TECHNICAL NOTE

Isabel López-Abadía,[1] *M.D.; Eduardo Arroyo,* [1] *B.S.; Ana Gremo,*[1] *B.D.; and J. M. Ruiz de la Cuesta,*[1] *M.D.*

An Improved Method for Subtyping Pi in Old Bloodstains

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ABSTRACT: Immunofixation procedures were used for detecting alpha-1 antitrypsin protease inhibitor (Pi) phenotypes in bloodstains. Neuraminidase elution of bloodstains, together with isoelectric focusing, immunofixation, and silver staining techniques, makes possible Pi subtyping in old bloodstains. No extra bands appear when the storage time is no longer than three months.

KEYWORDS: pathology and biology, genetic typing, human identification, bloodstains, alpha-1 antitrypsin, immunofixation, silver staining, protease inhibitor

Protease inhibitor (Pi) is a reliable genetic marker used in blood characterization and is responsible for most protease (trypsin) inhibitory activity in normal human serum. Its structure contains 12% carbohydrates, and its molecular weight ranges between 50 000 and 55 000 daltons.

Pi genetic variation was first detected by Laurell and Eriksson in 1963 using conventional acid starch gel electrophoresis [*1*]. In 1967 [*2*], the term Pi (protease inhibitor) was adopted for designating this genetic system. Then, several Pi variants were identified by this same technique. At first there were seven alleles (F, I, M, S, V, X, and Z), all of them autosomal and codominant, the most common of which was PiM. Since 1975, isoelectric focusing techniques in polyacrilamide gels have permitted the characterization of several subtypes of the PiM allele: M1, M2, M3, M4, and M5 [*3–6*].

In 1978, a subcommittee of the International Pi Committee met in Rouen, France, with the following objectives:

(*a*) to compare phenotyping methods and results,
(*b*) to clarify the relationships of the reported and new Pi variants to each other, and
(*c*) to decide upon a mutually acceptable system of nomenclature for both the old and the new variants.

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[1]Associate investigators, titular professor, and chief, respectively, Forensic Biology Service, Department of Legal Medicine, University Complutense, Madrid, Spain.

According to the nomenclature system that came out of the meeting, alleles should be designated by capital letters corresponding to their relative positions in isoelectric focusing. If there is no unused letter, a number or a place-of-origin name should be used with the letter of the closest anodal allele. Now, all variants anodal to the M band are designated with letters from B to L and those cathodal are designated with letters from N to Z.

The positions of the recognized variants as determined by isoelectric focusing are shown in Figs. 1 and 2.

The genetic variation of Pi has proved to be a valuable aid for individual identification of bloodstains. The present paper studies the application of immunofixation techniques, followed by silver staining after isoelectric focusing within narrow pH ranges to determine Pi alleles in bloodstains.

Materials and Methods

Gel Preparation

The polyacrylamide gel isoelectric focusing (PAGIEF) was carried out under the following conditions. The gel was 200 by 115 by 0.5 mm in size and contained 6.2% in total concentration of monomer and 3.2% in the total monomer due to the cross-linking agent [6 g of acrylamide (LKB), 0.2 g of bis-acrylamide (LKB), and 12 g of sucrose (Merck)].

The ampholyte concentration was achieved through the following mixture:

(*a*) 0.06% v/v ampholine, ph 4 to 6 (LKB),
(*b*) 0.02% v/v Pharmalyte, pH 4.5 to 5.4 (Pharmacia),
(*c*) 0.02% v/v Pharmalyte, pH 4.2 to 4.9 (Pharmacia), and
(*d*) 0.02% v/v Pharmalyte, pH 4 to 6.5 (Pharmacia).

Polymerization was carried out with 1% w/v ammonium peroxodisulfate (Merck) and 20 μL of TEMED (*N,N,N',N'*-tetramethylethylenediamine (Serva).

Sample Preparation

Blood was obtained from 100 normal donors with known Pi types. Stains were made by soaking pieces of clean cotton cloth in this whole fresh blood and storing them at room temperature until used. A neuraminidase solution (2% w/v) was used for extraction, and each sample consisted of a 0.5-cm² piece of stain. After a storage time of 24 h at 4°C in neuraminidase, the samples were centrifuged at 2000 rpm for 5 min and the supernatant was used for assays.

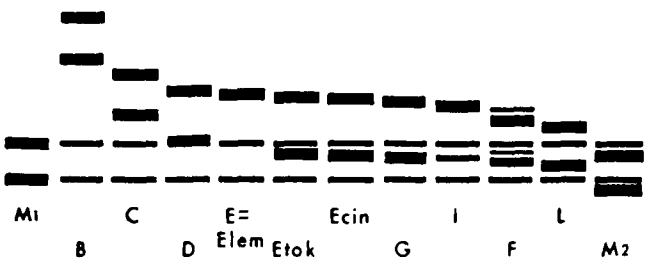

FIG. 1—*Pi variants anodal to M, showing two major bands of each variant obtained by isoelectric focusing in acrylamide. The narrow bands in each pattern indicate the position of M1.*

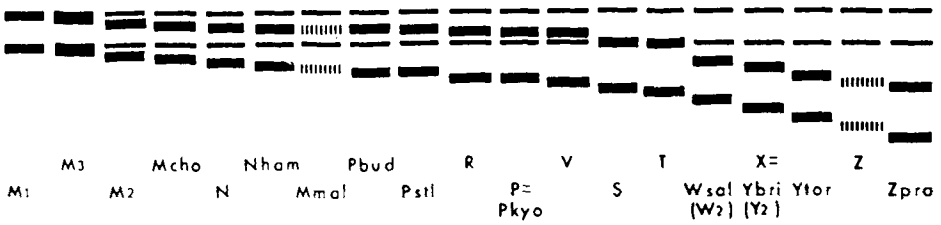

FIG. 2—*Pi variants cathodal to M, showing two major bands of each variant obtained by isoelectric focusing in acrylamide. The narrow bands indicate the position of M1. Dashed bands indicate deficiency alleles.*

Isoelectric Focusing Conditions

The gels were run on a flat-bed apparatus FBE-2000 (Pharmacia) at 4°C. The electrode gap was 100 mm. Acetic acid at 1% (anode) and ethanolamine at 1% (cathode) were used as the electrode solutions. The power supply (Pharmacia ECPS3000/150) was set at 1500 V, 7 W, and 150 mA and the volt-hour integrator (Pharmacia VH-1) was set at 6000 V/h. The prefocusing period (45 min) was run over 500 V/h at 1500 V, 8 W, and 150 mA. Whatman No. 3 filter paper application pieces, 5 by 3 mm in size were placed 250 mm from the cathode. Once the application papers had been removed (30 min), the gel was focused at 1500 V, 7 W, and 150 mA to the end (4 h).

Immunofixation

The antibody [purified immunoglobulin (lg) fraction of rabbit anti-human Pi (Dako) was diluted 1:4 in buffered saline (0.9% sodium chloride in distilled water). The dilution was spread over the surface of the gel and covered at 37°C for 30 min. Then the gel was rinsed overnight in buffered saline. The development method was that used for silver staining [7,8].

Results and Discussion

The authors purpose in this study was to test immunofixation procedures in old bloodstains. Pi phenotypes from 100 bloodstains were determined by isoelectric focusing techniques every week for a 4½-month period.

In our early experiments we tried several solutions for eluting bloodstains, including the following:

(*a*) distilled water,
(*b*) dithiothreitol (DTT), 0.05*M*;
(*c*) urea, 6*M*; and
(*c*) neuraminidase, 2% w/v.

The neuraminidase solution happened to be the best solution and the only one that could subtype M1 and M2 alleles.

The most common band patterns: M1, M2, and S (Fig. 3) could be clearly read in bloodstains up to 10 weeks old (Fig. 4). After this time, the S allele band tended to disappear, while the other bands (M1 and M2) kept the same intensity; thus, the subtyping lost reliability.

Bloodstains older than 14 weeks could not be subtyped since several extra bands appeared within the rank of focusing (Fig. 5). The appearance of additional irreversible anodal bands may erroneously suggest the presence of anodal variants. The Pi pattern was completely altered after 15 weeks at room temperature.

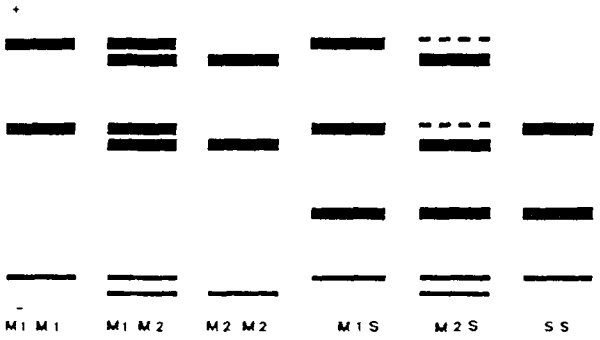

FIG. 3—*Schematic representation of the most common Pi phenotypes.*

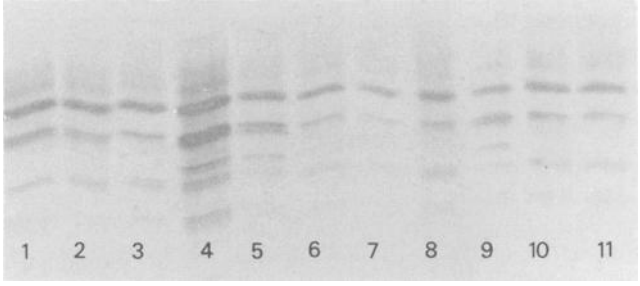

FIG. 4—*Pi phenotypes in 10-week-old bloodstains. The anode is at the top. The phenotypes can be read as follows: Lanes 3, 6, 7, 10, and 11—M1M1; Lanes 1, 2, and 8—M1M2; Lanes 5 and 9—M2S, and Lane 4—M1S.*

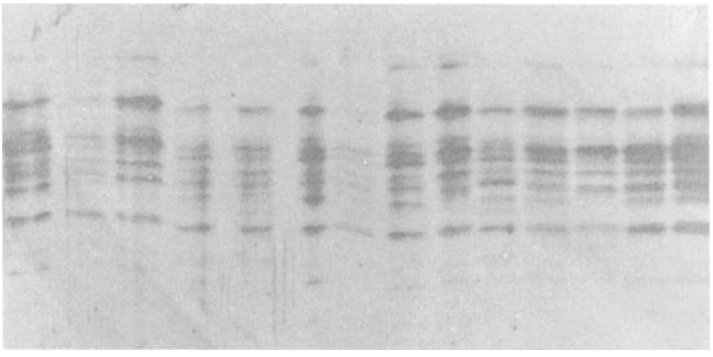

FIG. 5—*Pi phenotypes in 16-week-old bloodstains, showing additional irreversible bands due to degradation of protein. Identification of different variants of Pi is not possible under these circumstances. The anode is at the top.*

Conclusion

We can conclude that this method is reliable for subtyping bloodstains up to 12 weeks old and useful for routine case work. It affords the advantage of great sensitivity in Pi subtyping of old bloodstains.

References

[1] Laurell, C. B., "The Electrophoretic Alpha-1 Globulin Pattern of Serum Alpha-1 Antitrypsin Deficiency," *Scandinavian Journal of Clinical and Laboratory Investigation*, Vol. 15, 1963, pp. 132–140.

[2] Fagerhol, M. K. and Laurell, C. B., "The Polymorphism of "Prealbumins" and Alpha-1 Antitrypsin in Human Sera," *Clinical Chemistry Acta*, Vol. 16, 1967, pp. 199–200.

[3] Arnaud, P., Chapuis-Cellier, C., and Creyssel, R., "The Pi System: Its Study by Means of Thin-Layer Electrofocusing in Polyacrylamide Gel," *Protides of Biological Fluids*, H. Peeters, Ed., Pergamon Press, Oxford, England, 1975, pp. 515–520.

[4] Görg, A., Postel, W., Weser, J., Weidinger, S., Patuschnick, W., and Cleve, H., "Isoelectric Focusing in Immobilized Ph Gradients for the Determination of the Genetic Pi Variants," *Electrophoresis*, Vol. 4, 1983, pp. 153–157.

[5] Frants, R. R. and Eriksson, W., "Reliable Classification of Six Pi M Subtypes by Separator Isoelectric Focusing," *Human Heredity*, Vol. 28, 1978, pp. 201–209.

[6] Constants, J., Viau, M., and Govillard, C., "Pi M4: An Additional Pi M Subtype" *Human Genetics*, Vol. 55, 1980, pp. 119–121.

[7] Carracedo, A., Concheiro, L., Requena, I., and López-Rivadulla, M., "A Silver-Staining Method for the Detection of Polymorphic Proteins in Minute Bloodstains After Isoelectric Focusing," *Forensic Science International*, Vol. 23, 1983, pp. 241–248.

[8] Carracedo, A., Concheiro, L., Estefanía, J., and Suárez, C., "Isoelectric Focusing of Pi Subtypes in Bloodstains," *Forensic Science International*, Vol. 26, 1986, pp. 425–431.

Address requests for reprints or additional information to
Isabel López-Abadía
Sección de Biología Forense
Departamento de Toxicología y Legislacíon Sanitaria
Facultad de Medicina, Pabellon VII
Universidad Complutense
28040 Madrid, Spain