Z Rechtsmed (1989) 102:391–397

Zeitschrift für
Rechtsmedizin

Haptoglobin subtyping by isoelectric focusing in ultrathin-layer polyacrylamide gels Population genetic data for Hanover and Lower Saxony

T. Rothämel, H.-J. Krüger, and H. D. Tröger

Institut für Rechtsmedizin der Medizinischen Hochschule, Konstanty-Gutschow-Strasse 8, D-3000 Hannover 61, Bundesrepublik Deutschland

Summary. This report describes a method for subtyping haptoglobin by means of isoelectric focusing in 0.2-mm ultrathin-layer polyacrylamide gels. Haptoglobin (Hp) is purified by ion-exchange chromatography and reduced. The well-known advantages of ultrathin-layer gels combine high isoelectrophoretic resolution of the Hp subtypes with less demands for time and material and make sequential visualization by fixation and protein staining possible. The distribution of the Hp subtypes in 1500 unrelated adults from Hanover and Lower Saxony is presented. Allelic frequencies are calculated to be: Hp*2FF = 0.0030; *2FS = 0.5620; *2SS = 0.0290; *1F = 0.1537; *1S = 0.2523. Segregation analysis for 68 matings shows an autosomal codominant mode of transmission in all cases. For the population investigated the chance of isolated paternity exclusion with the subtyped Hp system amounts to 33.91%.

Key words: Forensic serology, Hp subtypes – Haptoglobin subtyping, by isoelectric focusing

Zusammenfassung. Beschrieben wird eine Methode zur Haptoglobin-Subtypisierung mittels isoelektrischer Fokussierung im 0,2 mm Ultradünnschicht-Polyacrylamidgel nach ionenaustauschchromatographischer Isolierung und reduktiver Spaltung des Haptoglobins. Durch die Anwendung ultradünner Gele wird bei geringem Zeit- und Materialaufwand eine hohe isoelektrophoretische Auftrennung der Hp-Subtypen erreicht sowie eine sequentielle Visualisierung durch Fixierung und Protein-Färbung ermöglicht. Die Subtypenverteilung in einer Stichprobe von 1500 unverwandten Erwachsenen aus Hannover und Niedersachsen wird mitgeteilt, aus der sich folgende Allelfrequenzen errechnen: Hp*2FF = 0,0030; *2FS = 0,5620; *2SS = 0,0290; *1F = 0,1537; *1S = 0,2523. Die Segregationsanalyse bei 68 Familien zeigt keine

Sonderdruckanfragen an: T. Rothämel

Abweichung vom autosomal-kodominanten Erbgang. Für die untersuchte Population beträgt die isolierte Vaterschaftsausschlußchance des subtypisierten Hp-Systems 33,91%.

Schlüsselwörter: Forensische Serogenetik, Hp-Subtypen – Haptoglobin-Subtypisierung

Introduction

Methods for practicable subtyping of Hp by means of isoelectric focusing in 0.5 to 1.0-mm thin-layer polyacrylamide gels (PAGIF) have recently been described [1–3]. Ultrathin-layer gels have technical advantages over thin-layer polyacrylamide gels, because they increase the resolving power, improve the heat convection, shorten all periods of fixation, staining and destaining and need smaller amounts of material [4, 5]. These properties have proved to have practil advantages in isoelectrophoretic typing of human transferrin [6, 7] and the C6 system [8], for example. In this report, we now present a method that exploits the advantages of ultrathin-layer polyacrylamide gels for Hp subtyping by isoelectrophoresis. Preliminary results have already been published [9].

Materials and methods

Sera were obtained from routine casework. Haptoglobin was partially purified by means of selective adsorption by anion exchange cellulose [10] according to the protocol of Patzelt and Schröder [2], but with all quantities reduced by half. The intramolecular disulphide bonds of Hp were cleaved as described by Smithies et al. [11]. Ultrathin-layer polyacrylamide gels were cast using the "horizontal sliding technique" introduced by Ansorge and de Maeyer [12], adapted to the demands of Tf typing by Krüger [6, 7]. A summary protocol for Hp subtyping is listed in Table 1.

Results

Band patterns of Hp subtypes detected by 0.2 mm PAGIF (Fig. 1) resemble those produced by thin-layer PAGIF [1, 2]. In addition to each major Hp band we found two or even three (e.g., Hp 2SS) minor bands that were reproduced throughout all our runs and are specific for each Hp polypeptide. Only Hp 2FF revealed no additional bands. Heterozygous Hp subtypes present a minor band pattern composed of the minor bands specific for the two corresponding homozygous subtypes (Fig. 2).

Table 2 shows the observed and expected Hp subtypes for an extensive population sample of 1500 random unrelated individuals from Hanover and Lower Saxony. The observed distribution of the Hp subtypes is in good agreement with the Hardy-Weinberg equilibrium. Segregation analysis of the Hp alleles for 68 matings and their offspring revealed transmission by autosomal codominant mode in all cases (Table 3). Using the formulae of Abbozzo et al.

Table 1. Protocol for Hp subtyping

Hp purification:
- equilibrate DEAE SS cellulose (Serva) in sodium acetate buffer (0.01 *M*, pH 4.7);
- mix 25 µl serum with 1 ml cellulose suspension (0.5% w/v) for some minutes;
- shortly centrifuge and discard excess buffer;
- resuspend the cellulose sediment with 1 ml sodium acetate buffer;
- shortly centrifuge and discard excess buffer again;
- elute Hp by stirring the sediment with 25 µl ammonium acetate solution (0.125 *M*)!

Hp cleavage:
- mix 20 µl Hp eluate with 20 µl cleavage reagent (8 *M* urea in 0.1 *M* boric acid, 0.04 *M* sodium hydroxide, pH 8.8, 20 µl β-mercaptoethanol added per 1 g urea) – all in all 8 *M* urea;
- alkylate with 4 µl iodoacetamide solution (0.5 *M*) after 20′ at room temperature!

Gel:
- casting procedure: "Horizontal sliding technique" using 0.2 mm spacers
- dimensions: 230 × 100 × 0.2 mm, Composition: PAG T_5C_3, carrier ampholytes pH 5–7 (4% w/v, LKB)

Isoelectrophoresis equipment:
- Pharmacia ECPS 3000/150; LKB 2117 Multiphor II; LKB 2219 Multitemp II

Electrolytes:
- anolyte: 1 *M* H_3PO_4, catholyte: 1 *M* NaOH

Running conditions:
- prerun; 1600 V, 5 mA, 5 W, 45′; run: 1600 V, 5 mA, 5 W, 2 h 45′; temperature 8°C

Sample application:
- soak strips of filter paper (5 × 10 mm) with 18 µl of the alkylated cleavage products;
- apply strips side by side 2 cm supra-cathodically and remove them after 30′!

Visualization:
- fixation: 20% TCA (w/v); 30′
- staining: 27% isopropanol, 10% acetic acid, 0.04% Coomassie Brilliant Blue R 250 and 0.05% Crocein Scarlet (Bio-Rad, w/v), 0.50% Cu(II)SO_4 (Fluka, w/v); 30′
- destaining: 12% Isopropanol, 7% Acetic Acid, 0.50% Cu(II)SO_4 (Fluka, w/v); 10′
- after visualization gels are simply air-dried.

[13] we were able to calculate the chance of isolated paternity exclusion of the subtyped Hp system for the Lower Saxonian population sample at 33.91%.

Discussion

Additional components in IEF patterns of the Hp bands which are quite similar to those reported here have already been described by Teige et al. [3]. Zischler et al. [14] observed single minor bands of each Hp 2FS and Hp2SS, respectively, in positions that show double minor bands in the 0.2 mm polyacrylamide gel (Fig. 2). Detection and identification of Hp subtypes is not affected by the minor

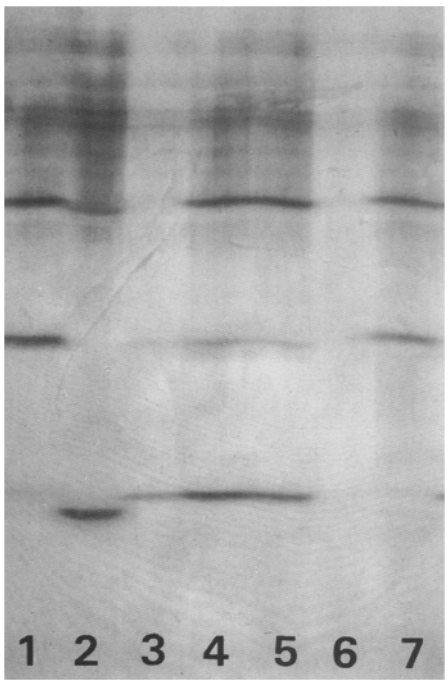

Fig. 1. Hp subtypes displayed by 0.2-mm PAGIF. Section of a gel showing the middle and upper parts but not the Hp 2FF region. *Lane 1,* Hp 2SS-1S; *lane 2,* Hp 1F; lane 3, Hp 2FS; lane 4, Hp 2FS-1S, lane 5, Hp 2FS-1S; lane 6, "Ahp"; lane 7, Hp 2SS-1S. The anode is at the top

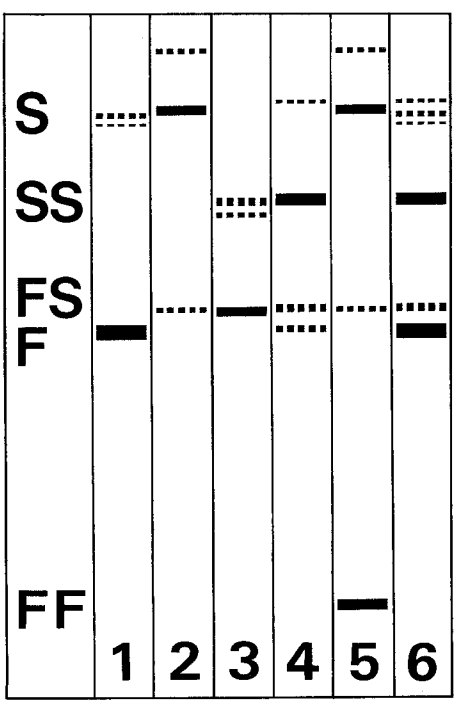

Fig. 2. A diagrammatic representation of the major and minor Hp bands. Lane 1, Hp 1F; lane 2, Hp 1S; lane 3, Hp 2FS; lane 4, Hp 2SS; lane 5, Hp 2FF-1S; lane 6, Hp2SS-1F. Major bands are drawn as continuous lines, the anode is at the top

Table 2. Hp subtype distribution and allelic frequencies in 1500 unrelated adults from Hanover and Lower Saxony (FRG)

Hp subtype	Observed		Expected	Allelic frequencies
	(%)	*n*		
2FF	0	0	0.0135	
2FF-2FS	0.20	3	5.0580	
2FF-2SS	0	0	0.2610	
2FS	31.13	467	473.7660	Hp*2FF = 0.0030
2FS-2SS	3.87	58	48.8940	*2FS = 0.5620
2SS	0.07	1	1.2615	*2SS = 0.0290
2FF-1F	0.27	4	1.3833	*1F = 0.1537
2FF-1S	0.13	2	2.2707	*1S = 0.2523
2FS-1F	17.13	257	259.1382	
2FS-1S	28.93	434	425.3778	$\sum$ = 1.0000
2SS-1F	0.40	6	13.3719	
2SS-1S	1.40	21	21.9501	Chi^2 = 1.0178
1F	2.53	38	35.4355	*df* = 4
1F-1S	7.87	118	116.3355	$95 \geq P \geq 90$
1S	6.07	91	95.4829	
$\sum$	100.00	1500	1499.9999	

Table 3. Inheritance of Hp subtypes

Parental subtypes		No. of families	No. of children	Offspring subtypes							
				2FS	2FS-2SS	2FS-1F	2FS-1S	2SS-1S	1F	1F-1S	1S
2FS	/2FS	5	5	5	–	–	–	–	–	–	–
2FS	/2FS-2SS	3	3	1	2	–	–	–	–	–	–
2FS	/2FS-1F	11	11	6	–	5	–	–	–	–	–
2FS	/2FS-1S	16	16	9	–	–	7	–	–	–	–
2FS	/1F-1S	4	4	–	–	2	2	–	–	–	–
2FS	/1S	2	2	–	–	–	2	–	–	–	–
2FS-2SS	/2FS-1S	3	3	–	–	–	2	1	–	–	–
2FS-2SS	/1S	1	1	–	–	–	–	1	–	–	–
2FS-1F	/2FS-1F	3	3	–	–	2	–	–	1	–	–
2FS-1F	/2FS-1S	5	7	–	–	2	3	–	–	2	–
2FS-1F	/1F	2	6	–	–	2	–	–	4	–	–
2FS-1F	/1S	1	1	–	–	–	–	–	–	1	–
2FS-1S	/2SS-1F	1	1	–	–	–	–	1	–	–	–
2FS-1S	/2SS-1S	1	1	–	1	–	–	–	–	–	–
2FS-1S	/1F	1	1	–	–	1	–	–	–	–	–
2FS-1S	/1F-1S	4	4	–	–	1	1	–	–	–	2
2FS-1S	/1S	3	3	–	–	–	2	–	–	–	1
1F	/1S	1	1	–	–	–	–	–	–	1	–
1F-1S	/1S	1	1	–	–	–	–	–	–	1	–
Total		68	74	21	3	15	19	3	5	5	3

bands, whose specific and reproducible isoelectrophoretic patterns may even help to identify the major bands. Focusing of minor components, however, represents the resolving power of ultrathin-layer polyacrylamide gels [5].

We compared the allelic frequency data recorded in the Lower Saxonian population sample (North Germany) with the average of the corresponding data for other regions of the Federal Republic of Germany (South West Germany, Rhine-Ruhr area) [14, 15] and Berlin (German Democratic Republic) [2]: In the Lower Saxonian population sample Hp*2FF and Hp*1S were found to appear with frequencies comparable to those in the other German regions, and Hp*2SS to slightly exceed the average (+ 0.0064; $\delta = 0.0028$). Hp*1F, however, shows a clearly higher value in the Lower Saxonian population sample compared with the average (+ 0.0103; $\delta = 0.0033$). The increase of both allelic frequencies is mainly at the cost of Hp*2FS, which is diminished accordingly (− 0.0155; $\delta = 0.0040$). These results may reflect genetic characteristics of different population samples. On the other hand, the higher value of Hp*1F might be due to the resolving properties of the 0.2-mm gel, since Hp 2FS and Hp 1F are clearly separated and Hp 1F is saved from being masked by the 2FS band.

There have been reports [16, 17] of deleting small proteins and polypeptides by acid-alcohol treatment, which is necessary if sequential visualization techniques [18] are applied (successive fixation, staining and destaining). Special simultaneous visualization techniques (simultaneous fixation and staining) have been developed to cope with this problem [16, 17] and have been applied in Hp subtyping by thin-layer PAGIF [1, 2]. We conducted trials with the simultaneous visualization technique according to Blakesley and Boezi [16], which combines a high signal-to-noise ratio with good fixing properties but takes 5–8 h to reach maximum sensitivity. The technical advantages of ultrathin-layer polyacrylamide gels enabled us to use a convenient method of sequential visualization that has been published by Bio-Rad Laboratories [19]. It is based on sensitive "Crowle's double stain" [20] on the one hand, and on effective background clearing by copper(II) sulphate according to Righetti and Drysdale on the other [21]. Modifying the original stain recipe [19], we used trichloroacetic acid (TCA) as fixative [5]. Applied to 0.5-mm polyacrylamide gels with their demand for prolonged acid-alcohol destaining, this visualization technique did not work satisfactorily, since it caused deletion of sample material and broadening of Hp bands.

Hp subtyping by 0.2 mm PAGIF can readily be implemented into many laboratories and, besides the general advantage of ultrathin-layer polyacrylamide gels for isoelectophoresis, is practicable because of its quick and convenient visualization.

References

1. Shibata K, Constans J, Viau M, Matsumoto H (1982) Polymorphism of the haptoglobin peptides by isoelectric focusing electrophoresis and isoelectric point determinations. Hum Genet 61:210–214
2. Patzelt D, Schröder H (1985) Haptoglobin subtypes in Berlin, GDR. A simple procedure for haptoglobin purification and subtyping. Z Rechtsmed 94:207–212

3. Teige B, Olaisen B, Pedersen L (1985) Subtyping of haptoglobin – presentation of a new method. Hum Genet 70:163–167
4. Görg A, Postel W, Westermeier R (1978) Ultrathin-layer isoelectric focusing in polyacrylamide gels on cellophane. Anal Biochem 89:60–70
5. Radola BJ (1980) Ultrathin-layer isoelectric focusing in 50–100 µm polyacrylamide gels on silanized glass plates or polyester films. Electrophoresis 1:43–56
6. Krüger H-J, Hummel K (1983) TF B, D, C1...7 in 0,2 mm PAGIF. Populationsgenetische Daten für Südwestdeutsche (N = 1026), Stadtmexikaner (N = 379) und Türken (N = 267). Proc 10th Int Congr Soc Forens Haemogenet, Munich 1983. Schmitt & Meyer, Würzburg, pp 411–413
7. Krüger H-J, Hummel K (1984) Large scale TF (Sub-C, B, D) typing by 0.2 mm PAG isoelectrophoresis. Phenotype frequencies in Mexicans (N = 956) and South-West Germans (N = 2124). Abstr 18th Congr Int Soc Blood Transfus, Munich 1984. Karger, Basel, p 60
8. Krüger H-J, Tabasaran M, Tröger HD (1988) Darstellung der Typen des C6-Systems mittels 0,2 mm PAG-Isoelektrophorese und Immunoblotting. In: Bauer G (ed) Gerichtsmedizin. F Deuticke, Vienna, pp 421–425
9. Rothämel T, Krüger H-J, Tröger HD (1986) HP-Subtypen in 0,2 mm PAGIF. Frequenz- und Segregationsanalysen für Hannover und Niedersachsen. Zbl Rechtsmed 29:108
10. Connell GE, Shaw RW (1961) The purification of haptoglobin. Can J Biochem 39:1013–1019
11. Smithies O, Connell GE, Dixon GH (1966) Gene action in the human haptoglobins. I. Dissociation into constituent polypeptide chains. J Mol Biol 21:213–224
12. Ansorge W, Maeyer L de (1980) Thermally stabilized very thin (0.02–0.3 mm) polyacrylamide gels for electrophoresis. J Chromatogr 202:45–53
13. Abbozzo G, Mattiuz PL, Ronchi L (1977) A priori probability of paternity exclusion. General formulas. Proc 7th Int Congr Soc Forens Haemogenet, Hamburg 1977. Schmitt & Meyer, Würzburg, pp 541–552
14. Zischler H, Kömpf J, Ritter H (1987) IEF of Hp-subtypes: presentation of population genetic data and variants. Ärztl Lab 33:85–87
15. Bertrams J, Müller B, Luboldt W (1988) Haptoglobin (Hp) subtypes in the German Rhine-Ruhr area. In: Mayr WR (ed) Advances in forensic haemogenetics, vol 2. Springer, Berlin Heidelberg New York, pp 158–162
16. Blakesley RW, Boezi JA (1977) A new staining technique for proteins in polyacrylamide gels using Coomassie Brilliant Blue G250. Anal Biochem 82:580–582
17. Steck G, Leuthard P, Bürk RR (1980) Detection of basic proteins and low molecular weight peptides in polyacrylamide gels by formaldehyde fixation. Anal Biochem 107:21–24
18. Gianazza E, Chillemi F, Duranti M, Righetti PG (1983) Analytical and preparative isoelectric focusing of peptides in immobilized pH gradients. J Biochem Biophys Methods 8:339–351
19. Ultra thin layer electrofocusing in polyacrylamide gels. Instruction manual. Bio-Rad Laboratories, 32nd & Griffin Ave, Richmond, CA 94804, p 12
20. Crowle AJ, Cline J (1977) An improved stain for immunodiffusion tests. J Immunol Methods 16:379–381
21. Righetti PG, Drysdale JW (1974) Isoelectric focusing in gels. J Chromatogr 98:271–321

Eingegangen am 25. Mai 1988