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Haptoglobin Phenotyping by Polyacrylamide Gel Isoelectric Focusing and its Application to Simultaneous Typing of Serum Proteins*

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Summary. A simple isoelectric focusing method for haptoglobin (HP) typing is described. Serum was pretreated first with C. perfringens neuraminidase (CPN) and then with dithiothreitol (DTT). The treated serum was subjected to polyacrylamide gel isoelectric focusing (PAGIF), and the band patterns were detected by immunoblotting. The method could be successfully applied to HP typing of bloodstains as old as 2 months. A slight modification of it enabled HP, complement component C81, and factor I (IF) to be typed simultaneously. The immunoblotting facilitated preservation of HP patterns. Thus, the PAGIF method for HP typing is suitable for routine use in the forensic laboratory.

Key words: Haptoglobin typing, polyacrylamide gel isoelectric focusing – Blood groups, HP-phenotyping

Zusammenfassung. Es wird eine einfache Methode zur Typisierung von Haptoglobin (HP) mittels Isoelektrofokussierung beschrieben. Das Serum wird mit Clostridium perfringens Neuraminidase (CPN), sodann mit Dithiothreitol (DTT) vorbehandelt und anschließend der Polyacrylamidgel-Isoelektrofokussierung (PAGIF) unterworfen. Die Banden wurden durch Immunoblotting sichtbar gemacht. Die Methode erwies sich als erfolgreich bei der HP-Darstellung von selbst 2 Monate alten Blutspuren. Durch eine leichte Modifizierung ist es möglich, neben HP auch die Komplement-Komponente C81 und Faktor I (IF) darzustellen. Immunoblotting ergab außerdem eine dauerhafte Darstellung der HP-Banden. PAGIF ist daher als forensische Routinemethode zur HP-Typisierung geeignet.

Schlüsselwörter: Haptoglobin-Typisierung, Isoelektrofokussierung in Polyacrylamidgel – Blutgruppen, HP-Typisierung

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Introduction

Classical starch gel electrophoresis (Smithies and Walkes 1956) or polyacrylamide gel electrophoresis has been used for routine serum HP typing. Bloodstains have been HP-typed by polyacrylamide gel electrophoresis. However, electrophoretic results are heavily streaked with deteriorated hemoglobin present in dried bloodstains. Stolorow and Wraxall (1979) developed a chloroform extraction method to eliminate the streaking effect of deteriorated hemoglobin. Bloodstains up to 2 years old were correctly HP-typed by this method. However, the chloroform extraction method involves a handling problem in the case of very small amounts of bloodstains. Shibata et al. (1982) and Patzelt and Schröder (1985) described PAGIF methods for HP subtyping. These IEF methods are free from hemoglobin interference because of the difference in pI between HP and hemoglobin, but require laborious steps for purification of HP with DEAE-cellulose and hydrolysis of HP with a β-mercaptoethanol/urea/iodoacetamide mixture.

Recently, Teige et al. (1985) devised a PAGIF method of HP subtyping without prior purification of HP. In our experience, however, the method appears to require much technical skill for reproducible results. Moreover, in some populations, especially the Japanese, the *HP*1F* and *HP*2SS* alleles are extremely rare, with *HP*1F* = 0.0017 and *HP*2SS* = 0.0172 (Nakada et al. 1986). Therefore, HP subtyping is rarely necessary for forensic casework in Japan. To include the HP system in bloodstain analysis and in the simultaneous phenotyping of hemogenetic markers for paternity testing, we developed a simple method of HP typing by PAGIF followed by immunoblotting as described briefly elsewhere (Tamaki et al. 1987). The present paper describes the detailed procedure for HP typing of serum and bloodstains and its modification for simultaneous typing of HP and other genetic markers.

Materials and Methods

Blood Collection and Bloodstain Preparation

Eigthy-five blood samples were obtained from Japanese residents of Oita Prefecture, located in the Southwestern area of Japan. The sera were HP-typed by the classical starch gel electrophoretic method. Bloodstains were prepared from the 85 samples on Whatman no. 3 filter paper. The stains were air-dried and left at room temperature for up to 2 months.

Reagents and Equipment

Anti-HP serum was obtained from DAKO-immunoglobulins; anti-IF serum from Cytotech; and anti-C81 and horseradish peroxidase-labeled anti-rabbit (or goat) IgG sera from Cappel Laboratories. The other chemicals used were of analytical grade. Nitrocellulose membranes (Trans-Blot Transfer Medium, Bio-Rad Laboratories) were used for immunoblotting.

A Flat Bed Electrophoresis Apparatus FBE 3000 (Pharmacia) and a Power Supply 2103 (LKB) were used for isoelectric focusing (IEF).

Pretreatment

For HP typing, serum samples were treated successively with CPN, DTT, and iodoacetamide as described previously (Tamaki et al. 1987), except that the serum samples were treated with DTT for 30 min.

Dried bloodstains on pieces of filter paper (3 × 5 mm) were extracted in 25 µl distilled water at RT overnight. Of the extracts 15 µl was treated with 3 µl CPN (5 units/100 µl) at RT overnight. A 0.1 *M* DTT solution (4 µl) was added, and the mixture was incubated at RT for 30 min. A 0.12 *M* iodoacetamide solution (5 µl) was added, and the resulting mixture was incubated at 4°C for 1 h

PAGIF

Polyacrylamide gels (T = 4.5%; C = 3%, 140 × 100 × 0.5 mm) containing 2.7% carrier ampholytes were prepared by chemical polymerization. The gel was prefocused for 30 min. Samples were applied with filter paper applicators (Whatman no.3), and focusing was carried out at 10°C for 3 h at 1500 V_{max}, and 9 W_{max}.

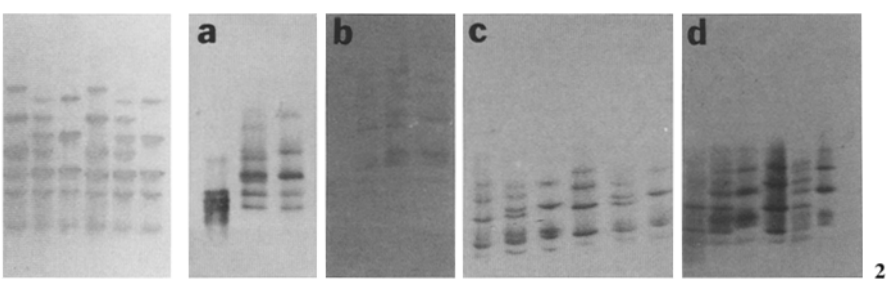
HP typing of Serum and Bloodstains

The PAG used contained 3.6 g urea and 2.7% Ampholine pH 6–8 (LKB). The anolyte and the catholyte were 0.04 *M* L-glutamic acid and 1 *M* sodium hydroxide, respectively. Serial double dilutions in saline of the pretreated serum samples on 3 × 3 mm applicators or bloodstain extracts on 3 × 7 mm applicators were placed at the cathodal side. After focusing, HP was detected by immunoblotting as previously described (Tamaki et al. 1985).

In addition, carrier ampholytes of different pH ranges and gradients were tested: a 1:1 or 2:1 mixture of Ampholine pH 6–8 and Ampholine pH 3.5–9.5, Ampholine pH 5–8 only, a 4:1 mixture of Ampholine pH 5–8 and Ampholine pH 3.5–9.5. The gels contained 4 *M* urea. The anolyte and the catholyte were 0.04 *M* L-glutamic acid and 1 *M* sodium hydroxide, respectively, or 0.5 *M* phosphoric acid and 0.5 *M* sodium hydroxide, respectively.

Simultaneous PAGIF of HP and Other Genetic Markers

Simultaneous typing of HP, C81, and IF and of HP and transferrin (TF) were carried out on various gels.



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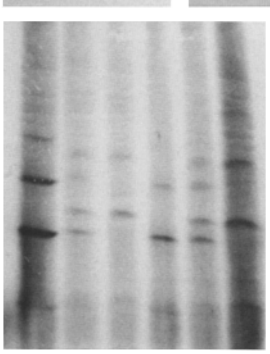

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Fig. 1. Phenotype patterns of serum HP after IEF (pH 6–8) in isolation. Anode at *top*. From *left* to *right:* HP 2, 2-1, 1, 2, 2-1, 1

Fig. 2a–d. Phenotype patterns of serum HP in gels of different pH ranges and gradients. Anode at *top*. From *left* to *right:* **a** (pH 6–8/3.5–9.5 = 1/1) HP 2, 2-1, 1; **b** (pH 6–8/3.5–9.5 = 2/1) HP 2, 2–1, 1; **c** (pH5–8) HP 2, 2–1, 1, 2, 2–1, 1; **d** (pH5–8/3.5–9.5 = 4/1) HP 1, 2-1, 2, 1, 2-1, 2

Fig. 3. IEF patterns of HP in bloodstains. Anode at *top*. From *left* to *right:* HP 2, 2-1, 1, 2, 2-1, 1

Results and Discussion

Figure 1 shows phenotype patterns of serum HP after IEF in isolation. The three common phenotypes, HP 1, HP2-1, and HP 2, could be identified readily. HP in up to 64-fold diluted serum was typable, but a 1:2 dilution of the pretreated serum was found most suitable for routine HP typing.

Figure 2 shows IEF patterns of serum HP in gels of different pH ranges and gradients. HP could be typed in all pH ranges tested. The distance between two bands of HP 2-1 tended to be inversely proportional to the width of the pH range. Ampholine pH 6–8 was most suitable for HP typing in isolation.

In simultaneous PAGIF of HP, C81, and IF, a 1:1 mixture of Ampholine pH 3.5–9.5 and Ampholine pH 6–8 gave the most readable patterns as described previously (Tamaki et al. 1987). In addition, HP could be typed simultaneously with TF on a PAG containing Pharmalyte 5–8 (Pharmacia) and 4 *M* urea.

All bloodstains as old as 2 months could be HP-typed correctly and clearly by the PAGIF-immunoblotting method (Fig. 3). Since practically no interference from hemoglobin was noted, bloodstain extracts did not need pretreatment with chloroform. A follow-up study on HP typing of aged bloodstains is in progress.

The present study demonstrates that our method is simple, sensitive, reproducible, convenient for permanent record, and hence suitable not only for paternity testing but also for bloodstain analysis.

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