# TECHNICAL NOTE

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# Determination of C3 Phenotype in Dried Bloodstains Using Isoelectric Focusing

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**ABSTRACT:** Fresh whole blood and bloodstains were analyzed by isoelectric focusing (IEF) to determine the C3 phenotype of the blood donor. Three common phenotypes exist as a result of two autosomal alleles. The three phenotypes can be identified in fresh serum or in serum samples which had been stored at −20°C for more than a year. Bloodstains maintained in a desiccator at 25 or at 37°C retained the native form of C3 which could be detected for at least two weeks. Beyond two weeks of storage, stains became difficult to phenotype due to decreased banding intensity. Bloodstains aged longer than one month could not be phenotyped. C3 could not be detected in human semen by the serological methods employed.

**KEYWORDS:** pathology and biology, genetic typing, blood, complement (biology), isoelectric focusing, C3, serology

Human C3, the third component of complement, is a serum protein which plays a vital role in both classical and alternate pathways of complement. This polymorphic protein is found in human serum at a concentration of approximately 1.5 to 1.6 mg/mL. Its activation results in release of chemotactic, opsonic, and anaphylactic serum factors. C3 has been purified from plasma and physicochemically characterized [*1*]. It consists of two polypeptide chains having molecular weights of approximately 120 000 and 75 000 daltons, respectively, which are linked by two intermolecular disulfide bonds. Three common and fifteen rare variant phenotypes of C3 have been identified. The three common phenotypes resulting from the control of two autosomal codominant alleles have been designated FF, FS, and SS by Alper and Propp [*2*] and as 1-1, 1-2, and 2-2 by Azen and Smithies [*3*]. The gene frequencies of $C3^S$ and $C3^F$ differ significantly among Caucasians, blacks, and Orientals, with the $C3^F$ allele almost nonexistent in the Oriental population. The gene frequency of the $C3^F$ allele is only about 10% in the U.S. black population.

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Analysis of C3 and its spontaneous cleavage products in whole blood by crossed immunoelectrophoresis has been used to estimate the postmortem interval [4]. Furthermore, C3 in dried bloodstains has been analyzed by agarose gel electrophoresis and found to be sufficiently stable to phenotype a bloodstain maintained at room temperature for at least two weeks [5]. Thus far, C3 has been analyzed by high-voltage starch gel electrophoresis, by electrophoresis on cellulose acetate membranes, and by electrophoresis on agarose gels. In this paper we describe an IEF procedure that permits a more rapid analysis and better resolution of the C3 banding pattern than that obtained using standard electrophoretic methods.

## Materials and Methods

### *Blood Samples*

Blood samples were obtained from volunteers by finger prick. Serum was prepared by allowing whole blood to clot and retract at room temperature for 2 h, followed by centrifugation to separate serum from hematocrit. The serum was then aliquoted and frozen at −20°C until analyzed. Reference serum samples for C3 phenotyping which were provided by the New York City Blood Bank were kept frozen at −20°C. Bloodstains were phenotyped by comparing their banding pattern with those of the reference sera. Whole blood samples used for bloodstain preparation were initially placed on Whatmann 3MM filter paper and allowed to dry at room temperature for 2 h and then placed either in a freezer set at −20°C, in a desiccator maintained at room temperature (25°C), or in an incubator, desiccated or humidified, at 37°C for periods up to two months. Stains were also maintained at room temperature under ambient humidity conditions (average 73% relative humidity) for up to two months. Ten stains of unknown phenotype were prepared for each of the environmental conditions described. Two of these were removed for analysis after three days, one week, and weekly thereafter up to two months. Stains were gently extracted with saline for 1 to 2 h before isoelectric focusing.

### *Semen Samples*

Semen samples were donated by individuals who were under study at the New York Fertility Research Foundation. Samples were maintained at 4°C for 24 to 48 h before analysis for C3 phenotype.

### *Isoelectric Focusing*

Thick (1.0-mm) agarose gels were prepared by addition of 0.4-g, isoelectric focusing (IEF) grade agarose (IsoGel, EEO = 0) (FMC Corp., Rockland, Maine) and 4.8-g D-sorbitol to 35-mL deionized-distilled water. The mixture was stirred and heated until the solution clarified. When the temperature decreased to 60°C, 2.5 mL of ampholine, pH range 5 to 7, (LKB Instruments Inc., Gaithersburg, Maryland) were added and the mixture was poured into a 15-cm by 20-cm by 1.0-mm gel mold. The gel was allowed to harden for at least 2 h, but preferably overnight, before use. Wicks were cut from Whatmann No. 7 filter paper and soaked in either 0.05*M* sulfuric acid ($H_2SO_4$) (anodal wick) or 1.0*M* sodium hydroxide (NaOH) (cathodal wick). Ten microlitres of bloodstain extract or liquid semen were applied to Whatmann No. 3 filter paper strips (3 by 5 mm). These strips were placed 1 cm from the anodal wick. The gel was run for 2 h at a constant power of 3 W. The cooling platform was maintained at a constant temperature of 4°C. Prefocusing the gel before sample application for 15 min at 1 W did not enhance the focusing results.

*Immunofixation and Staining*

Following electrofocusing, immunofixation of C3 was performed using the method of Ritchie and Smith [6]. Cellulose acetate strips were soaked in goat anti-human C3 which had been diluted 1:6 with phosphate buffered saline, pH 7.2. This antiserum was purchased from Cappel Laboratories (Cochranville, Pennsylvania). The strips were applied to the surface of the gel and the plate was incubated in a humidified chamber at 37°C for 30 to 45 min or overnight at 23 to 25°C. The gel was then allowed to soak in 1 L of saline for approximately 3 h and then in 1 L of distilled water for an additional 4 h to remove any nonprecipitated protein in the gel. After drying, the gel was stained with Coomassie Blue (0.1% w/v) in methanol:water:glacial acetic acid (5:4:1). Destaining solution consisted of the same solution but without the dye.

*Densitometry*

A scanning laser densitometer purchased from LKB Instruments, Inc. (Gaithersburg, Maryland), and interfaced with a Hitachi integrating chart recorder was used to scan the stained gels and obtain tracings of the C3 banding profiles. This system can be used to compare the relative position and staining intensity of selected bands within an individual sample and can also be used to compare these characteristics within the banding patterns obtained with different samples. A more complete description of the use of laser densitometry for qualitative and quantitative analysis of electrophoretic separations of proteins can be found in Ref 7.

## Results

The three common phenotypes resulting from the two common codominant alleles $C3^S$ and $C3^F$ were easily resolved when fresh sera were analyzed. The resulting banding patterns for these serum samples were compared to those of reference sera that had been kept at −20°C. Three serum samples that had been stored at −20°C for approximately one year could also be phenotyped. All bloodstains that were prepared on filter paper and stored, desiccated at 25°C for up to two weeks, could be successfully phenotyped. Similar results were obtained with stains maintained at room temperature and 73% relative humidity. Bloodstains maintained at 37°C in a desiccator could also be phenotyped; however, none of the stains that were kept at 37°C under humidified conditions could be typed due to the absence of the relevant bands (a, b, and c bands in Fig. 1). All stains that were maintained desiccated at room temperature for more than two weeks were, at best, difficult to phenotype as a result of a decrease or complete loss in the staining intensity of the relevant bands. Bloodstains stored under these conditions for one month could not be phenotyped. The IEF pattern of several two- and four-week-old bloodstains is illustrated in Fig. 1. One phenotype (SS) is found in Lanes 3 and 4; a second (FF) is found in Lanes 5 and 8; the third (FS) is found in Lanes 6, 7, 9, 10. The four-week aged stains cannot be phenotyped in this system because they do not produce any of the cathodal (a, b, or c) bands. Densitometric tracings of the banding patterns for the three different phenotypes are shown in Fig. 2. The trace is started near the "a" band and the laser scans cathodally. Differences in band positions and relative band intensities can easily be observed on the tracing. We were unable to find C3 in fresh semen using IEF, in agreement with Tauber et al. [8] and our previous electrophoretic study of C3 in semen [5].

## Discussion

The use of isoelectric focusing for the analysis of serological evidence has gained importance over the past few years primarily because of its detection sensitivity, resolving power,

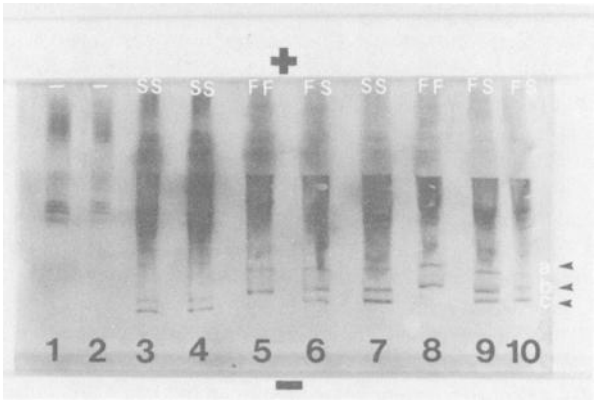


FIG. 1—*Banding pattern resulting from isoelectric focusing of individual human bloodstains illustrating three major phenotypes of C3. Lanes 1 and 2: bloodstains aged for one month in a desiccator at room temperature. Lanes 3 to 10: bloodstains aged for two weeks under same conditions.*

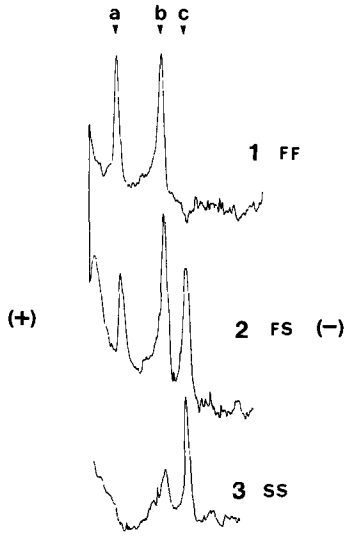


FIG. 2—*Scanning laser densitometer tracings of banding patterns representing three common C3 phenotypes as seen in Fig. 1. Tracing 1 corresponds to Lane 8, Tracing 2 corresponds to Lane 9, and Tracing 3 corresponds to Lane 3. Tracings for each phenotype were aligned for easier comparison of intensities of bands a, b, and c.*

reproducibility, and speed. It has been used to type erythrocyte acid phosphatase, transferrin, group-specific component, esterase D, alpha 1-antitrypsin, and haptoglobin, and has also been used to subtype phosphoglucomutase 1 ($PGM_1$) [9]. For the analysis of C3, in particular, we have found substantial improvements in resolution and therefore phenotyping when using IEF as compared to electrophoresis. The sharp bands that result from the former approach and which can be seen in Fig. 1 are in marked contrast to the diffuse bands that result from conventional electrophoresis [5]. Because the bands resulting from IEF are more

sharply focused, the analyst is able to phenotype stains with greater confidence. It should also be noted that the electrophoretic procedure takes approximately 6 h to run, compared with 2 h using IEF. Thus the latter procedure has several advantages over the former.

We had hoped that the improved resolution available with the IEF procedure would have allowed for the phenotyping of stains older than two weeks, which is the apparent age limit for bloodstain analysis using electrophoresis. Unfortunately, the lack of stability of C3 under non-frozen conditions prevents this. Although the C3 polymorphic system is of significant usefulness to clinical scientists and geneticists, this instability tends to limit its value for forensic serologists. Nevertheless, note that C3 is potentially a valuable ethnic marker since 1 of the 2 codominant alleles, $C3^F$, is completely absent among Japanese and U.S. Orientals. In a study of 464 Japanese, not a single individual was phenotyped as FF or as FS [*10*]. Also, a lower frequency (10%) of this gene is found in the U.S. black population than in the U.S. Caucasian population [*2*].

We were unable to detect the presence of C3 in semen by IEF. This confirms our previous observations using electrophoresis as well as those of Blake et al [*11*]. The lack of C3 in semen is not surprising. The biological activities resulting from its cleavage include immune adherence (opsonization), leukocyte chemotaxis, anaphylatoxin, and lymphocyte activation. The activation of the complement system and the subsequent generation of these biological effector activities would serve no obvious purpose in semen. In contrast, C3 is present in serum in the highest concentration (1500 $\mu$g/mL) of all other complement components. This too is not surprising since C3 plays a central role in both the classical and alternate (amplification) pathways of complement activation.

Agarose rather than polyacrylamide was chosen as the gel medium for IEF analysis of C3 because the former is safer to handle, easier to manipulate, chemically unreactive, and produces suitable gels more rapidly than the latter. The banding pattern that results from agarose IEF is sharper and clearer than that obtained from agarose electrophoresis since electroendosmosis effects and diffusion effects are minimized by the former technique.

In a study of spontaneous C3 cleavage in vitro at several different temperatures (14, 23, and 37°C), Kominato et al found that incubation of whole blood at a higher temperature led to faster conversion of C3 or C3c [*4*]. They also showed that C3 in whole blood stored at 37°C converted slowly to its cleavage product. These observations, as well as our own, indicate that storage temperature is an important factor in protein stability and suggest that serological casework samples should be refrigerated or frozen to preserve activity.

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