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The examination of the ITI system in disputed paternities

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Summary. 106 paternity cases with a total of 114 putative fathers were examined in the inter-α-trypsin inhibitor (ITI) system. Analysis was performed by isoelectric focusing (IEF) of untreated sera on polyacrylamide gels. From 39 paternity exlusions, determined in other genetic systems, 7 were confirmed in the ITI system. In 75 expertises the alleged man was not excluded from fatherhood; in 68 cases the probability of paternity was W > 99.73%. The practical exclusion rate in the ITI system was therefore calculated to be 10.45%. The theoretical exclusion rate was determined to be 19.3%. In one paternity case the alleged father and the child showed inverse homozygosity in the ITI system, while the man was not excluded from fatherhood in 28 additional marker systems. The calculated probability of his paternity was 99.99%. The assumption of an incomplete expression of the ITI phenotypes in infants is supported by a significant deviation between the observed and expected ITI distributions at population equilibrium.

Key words: Inter-α-trypsin inhibitor (ITI) system – Paternity testing – IEF – Immunoelectrophoresis (IEP)

Zusammenfassung. 106 Vaterschaftsgutachten mit insgesamt 114 Putativvätern wurden im Inter-α-Trypsin Inhibitor (ITI) System untersucht. Von 39 in anderen genetischen Systemen bestimmten Vaterschaftsausschlüssen wurden 7 im ITI System bestätigt. In 75 Gutachten war der Putativvater von der Vaterschaft nicht ausgeschlossen, in 68 Fällen lag die Vaterschaftswahrscheinlichkeit bei W > 99,73%. Der errechnete Wert für die praktische Ausschlußwahrscheinlichkeit im ITI System liegt bei 10,45%, der Wert der theoretischen bei 19,3%. In einem Vaterschaftsgutachten wiesen der Putativvater und das Kind entgegengesetzte Reinerbigkeit im ITI System auf, obwohl er in 28 anderen Marker Systemen von der Vaterschaft nicht ausgeschlossen war. Die errechnete Wahrscheinlichkeit seiner Vaterschaft lag bei 99,99%. Die Annahme einer unvollständigen Expression der ITI Phänotypen bei Kleinkindern wird durch eine signifikante Abweichung von der beobachteten und der erwarteten ITI Phänotypenverteilung bei Populationsgleichgewicht erhärtet.

Schlüsselwörter: Inter-α-Trypsin Inhibitor (ITI) System – Vaterschaftsbegutachtung – IEF – Immunelektrophorese (IEP)

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Introduction

We recently described a genetic polylmorphism for human inter-α-trypsin inhibitor using isoelectric focusing (Vogt and Cleve 1990). The inheritance of two common (ITI*1, ITI*2) and one rare (ITI*3) allele was confirmed by the study of 36 families. Gene frequencies of the ITI system were determined by 2 population studies: ITI*1, ITI*2 and ITI*3, were found to be 0.575, 0.417 and 0.008, respectively in Southern Germany ($n = 248$), and 0.577 and 0.423, for ITI*1 and ITI*2, respectively in Tyrol, Austria ($n = 124$). Analyses were performed by IEF in agarose gels using sera, pretreated with neuraminidase, followed by immunoblotting or immunfixation with specific ITI-antiserum.

In the present study, we examined the usefulness of the ITI system in cases of disputed paternity by a simplified and rapid IEF procedure. ITI phenotypes were classified by IEF of sera without prior enzyme digestion. IEF was carried out on polyacylamide gels with the Multiphore II system and the PhastSystem. ITI patterns were developed by immunoblotting. In addition, serum samples from children ($n = 109$) and adults ($n = 63$), involved in these cases of disputed paternity were investigated for quantitation of the ITI protein by the rocket immunoelectrophoretic technique (Laurell 1966; Wallenborg and Andersson 1978).

Materials and methods

Serum samples from 106 paternity cases (106 mothers with 109 children and 114 alleged fathers) were examined from the routine case material of Prof. Dr. Dr. F. Schwarzfischer, Institute of Anthropology and Human Genetics, University of Munich. The paternity testing comprised a total of 28 systems: 8 blood group antigens, 9 red cell enzyme markers, 11 serum protein markers.

I. Isoelectric focusing (IEF). For IEF on polyacrylamide gels (PAG) two electrophoresis systems were employed; 1.) the Multiphor II system and 2.) the PhastSystem.

1.) Polyacrylamide gels, cast on GelBond PAG films (250 × 125 × 0.5 mm), consisted of 2.8 ml acrylamide stock solution (30% T, 3% C), 0.6 ml carrier ampholytes (40% w/v) pH 3.5–9.5, 0.5 ml carrier ampholytes (40% w/v) pH 4.0–6.5, 17 μl TEMED and 12.8 ml distilled water. After degassing 29 μl ammonium persulfate (40% w/v) was added. Electrode solutions were 0.25 *M* acetic acid (anode) and 0.25 *M* NaOH (cathode). Serum samples (4 μl) were applied with an applicator strip near the anode. IEF was carried out for 4 h as follows: 30 min at 0.2 W; 10 min at 0.8 W and 2 h 50 min at 10 W. The cooling temperature was 10°C.

2. Polyacrylamide gels used in the PhastSystem were identical to these described under 1.). For an appropriate size of 45 × 50 mm, 10 gel pieces were cut from the larger slabs. The PhastSystem separation method was programmed in the memory unit in 3 steps; 1: 30 Vh at 2000 V, 2.0 mA and 3.5 W; 2: 30 Vh at 200 V, 2.0 mA and 3.5 W; 3: 410 Vh at 2000 V, 2.0 mA and 3.5 W. All steps were performed at 15°C. Two gels were run simultaneously. Sera were loaded on 2 PhastGel sample applicators, each providing for 12 samples and a loading capacity of 0.3 μl per well. They were placed inside the PhastSystem during step 1 and were raised to the gel surface between step 1 and 2, at 0 Vh.

After IEF with Multiphor II and PhastSystem, proteins were detected by immunoblotting with specific ITI-antiserum (Dakopatts, Denmark).

Western blotting was carried out as described previously (Vogt and Cleve 1990). For visualization 98 ml (25 ml) PBS, pH 7.3, 60 mg (15 mg) 4-chloro-1-naphthol in 20 ml (5 ml) ethanol and 2 ml (1 ml) 0.06% H_2O_2 were used. The values for the visualisation using the PhastSystem are shown in brackets.

II. Immunoelectrophoresis (IEP). Quantitative analysis by IEP according to Laurell was performed on GelBond film (Pharmacia/LKB) with agarose gels (0.5 × 250 × 120 mm). For gel preparation, a solution containing 200 mg Agarose M (Pharmacia/LKB) in 20 ml Tris-barbiturate buffer, pH 8.6 was heated in a boiling water bath. After degassing and cooling to 55°C, 100 μl monospecific ITI-antiserum was added. For IEP, serum samples were diluted 1:5 with 0.9% NaCl and 4 μl serum was introduced into wells placed 3 cm from the gel margin. Tris-barbiturate solution, pH 8.6 was used as bridge buffer. Electrophoresis was carried out at constant voltage of 10 V/cm for 5 h at 10°C. After electrophoresis the gel was wetted with distilled water and covered with one moist and several dry sheets of filter paper, a glass plate and a 1 kg weight, for 3 min. Pressing was repeated twice. Before staining, the gel was washed in 0.9% NaCl (2 × 10 min), pressed and dried, then stained with 0.5% Coomassie Brillant Blue R-250 in 250 ml ethanol, 8 ml glacial acetic acid and 100 ml distilled water.

Results

Figures 1 and 2 show the different ITI phenotypes disclosed by IEF on the Multiphor II and on the PhastSystem, respectively.

Of the 106 cases examined, which included a total of 114 men, putative fathers were excluded in one or more systems in 39 cases. Of these 39 exclusions, 7 were confirmed by the ITI system (Table 1). In 32 cases the constellation in the ITI system was compatible with fatherhood, however, the men were excluded as fathers in one or more of the other genetic systems (not shown).

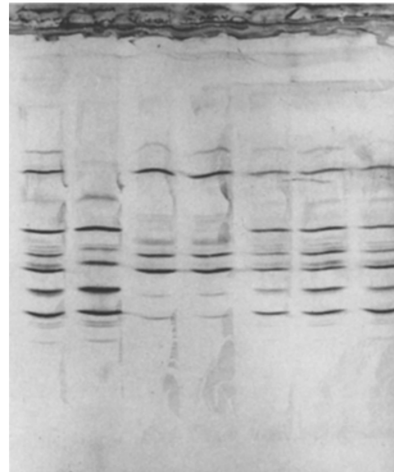

Fig. 1. Common ITI phenotypes, demonstrated by IEF on polyacrylamide gel (pH 3.5–9.5) and immunoblotting. Anode at the top. Shown are the phenotypes, ITI 1–2, 2, 1, 1, 1–2, 1–2, 1–2 (from left to right)

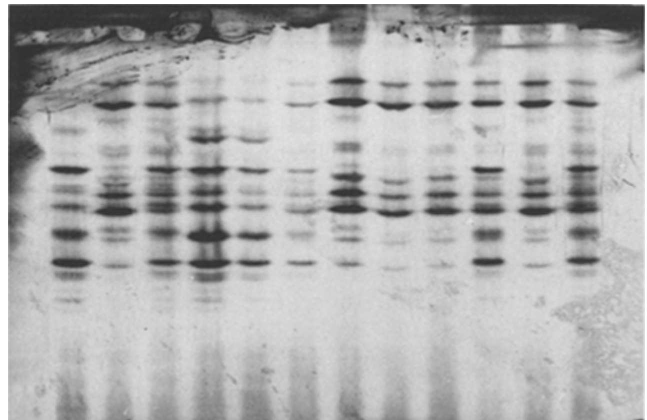

Fig. 2. 2:1 enlargement of the ITI phenotypes, disclosed by IEF with the PhastSystem on polyacrylamide gel (pH 3.5–9.5) and immunoblotting. Anode at the top. Shown are the ITI phenotypes, 2, 1, 1–2, 2–3, 2–3, 1–2, 1, 1, 1, 1–2, 1, 1–2 (from left to right)

In 75 expertises the alleged man was not excluded from fatherhood. In 7 cases the biostatistical probability for paternity was between 95% and 99.73%, in 68 > 99.73%. Of these latter cases, 67 were used for the calculation of the practical exclusion rate in the ITI system, which was 10.45% (Table 2).

From the observed distribution of ITI-phenotypes in the parents from this sample study the theoretical exclusion rate was calculated to be 19.3%.

As shown in Table 1, one exceptional case with an apparent exclusion constellation in the ITI system was

Table 1. Exclusions in the ITI system

Case	Mother	Child	Accused man	Further exclusions
1)	2	2	1	Rh, GPT, Gc, α_2HS, F13B, ORM
2)	2	2	1	Tf, Gc, F13B, ORM
3)	1–2	1	2	MN, ACP, PGM, ADA, Gc, GPT, α_2HS
4)	2	2	1	ABO, Fy, Gm, ACP
5)	1	1	2	MN, Rh, Gm, ACP, AK, Tf, ORM
6)	1–2	2	1	PGM, GLO, Gt, Hp
7)	1–2	1	2	ABO, MN, PLG, ACP, EsD, C3, α_2HS, Gm, ORM
8)	2	2	1	–

Table 2. ITI phenotyping in 67 cases of disputed paternity with "practically proved" values (> 99.73%) of fatherhood

Mother	Child	Accused man	*n*
1	1	1	7
1	1	1–2	9
1	1–2	1–2	4
1	1–2	2	2
1–2	1	1	9
1–2	1–2	1	6
1–2	1	1–2	2
1–2	1–2	1–2	7
2	2	1–2	1
2	1–2	1–2	2
2	1–2	1	2
1–2	2	2	2
1–2	1–2	2	4
1–2	2	1–2	4
2	2	2	4
1	1	1–3	1
1–2	2–3	2–3	1
Total			67

found (case 8). The alleged man in this case was not excluded by any other markers. The calculated probability of paternity excluding ITI was 99.99% and it may be assumed that he is, indeed, the father of the child. This observation is not in agreement with the postulated mode of inheritance and prompted further investigations. We considered first age dependent differences of ITI phenotype expression. The distribution of the ITI phenotypes in adults and in children from our material is given in Table 3. Only the children below the age of 18 months were included. While the ITI distribution observed in adults is in agreement with the distribution expected at population equilibrium and corresponds to our previous results (Vogt and Cleve 1990), a significant deviation

Table 3. Distribution of ITI phenotypes in samples of adults and children ($\leq$ 18 months) in cases of disputed paternity. χ^2 (adults) = 0.407, $df = 2$, $0.1 < P < 0.25$; χ^2 (children) = 9.795, $df = 2$, $0.99 < P < 0.995$

	ITI phenotypes						
	n	1	1–2	2	2–3	1–3	3
Adults							
Observed	220	81	100	35	2	2	0
Expected	220	79.20	103.20	33.62	1.56	2.40	0.02
Children							
Observed	81	35	26	19	1	0	0
Expected	81	28.444	38.519	13.040	0.401	0.593	0.003

Table 4. Quantitative determination of ITI concentrations by rocket immunelectrophoresis compared to the serum from a healthy adult used as standard (= 100%)

	n	Mean value (%)	Range (%)
Adults	63	80.8	60–100
Children	109	80.8	56–105

was noticed in the children. A deficiency of the heterozygous ITI phenotype 1–2 and an excess of the homozygous phenotypes ITI 1 and 2 were found. In order to assess the possible influence of the serum concentrations as the reason for this discrepancy, we made a quantitative determination of ITI in the sera of children and of adults by rocket immunoelectrophoreses. We used the serum from a healthy adult as a 100% standard. The results obtained in sera from 109 children and from 63 adults are given in Table 4. The mean values in both adults and in children were found to be 81% and therefore lower than the standard. However mean values and ranges did not show any differences.

Discussion

Employing a simplified IEF method, reliable classification of the different ITI phenotypes has been accomplished. This method does not require digestion with neuraminidase prior to separation by IEF and an expanded pH range of 3.5–9.5 was used. Resolution of the ITI band pattern was achieved by addition of carrier ampholytes pH range 4.0–6.5 which permits reliable classification. In addition analysis with 2 different separation procedures, Multiphore II and PhastSystem gave comparable results for resolution and reproducibility. The latter system was found to be a particular economical procedure with regards to analytical time and reagents.

Family studies indicate a simple mode of inheritance: 3 ITI phenotypes are determined by 2 common alleles, further rare phenotypes are also present (Vogt and Cleve 1990; Vogt et al. 1991). While the genetic control of the ITI system appears to be clear cut, the application of this system to disputed paternity cases requires caution.

A significant departure from the Hardy Weinberg equilibrium was observed in children below the age of 18 months. This deviation is due to a deficiency of the heterozygous phenotype, ITI 1–2 and a concomitant increased incidence of the homozygous phenotypes, ITI 1 and ITI 2. Thus a difference between phenotype and genotype in infants cannot be excluded. We assume that infants with the heterozygous genotype, ITI*1–2, may type as homozygous ITI phenotype with the characteristics of ITI 1 or ITI 2. A relationship between this supposed incomplete expression of the heterozygous ITI phenotypes in children below the age of 18 months and a variation in their ITI serum concentration, however, could not be established.

From our results, we conclude that the application of the ITI system for paternity testing is restricted to cases involving older children. In all cases, with younger infants the constellation of inverse homozygosity between the child and the alleged father can not be considered as a reliable exclusion in the ITI system.

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