TECHNICAL NOTE

Arni S. Masibay,[1] *M.S.F.S. and Nicholas T. Lappas,*[1] *Ph.D.*

The Detection of Protein p30 in Seminal Stains by Means of Thin-Layer Immunoassay

REFERENCE: Masibay, A. S. and Lappas, N. T., **"The Detection of Protein p30 in Seminal Stains by Means of Thin-Layer Immunoassay,"** *Journal of Forensic Sciences*, JFSCA, Vol. 29, No. 4, Oct. 1984, pp. 1173-1177.

ABSTRACT: The detection of p30 by means of an indirect thin-layer immunoassay (TIA) is described. Extracts from 20 samples can be analyzed in approximately 2 h with a detection limit of approximately 50 ng. The p30 protein was detected in seminal stains which had been stored at room temperature for six months and at 130°C for 4 h. Blood, saliva, urine, perspiration, and tears did not interefere with the method. The reliability of the method was demonstrated in a blind study.

KEYWORDS: pathology and biology, semen, proteins, immunoassay

Several investigators [*1-12*] have described the isolation and characterization of apparently semen specific proteins. Many of these proteins have characteristics, for example, molecular weight, immunoelectrophoretic mobility, seminal specificity, and isoelectric point, similar to those of the protein described by Sensabaugh which he designated p30 [*13*]. Sensabaugh, who proposed the use of p30 as a marker for semen identification in forensic science laboratories, evaluated the use of Ouchterlony double diffusion, radial immunodiffusion, and "rocket" electrophoresis for its detection. Subsequently, the development of an enzyme-linked immunosorbent assay (ELISA) has been reported [*14, 15*].

The successful applications of thin-layer immunoassay (TIA) to the identification of human bloodstains [*16, 17*] and the detection of fetal hemoglobin in bloodstains [*18*] indicated that TIA could be applied to the detection of p30 in semen stains. This paper describes an inhibition-TIA technique for this purpose.

Materials and Methods

Equipment

Falcon Microtest III® flat bottom polyvinyl chloride (PVC) microtiter plates were obtained from Curtin Matheson Scientific. Microlitre quantities of reagents were delivered by means

Portions of this research were presented at the Forensic Science Symposium on the Analysis of Sexual Assault Evidence, FBI Forensic Science Research and Training Center, Quantico, VA, 8 July 1983. Received for publication 12 Nov. 1983; revised manuscript received 5 Jan. 1984; accepted for publication 10 Jan. 1984.

[1]Graduate student and associate professor, respectively, Department of Forensic Sciences, The George Washington University, Washington, DC.

of Oxford adjustable Sampler® micropipettes. Results were viewed and photographed with a Bausch and Lomb Stereozoom® 7 microscope fitted with a Polaroid camera.

Antisera

Anti-p30 serum (Ap30) was purchased from SERI, Emeryville, CA. The immunoglobulin G (IgG) fraction of an anti-rabbit IgG serum (AIgG) produced in sheep was purchased from Cappel Laboratories, Cochranville, PA.

Samples

Whole semen samples, which had been incubated at 37°C for 15 min following their collection, were obtained from 75 donars at a local clinic. They were stored at −20°C before their use. Seminal plasma, obtained by the centrifugation of whole semen for 30 min at approximately 2500 rpm, was stored at −20°C until used. Other human samples used in this research were obtained from volunteers. Stains, ranging in size from 0.25 to 2 μL, were prepared by applying the appropriate volume of biological material to cotton cloth and drying with a stream of cold air. These stains were stored at room temperature for periods of up to six months.

Semen stains, approximately 5 μL in size and prepared on filter paper with semen of known p30 concentrations, 145 to 1915 μg/mL, were analyzed to estimate the detection limit of the TIA method. These stains were extracted with either 100 or 200 μL of saline. These extracts were serially diluted with saline. Both the undiluted and diluted extracts were analyzed.

Analytical Method

The wells of the microtiter plates were washed with 70% ethanol and dried thoroughly prior to their use.

Except when otherwise noted, 2-μL stains were extracted with 25 μL of saline in a microtiter well for 60 min at room temperature. A 5-μL aliquot of the extract was incubated with 5 μL of appropriately diluted Ap30 for 60 min at room temperature. The final dilution of Ap30 in this inhibition mixture depended on the Ap30 titer but generally was 1:32.

While the above incubation was proceeding, antigen monolayers were prepared in the wells (two wells for each sample) of a second microtiter plate. Approximately 100 μL of a 1:100 dilution of seminal plasma were incubated in each well for 30 min at room temperature. After this incubation, the wells were emptied. Then they were filled with distilled water and emptied again. The wells were washed with water three additional times in rapid succession and then dried with a stream of cold air.

A 2-μL aliquot of the inhibition mixture was applied to the center of each of the two monolayer coated wells and incubated for 30 min at room temperature. The wells were covered with transparent tape during this incubation to minimize evaporation. Following this 30-min incubation period, the tape was removed and the wells, with the applications still in place, were inverted and exposed to vapor from a 60°C water bath for 1 min. The wells then were washed and dried immediately as described above.

Approximately 75 μL of a 1:100 dilution of AIgG were added to the wells and incubated for 15 min at room temperature. After this incubation period, the wells were washed, dried, and exposed to water vapor as described above. The monolayer surface of each well was viewed microscopically (10 to ×20) and any positive reactions were recorded.

Ap30 Titer Determination

The Ap30 titer was determined by use of the analytical method described above with the exception that instead of incubating the Ap30 with a stain extract it was diluted with saline

before its incubation on the monolayer surface. The titer was the highest dilution of Ap30 that yielded unequivocal and reproducible positive reactions.

Blind Study

A total of 45, 2-μL stains were prepared on cotton cloth. The biological materials used included semen, as well as blood, urine, saliva, tears, and perspiration obtained from male and female volunteers. These stains were extracted with 25 μL of saline by the analyst who then assigned a code number to each extract. The extracts were given to a second person who changed their code numbers and resubmitted them to the analyst who conducted the analysis as described above. The codes were broken only after all the analytical results had been recorded and interpreted.

Results and Discussion

Typical results obtained from the TIA analysis of human fluid stains are presented in Fig. 1. The result obtained from the analysis of a nonsemen-containing stain is shown in Fig. 1*a* and demonstrates that in the absence of semen the Ap30 was not inhibited. As a result of the reaction of Ap30 with the seminal plasma monolayer, a thicker protein layer was produced in the area of the Ap30 application. Because of its increased hydrophilicity, this thicker protein layer is viewed as an area of large condensation droplets surrounded by smaller condensation droplets in the area that was not exposed to Ap30. The result obtained from the analysis of a semen stain is shown in Fig. 1*b*. The inhibition of the Ap30, with the stain extract, prevented it from reacting with the monolayer. Therefore, since no antigen-antibody (Ag-Ab) reaction occurred, an area of increased hydrophilicity did not occur. The p30 protein was detected in each of 75 known semen stains, 0.5 μL or larger, analyzed by the TIA method. It was not detected in stains prepared from blood, urine, saliva, tears, or perspiration.

The p30 protein was detected in semen stains that were up to six months of age. This finding is in agreement with the stability of p30 reported by Sensabaugh [*13*]. In addition, p30 was detected in 2-μL semen stains which had been incubated at 130°C for up to 4 h.

The p30 protein was detected in undiluted extracts prepared from semen stains with p30 concentrations as low as 145 μg/mL. It was also detected in diluted extracts prepared from

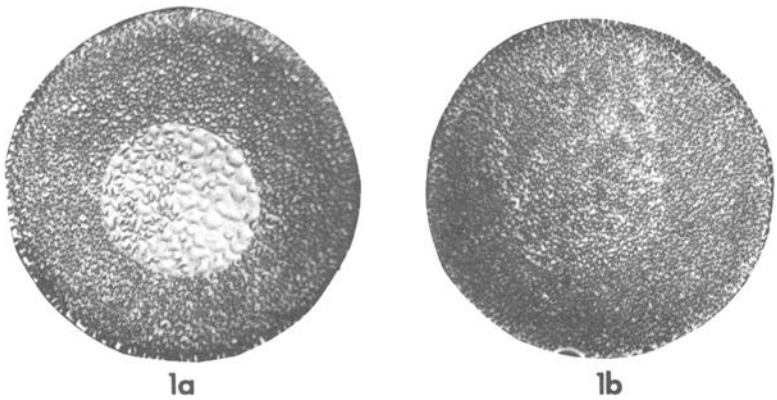

FIG. 1—*Typical results obtained from the TIA analysis of human fluid stains where* (a) *is the result obtained from the analysis of a nonsemen-containing stain and* (b) *is the result obtained from the analysis of a semen stain.*

semen stains of known p30 concentrations when the concentrations of p30 in these diluted samples was at least 10 μg/mL. If an extraction efficiency of 100% is assumed, this represents a detection limit of approximately 50 ng, since only 5 μL of the sample was required for analysis.

The blind study demonstrated the reliability of the TIA method. There were no false-positive or false-negative results, that is, p30 was only detected in each of the 18 semen samples analyzed.

Conclusion

TIA has been demonstrated to be an acceptable method for the detection of p30 in semen stains. The reliability of the method determined in a blind study as well as the ease of the method and the low detection limit suggest that TIA may be a useful component of an analytical protocol for the identification of semen stains.

Acknowledgments

The authors are grateful to and thank Dr. G. Sensabaugh, University of California, Berkeley, CA, for providing semen stains with known p30 concentrations; Dr. S. Leto, Washington Fertility Study Center, Washington, DC, for providing semen samples; Dr. C. McWright for valuable discussions concerning this research; and Ms. K. Cooper for her excellent secretarial assistance in the preparation of the manuscript.

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Address requests for reprints or additional information to
Nicholas T. Lappas, Ph.D.
Department of Forensic Sciences
The George Washington University
Washington, DC 20052