TECHNICAL NOTE

Reena Roy,[1] *Ph.D. and Ina C. Roy,*[2] *A.B.*

Haptoglobin Phenotyping from Older Bloodstains by Enzyme Immunoassay and Haptoglobin Phenotypes Within a Nebraska Caucasian Population

REFERENCE: Roy, R. and Roy, I. C., **"Haptoglobin Phenotyping from Older Bloodstains by Enzyme Immunoassay and Haptoglobin Phenotypes Within a Nebraska Caucasian Population,"** *Journal of Forensic Sciences*, JFSCA, Vol. 36, No. 2, March 1991, pp. 571–575.

ABSTRACT: Enzyme immunoassay and Western blotting (electrophoretic) techniques were used to determine haptoglobin (HP) phenotypes from older bloodstains.

Serum was collected from liquid blood and the HP phenotypes were determined. Bloodstains were prepared from these specimens and stored at various temperatures for several months. The stains were extracted and applied to gradient polyacrylamide gels.

The Western blotting technique was used to achieve the transfer of HP bands from the gels to the nitrocellulose membranes. Enzyme immunoassay with goat anti-HP antiserum and rabbit anti-goat immunoglobulin peroxidase were used to identify the HP bands from the extracted samples.

Enzyme immunoassay was found to be clearly more sensitive than *o*-dianisidine or *o*-tolidine in detecting HP bands from diluted serum samples.

The haptoglobin frequency in a Caucasian population in Nebraska was calculated. The frequencies of Phenotypes 1, 2-1, and 2 were found to be 15.8, 48.4, and 35.8%, respectively.

KEYWORDS: pathology and biology, genetic typing, haptoglobin, enzyme immunoassay, Western blotting, phenotype frequency

Haptoglobin (HP) is an α_2 serum glycoprotein. It is a hemoglobin-binding protein which has three main phenotypes: HP 1, HP 2-1, and HP 2 [*1*]. HP has been shown to remain stable for a prolonged period of time [*2*] and has a high discriminatory power of 0.60 [*3,4*].

Several procedures have been described for separating and identifying the phenotypes of HP [*5–12*]. The polymorphism is usually detected by using *o*-tolidine or *o*-dianisidine dyes, which indicate where the peroxidase activity of hemoglobin is complexed with HP.

This study was undertaken to determine HP phenotypes from older bloodstains and to detect the sensitivity of the enzyme immunoassay procedure. The haptoglobin frequency in a Caucasian population in Nebraska was also determined.

Received for publication 4 May 1990; revised manuscript received 2 June 1990; accepted for publication 5 June 1990.

[1]Forensic serology supervisor, Nebraska State Patrol Crime Laboratory, Lincoln, NE.

[2]Student, Stanford University Medical School, Stanford, CA.

Materials and Methods

The tank buffer for electrophoresis was prepared by dissolving 21.8 g/L glycine (0.29*M*) and 4.5 g/L (0.037*M*) of Tris base [tris(hydroxymethyl)aminomethane] in deionized water. The resultant pH of the buffer was 8.3. Polyacrylamide gradient gels, 2 to 16%, from Pharmacia-LKB (PAA 2/16) were run as horizontal slab gels on a SERI electrophoresis apparatus. The gel size was 75 by 75 mm with a thickness of 2.7 mm.

Trans-blot tank buffer and gel equilibration buffer (Tris-glycine buffer) were prepared by dissolving 7.27 g/3 L Tris base (20m*M*) and 33.78 g/3 L glycine (150m*M*) in 600 mL of methanol and deionized water. The resultant pH of the buffer was 8.4. Tris buffered saline (TBS) was prepared by dissolving 1.21 g/1 L Tris base (10m*M*) and 29.22 g/1 L sodium chloride (NaCl) in deionized water. The pH of the buffer was adjusted to 8.0 with HCl.

Tween/tris buffered saline (TTBS) (0.1% Tween-TBS) was prepared by adding 1 mL of Tween 20 (Bio-Rad) to 1 L of TBS. Blocking solution [1% gelatin-TBS or 0.5% bovine serum albumin (BSA)-TBS] was prepared by dissolving 1 g of gelatin in 100 mL of TBS. (Gelatin requires slight heat to dissolve.) Also, 0.5 g of BSA in 100 mL of TBS can be used (available from Bio-Rad and Ortho-Diagnostics).

The antibody buffer was 1% gelatin-TBS or 0.5% BSA-TBS. The first antibody was used as a 1:100 dilution of anti-human HP (goat) in antibody buffer (ATAB). The second antibody was used as a 1:250 dilution of rabbit anti-goat immunoglobulin horseradish peroxidase conjugate (HRP).

For color development, the following buffer and reagents were used. HRP color-developing solution was prepared by dissolving 60 mg of 4-chloro-1-naphthol (Bio-Rad) in 100 mL of TBS and 20 mL of ice-cold methanol. Hydrogen peroxide 30% (60 μL) was added to this solution immediately prior to color development.

Serum was collected from liquid blood samples, and the HP phenotypes were determined by methods described previously [*11,12*]. Bloodstains from individuals with HP Phenotypes 1, 2-1, and 2 were prepared in duplicate by taking approximately 500 μL of each sample and applying this amount to the center of a square piece of cotton cloth. After being air-dried, each bloodstained cotton cloth piece was put in a labeled coin envelope. One group was stored at −20°C and the other was stored at room temperature. Serum samples were collected in 1.5-mL centrifuge tubes and stored at −20°C.

Pieces of cloth approximately 5 by 5-mm with dried bloodstains were cut and placed in 1.5-mL microfuge tubes. Distilled water (30 μL) was added to each tube. The tubes were gently agitated on a rotator for 30 min at room temperature. The cuttings were then removed from the tubes and placed inside micropipette tips (1 to 250 μL in size). Each tip was then returned to its original 1.5-mL microfuge tube, and this assembly was centrifuged at 1200 × *g* for 5 min. The micropipette tips containing the cuttings were then discarded. The extracts were now at the bottoms of the microfuge tubes. Volumes of 300 μL of chloroform and 10 μL of ethanol were added to each extract. The tubes were vortexed vigorously for 2 min and centrifuged for 5 min at 9000 × *g* in a microfuge. The clear uppermost layer containing the HP protein was removed for subsequent typing.

The bloodstain extracts, in volumes of 5 μL, were applied onto No. 3 Whatman filter paper strips (5 by 1 mm) and gently placed on top of the gel surface approximately 1 cm from the 2% cathodic edge. Two sponges, one on each end of the gel, were used as wicks to provide contact with the buffer in the tanks. The serum samples stored at −20°C were used without extraction. These were serially diluted and applied in amounts of 3 μL onto the filter strips as was described for the bloodstain extract application.

After this, electrophoresis was performed on the applied samples at 4°C using a Pharmacia-LKB electrophoresis power supply at 125 V for 17 h or until the hemoglobin bands were at the 16% anodic end of the gel.

After electrophoresis, the following procedures for Western blotting and enzyme im-

munoassay were carried out at room temperature. The gel was equilibrated with gentle shaking on a rotator in Tris-glycine buffer for 60 min, with one buffer change after 30 min. The gel was then transferred onto the gel holder of a Bio-Rad trans-blot apparatus. A nitrocellulose membrane (Bio-Rad, 0.45 μm, 75 by 75 mm) previously soaked in the Tris-glycine buffer was laid on top of the gel, and care was taken to remove any air bubbles trapped between the gel and the membrane. Western blotting was achieved by overnight (16 h) transfer in a Bio-Rad trans-blot apparatus containing Tris/glycine/methanol buffer at 25 V with a Bio-Rad power supply.

After transfer, the blot (nitrocellulose membrane) was removed and the free sites were blocked by incubating the blot in 0.5% BSA-TBS or 1% gelatin-TBS for 1 h. The blot was then incubated for 2 h with the first antibody in 0.5% BSA-TBS or 1% gelatin-TBS and washed in four changes of TTBS for 15 min each. The blot was next incubated for 2 h with the second antibody in 0.5% BSA-TBS or 1% gelatin-TBS and subsequently washed in four changes of TTBS for 15 min each.

The peroxidase activity was visualized by submerging the blots in the HRP color-developing solution and rinsing them in distilled water to stop the reaction. The blots can be left in the color-developing solution for from a few minutes to overnight, depending on the desired intensity of the HP bands, since the intensity of the bands increases with the increase in the time submerged in the developing solution. The nitrocellulose blots can be air-dried and stored in the dark at room temperature.

Results and Discussion

HP polymorphism was determined from fresh serum samples after the samples had been stained with *o*-tolidine prior to being stored at −20°C and from bloodstain preparations. Figure 1 illustrates HP Types 2-1, 1, and 2, determined from fresh serum samples on a 2 to 16% gradient polyacrylamide gel. Bloodstains were prepared and stored at both room temperature and at −20°C for several months. Enzyme immunoassay was used to phenotype haptoglobin correctly from bloodstains as old as 15 months when

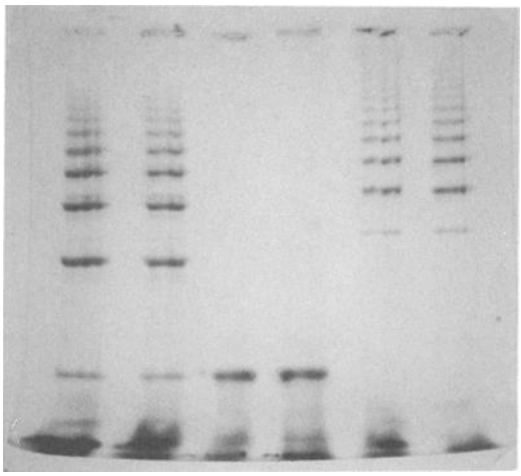

FIG. 1—*Separation of HP phenotypes from fresh serum samples on a 2 to 16% gradient polyacrylamide gel after staining with* o-*tolidine. The phenotypes from left to right are as follows:* (Lane 1) *HP 2-1, diluted 1:5;* (Lane 2) *HP 2-1, diluted 1:10;* (Lane 3) *HP 1, diluted 1:5;* (Lane 4) *HP 1, diluted 1:10;* (Lane 5) *HP 2, diluted 1:5; and* (Lane 6) *HP 2, diluted 1:10. The cathode is on top. The direction of migration is from cathode to anode.*

stored at room temperature and as old as 22 months when stored at −20°C. In addition, serum samples stored at −20°C for up to 30 months were also successfully typed (Fig. 2).

Enzyme immunoassay demonstrated a higher degree of sensitivity in detecting HP in diluted serum samples than *o*-tolidine. HP polymorphism could be detected from fresh serum samples only at a 1:10 dilution with *o*-tolidine (Fig. 1). Enzyme immunoassay with peroxidase can accurately detect HP polymorphisms in fresh serum diluted 1:60 (Fig. 2). Enzyme immunoassay with alkaline phosphatase has been reported to be the most sensitive method of detection of HP [*13*]. Although a strong background was observed, serum diluted 1:256 has been typed. Attempts are being made in this laboratory to use alkaline phosphatase immunoassay on older blood samples.

Frequency data for HP are available for a number of populations, including the population of the United States [*14*]. The frequency for the HP system reported in Table 1 is for a Nebraska Caucasian population sample collected from forensic laboratory casework and volunteer donors. Nebraska's population is approximately 95% Caucasian, according to the most recent census [*15*].

Data for HP phenotypes were converted to frequencies (assuming that phenotype frequencies accurately reflect genotype frequencies in this population), and the indirect test was used to determine whether the population is in Hardy-Weinberg equilibrium with respect to the HP locus. The χ^2 value of 0.0673 indicates no significant deviation from the expected frequencies of these genotypes in a population at Hardy-Weinberg equilibrium at this locus (significant deviation is implied by a χ^2 value > 3.841 for 1 degree of freedom and $P = 0.05$). The frequencies resemble those of other Caucasian populations in the United States.

Haptoglobin polymorphism is employed in forensic science laboratories for individualization of specimens and for inclusion or exclusion of donors of bloodstains. Enzyme immunoassay, while slightly more time-consuming than current procedures, can be used successfully in forensic science casework. Enzyme immunoassay clearly is superior to *o*-tolidine for phenotyping HP from older bloodstains and diluted serum. In addition, if *o*-tolidine is first used without success, the gel is still suitable for development with enzyme

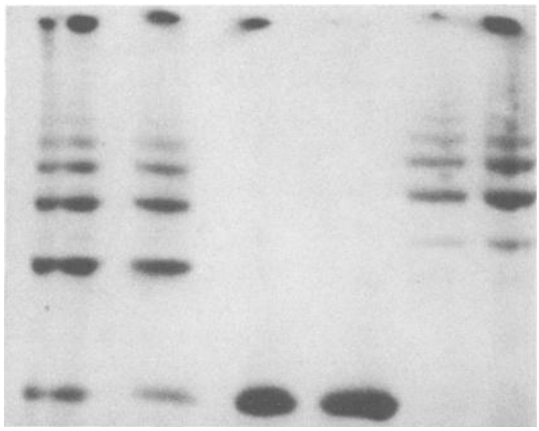

FIG. 2—*HP types on nitrocellulose membrane after Western blotting and enzyme immunoassay. The phenotypes from left to right are as follows:* (Lane 1) *HP 2-1, fresh serum diluted 1:40;* (Lane 2) *HP 2-1, bloodstain stored at room temperature for 15 months;* (Lane 3) *HP 1, bloodstain stored for 15 months at room temperature;* (Lane 4) *HP 1, bloodstain stored at −20°C for 22 months;* (Lane 5) *HP 2, fresh serum diluted 1:60; and* (Lane 6) *HP 2, serum frozen for 30 months and diluted 1:60. The cathode is on top. The direction of migration is from cathode to anode.*

TABLE 1—*Frequencies of haptoglobin phenotypes in a Nebraska population.*

	HP Phenotype		
	1[a]	2-1[b]	2
Observed frequency, %	0.158/15.8	0.484/48.4	0.358/35.8
Expected frequency, %	0.160/16.0	0.480/48.0	0.360/36.0
Number of individuals	104	320	236

[a]Observed allele frequency of HP* 1 = 0.40.
[b]Observed allele frequency of HP* 2 = 0.60.

immunoassay. In this study, *o*-tolidine was first used to detect the bands from the gels illustrated in Fig. 2. When the bands were not visible, enzyme immunoassay was performed on the same gel, and the HP bands were correctly identified.

References

[1] Jay, B. W. H., "Haptoglobin Polymorphism—Another System to Differentiate Blood Groups," *Canadian Society of Forensic Science Journal*, Vol. 8, 1975, pp. 21–26.

[2] Stolorow, M. D., Wraxall, M. S., and Wraxall, B. D. G., "An Efficient Method to Eliminate Streaking in Electrophoretic Analysis of Haptoglobin in Bloodstains," *Journal of Forensic Sciences*, Vol. 24, No. 4, Oct. 1979, pp. 856–863.

[3] Smalldon, K. W. and Moffat, A. C., "The Calculation of Discriminating Power for a Series of Correlated Attributes," *Journal of the Forensic Science Society*, Vol. 13, 1973, pp. 291–295.

[4] Giblett, E. R. and Brooks, L. E., "Haptoglobin Types: Haptoglobin Subtypes in Three Racial Groups," *Nature*, Vol. 197, 1963, pp. 576–577.

[5] Smithies, O. and Walker, N. F., "Genetic Control of Some Serum Proteins in Normal Humans," *Nature*, Vol. 176, 1955, pp. 1265–1266.

[6] Smithies, O. and Walker, N. F., "Notation for Serum Protein Groups and the Genes Controlling Their Inheritance," *Nature*, Vol. 178, 1956, pp. 694–695.

[7] Blackwell, R. Q. and Liu, C. S., "Haptoglobin Type Determination by Cellulose Acetate Zone Electrophoresis," *Clinica Chimica Acta*, Vol. 8, 1963, p. 868.

[8] Culliford, B. J., *The Examination and Typing of Bloodstains in the Crime Laboratory*, U.S. Government Printing Office, Washington, DC, 1971, pp. 219–228.

[9] Hoppe, H. H., Hennig, W., and Brinkmann, B., "Horizontal Polyacrylamide Electrophoresis for the Determination of Serum Protein (Haptoglobin) and Red Cell Enzyme Polymorphisms," *Humangenetik*, Vol. 14, 1972, pp. 224–231.

[10] Felix, R. T., Boenisch, T., and Giese, R. W., "Haptoglobin Phenotyping of Bloodstains by Non-gradient Polyacrylamide Electrophoresis," *Journal of Forensic Sciences*, Vol. 22, No. 3, July 1977, pp. 580–584.

[11] Grunbaum, B. W., "Phenotyping of Haptoglobin on Gradient Acrylamide Gel Slabs Using the Beckman Microzone System," *Journal of Forensic Science Society*, Vol. 15, 1975, pp. 229–234.

[12] Budowle, B. and Chowg, G. H., "Discontinuous Polyacrylamide Gel Electrophoresis for Typing Haptoglobin in Bloodstains," *Journal of Forensic Sciences*, Vol. 30, No. 3, July 1985, pp. 893–897.

[13] Hoste, B., "Advantages of Enzyme-Immunoassay After Blotting in Bloodstain Grouping: Application to the Haptoglobin Groups," *Electrophoresis*, Vol. 7, 1986, pp. 479–480.

[14] Gaensslen, R. E., Bell, S. C., and Lee, H. C., "Distributions of Genetic Markers in United States Populations: III. Serum Group Systems and Hemoglobin Variants," *Journal of Forensic Sciences*, Vol. 32, No. 6, Nov. 1987, pp. 1754–1774.

[15] *Characteristics of the Population*, Vol. I, Part 29, Bureau of the Census, U.S. Department of Commerce, Washington, DC, 1982, Chapter B.

Address requests for reprints or additional information to
Reena Roy, Ph.D.
Nebraska State Patrol Laboratory
3701 South 14th Street
Lincoln, NE 68502