	0.193	0.893	
IF	0.086	0.902	PAGIF
Hp	0.160	0.918	
ACP	0.138	0.929	
ESD	0.076	0.934	PAGIF
GPT	0.180	0.946	
PGD	0.073	0.950	SAGE
GLO	0.054	0.953	
Gm	0.407	0.972	HAI
Km	0.166	0.977	
ABO	0.192	0.981	HA
MNSs	0.235	0.986	HA
Rh	0.237	0.989	HA
P	0.078	0.990	HA
Jk	0.180	0.992	HA
Fy	0.084	0.992	HA
Di	0.044	0.993	HA

HAI = Hemagglutanation inhibition
HA = Hemagglutination

and on the other, HLA haplotypes have not yet been studied extensively enough in the Japanese. Therefore, it is our policy not to include the HLA system until the typing of the 27 systems has turned out to be unsatisfactory.

According to our routine typing program (Fig. 2), the paternity testing can be completed within 2 days of blood collection, if two examiners and two electrophoresis units are available. Of course, the typing schedule can be tailored to the needs and work capacity of the individual laboratory.

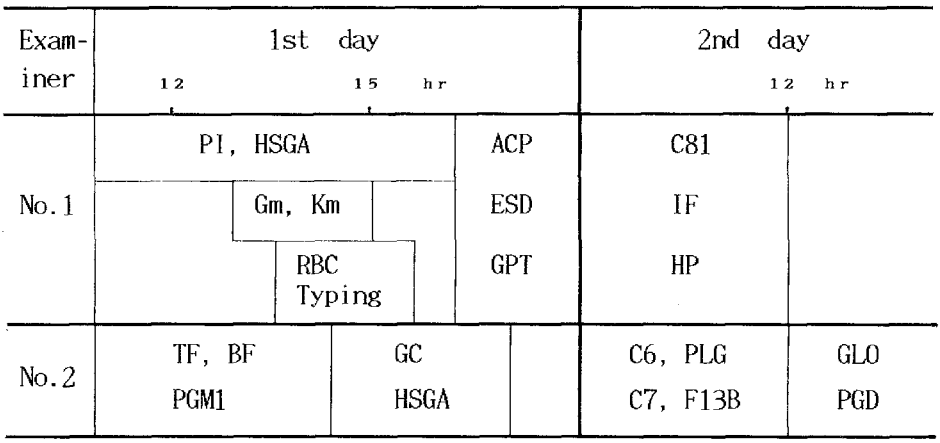

Fig. 2. A routine typing program showing work assignments to two examiners

The orosomucoid system was not included in the program despite its apparently high average probability of paternity exclusion because the OR alleles have not been well defined in the Japanese population. With other ethnic groups, it can be typed in combination with Gc and HSGA on the second day after overnight treatment of the sample with CPN.

Several modifications of the paternity testing protocol are possible. The IF system can be typed simultaneously with C6, C7, F13B, and PLG, if space permits. BF may be typed concurrently with C81 and IF in the pH 5–8/3–10 range. Details of the BF typing procedure and population data will be described elsewhere. If CPN-treated sera are preferred for HSGA or Tf typing, their typing must be scheduled for the second day. The merit of typing PI, Gc, HSGA, Tf, BF, PGM1, ACP, ESD, and GPT as well as red cell antigens and IgG on the first day is that it often provides sufficient information to justify omission of some or all of the typings scheduled for day 2. From a practical point of view, therefore, it is advisable to type as many systems as possible on day 1 and terminate the testing as soon as paternity has been excluded by more than one system or the probability of paternity has exceeded 0.9975. It depends on the philosophy of the forensic scientist whether or not he proceeds with the testing.

The methods described here appreciably reduce the time and cost of paternity testing and, in addition, facilitate preservation of electrophoretic patterns. For example, PGM1, GPT, and PGD patterns on cellulose acetate membranes, not to mention serum protein blots on NCM, can be filed for semipermanent record. These advantages make the methods suitable for forensic science practice.

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