Journal of Criminal Law and Criminology

Volume 57 | Issue 1 Article 15

1966

Immunological Identification of Human Seminal Stains

T. W. Mischler

E. P. Reineke

THE JOURNAL OF CRIMINAL LAW, CRIMINOLOGY AND POLICE SCIENCE
Vol. 57, No. 1

Printed in U.S.A.

IMMUNOLOGICAL IDENTIFICATION OF HUMAN SEMINAL STAINS

T. W. MISCHLER AND E. P. REINEKE

Terrence W. Mischler received the Bachelor of Science Degree in Zoology from Michigan State University following which he spent one year with the Indiana University, Department of Police Administration, conducting a survey of alcohol and road traffic problems in Grand Rapids, Michigan. Upon completion of this survey he returned to Michigan State University to pursue graduate work. The data reported herein are taken from a thesis submitted by Mr. Mischler in partial fulfillment of the requirements for the Master of Science Degree in Physiology.

E. P. Reineke is a Professor of Physiology at Michigan State University. Professor Reineke, who supervised Mr. Mischler's graduate work, has published extensively on physiological subjects.—EDITOR.

The only fully accepted way, at present, to identify seminal stains positively is the finding of human spermatozoa on the stain. A serious disadvantage of this method is that it is based on the assumption that sperm were originally present in the semen and it will not detect aspermic specimens. The finding of spermine, choline, and acid phosphatase have been used to identify human seminal stains. These tests are predicated on the fact that human semen has high concentrations of these substances, but the specificity of these tests is still being debated.

These difficulties have led to some limited use of immunological techniques for demonstrating the presence of organ and species specific proteins in seminal stains, thus using this as a means of seminal stain identification. Farnum (1901) first demonstrated that an antiserum to human semen would cause a precipitation reaction with an extract of a seminal stain 34 days old. Muller *et al.* (1958a) first described how immuno-diffusion tests could be applied to problems occurring in forensic science. These same authors (1958b) found that spots of sperm, urine, saliva, milk, cerebrospinal fluids, blood, and gastric fluid could be identified as being of human origin. Muller *et al.* (1959) stressed the importance of using the proper antiserum, stating that the rabbit should be used and repeated injections should be avoided, so as to eliminate species cross-reaction. Coombs *et al.* (1963) reported serological identification of human seminal stains by employing the Ouchterlony agar diffusion test. They used antiseminal plasma adsorbed with human serum and saliva. They found no cross-reaction with the dog, ram, or rabbit and a faint reaction with boar semen. Weil *et al.* (1959), using the complement fixation test, reported that semen could be detected on female genitalia after intercourse. This also indicated no cross-reaction between vaginal secretions and seminal plasma. Weil (1961) also found that antiserum to seminal plasma will not cross-react with semen from the ram, bull, or stallion.

These workers have all shown that it is possible to demonstrate seminal plasma proteins by immunological procedures and that this method, when combined with proper controls, is species and organ specific. The results presented here were obtained in an effort to characterize more fully proteins in seminal stains and to discover the conditions under which they remain immunologically reactive. It is hoped that these results will encourage a wider use of immunology in forensic science.

MATERIALS AND METHODS

The human semen used in this research was supplied by a local physician. The semen was approximately 32 hours old when taken to the laboratory, and it was kept under refrigeration for the greater part of this time. When received, the samples were immediately frozen and maintained in the frozen state until they were used experimentally. Human serum was obtained from a local hospital laboratory.

The preparation of antiserum to human serum (AHSr) and human semen (AHSp) was accomplished using the alum precipitation method. Five ml. of the sample was mixed with 16 ml. of water and then 18 ml. of 10% alum ($KAl(SO_4)_2 \cdot 12\,H_2O$) was added. The pH was adjusted to 6.5 with 5N NaOH; the resulting sediment was washed 3 times with isotonic saline containing 0.01% merthiolate (Eli Lilly and Co.) and made up to a final volume

TABLE 1

EXPERIMENTAL CONDITIONS IMPOSED ON SEMEN STAINS

1. Normal Stain:	Stains were extracted after drying at room temperature.
2. 42° C Stain:	The dried stains were subjected to a dry heat of 42° C for 30 minutes prior to extraction.
3. 62° C Stain:	The dried stains were subjected to a dry heat of 62° C for 30 minutes prior to extraction.
4. 68° C Humidified Stain:	The dried stains were subjected to 68° C for 30 minutes in a humid atmosphere prior to reaction.
5. Washed Stain:	The dried stains were washed vigorously, using a magnetic stirrer, for 5 minutes in soapy tap water and then while still moist submerged in the extraction solution.

of 20 ml. with this solution. Dutch black-belted rabbits were immunized by injecting 2 ml. of the alum precipitated protein intramuscularly once a week for 3 weeks in the thigh muscle of each rabbit. A control bleeding was done prior to the initial injection. The animals were bled during the fourth week by cardiac puncture. This blood and the control blood were allowed to clot at room temperature for 3 hours and then stored overnight at 10° C. The serum was decanted, centrifuged, 0.01% merthiolate added, and then frozen until needed. Human semen contains some serum proteins. Therefore AHSp was mixed with equal volumes of serum to remove antibodies produced against human serum proteins. This product was designated as adsorbed AHSp.

Human seminal stains were subjected to carefully chosen and controlled experimental conditions to determine whether the proteins in the stain remained immunologically reactive. The types of conditions that were used will be found in Table 1. Three different volumes of semen were pipetted on the cloth for each set of conditions. These amounts were 0.1 ml., 0.05 ml., and 0.03 ml. These graduations were to determine reasonable lower limits, with each set of conditions, for the finding of semen proteins in the stain. The stains were made on 1 cm^2 pieces of knit cotton cloth, dried at room temperature, and then subjected to various conditions already described. Extraction of the proteins was accomplished by submerging the cloth overnight at 10° C in 0.2 ml. of phosphate-buffered saline pH 7.4 containing 0.01% merthiolate. Coombs *et al.* (1963) indicated that extractions could be accomplished in a few hours. The extracts were analyzed using two methods. First, immunological double diffusion tests, in duplicate, were made on each extract. Second, each extract was examined microscopically to determine the presence or absence of spermatozoa.

The basic theory of immunological double diffusion was developed by Ouchterlony (1958). A layer of buffered agar has a number of holes called wells punched into it, often a series of 4 wells in a circle around a center well. The antigen is deposited into the center well and the antisera in the outer wells. The essential feature is the diffusion of antigen and antibody toward each other in the agar. There will be a meeting of diffusion fronts, and a zone of optimum proportions results somewhere in the over-lapping area. Antigen-antibody combination occurs in this zone, and a white precipitation line appears in the agar, indicating that at least one antigen-antibody system is present.

The procedure used in this research was to pipette 5 ml. of 1% oxoid Ionagar No. 2 (Colab No. L12) prepared in a phosphate buffer,[1] (pH 7.4, ionicity 0.15, merthiolate 0.01%) onto a 2 x 3 inch. glass slide. The agar was allowed to gel and then stored in a humid atmosphere for as long as one week before being used. A 5-well pattern (Fig. 1) with four wells, placed in a circle, each 5 mm. from the center well was drawn on paper. The slide was laid on this pattern and the wells were cut with a 10 mm. cork borer. The agar was removed with curved forceps, the wells charged with suitable reactants and left to diffuse in a humid atmosphere at 25° C.

Precipitation occurred overnight and was complete in 2 days. The slide was allowed to stand one day in phosphate buffered saline at pH 7.4 and another day in distilled water to remove protein not precipitated. Then a moistened piece of Whatman No. 40 filter paper was laid on the agar, and the preparation was dried to a film in an incubator. Staining was done with Amidoschwartz[2]

[1] Phosphate buffer pH 7.4 ionicity 0.15
Na_2HPO_4 12.8 gm.
$NaH_2PO_4 \cdot H_2O$ 2.62 gm.
H_2O 1000 ml.

[2] Amidoschwarz stain:
Amidoschwarz 0.5 gm.
12% Acetic acid 225 ml.
1.6% Sodium acetate 225 ml.
Glycerol 50 ml.

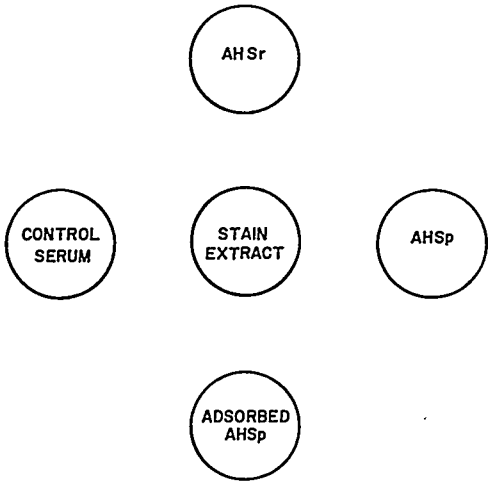

FIGURE 1
Arrangement of wells and reactants for immunological double diffusion tests.

and destaining with 5% acetic acid. This provided a permanent graphic record of the results.

RESULTS

It was found that appropriate controls must be set up to insure correct interpretation of the results. Serum taken from rabbits prior to immunization proved negative when diffused against semen. When adsorbed AHSp was diffused against human serum no precipitation resulted, indicating that antibodies to blood serum proteins had been removed. When pooled human saliva was diffused against adsorbed AHSp a very faint precipitin line resulted. A comparison of semen and saliva, with adsorbed AHSp revealed no serological identity between the saliva line and any of the 4 visible semen components. Therefore, no further adsorption was considered necessary. Neither human urine nor extracts of the cloth used as a vehicle for semen stains resulted in precipitation when diffused against adsorbed AHSp. Coombs *et al.* (1963) reported no reaction with human sweat. These results and those reported by others demonstrate that, with proper controls, adsorbed AHSp is organ and species specific.

Results of analysis of all stains are summarized in Table 2. The precipitin patterns of the 0.05 ml. stains were typical of those obtained from all stains and are presented in Figure 2. Table 2 shows that for each experimental condition and semen volume all 3 antisera reacted with stain extracts. This demonstrates that the stains contain seminal proteins that react immunologically with AHSp. These proteins were resolved into those common with serum and those specific to semen by their reaction with AHSr and adsorbed AHSp respectively. Except for the 62° C stain, each antiserum produced at least 2 precipitin lines and there never was any reaction with the control rabbit serum. Both within and between the several experimental conditions employed there was good consistency in the number of precipitin lines obtained with the three antisera. Finally, spermatozoa were found in the majority of stain extracts; the only exceptions were stains made with minimal quantities of semen.

TABLE 2
ANALYSIS OF HUMAN SEMINAL STAINS

Stain	Number of Precipitation Lines			Presence of Sperm
	AHSr	AHSp	Adsorbed AHSp	
Normal Stain				
.1 ml	2	5	3	Yes
.05 ml	3	6	3	Yes
.03 ml	2	6	3	Yes
42° C Stain				
.1 ml	4	6	2	Yes
.05 ml	2	5	2	Yes
.03 ml	3	3	2	No
62° C Stain				
.1 ml	3	6	1	Yes
.05 ml	1	8	1	Yes
.03 ml	1	6	1	No
68° C Humidified Stain				
.1 ml	2	3	3	Yes
.05 ml	3	5	3	No
.03 ml	4	6	4	No
Washed Stain				
.1 ml	2	3	3	Yes
.05 ml	2	3	3	Yes
.03 ml	2	3	3	No

DISCUSSION

It was possible to identify all of the prepared stains as human semen, no matter what experimental conditions were imposed on the stains or the amount of semen that was pipetted on the cloth. This was shown by the fact that adsorbed AHSp always produced a precipitin reaction. AHSr and AHSp also reacted in all tests, thus demonstrating that non-organ specific proteins

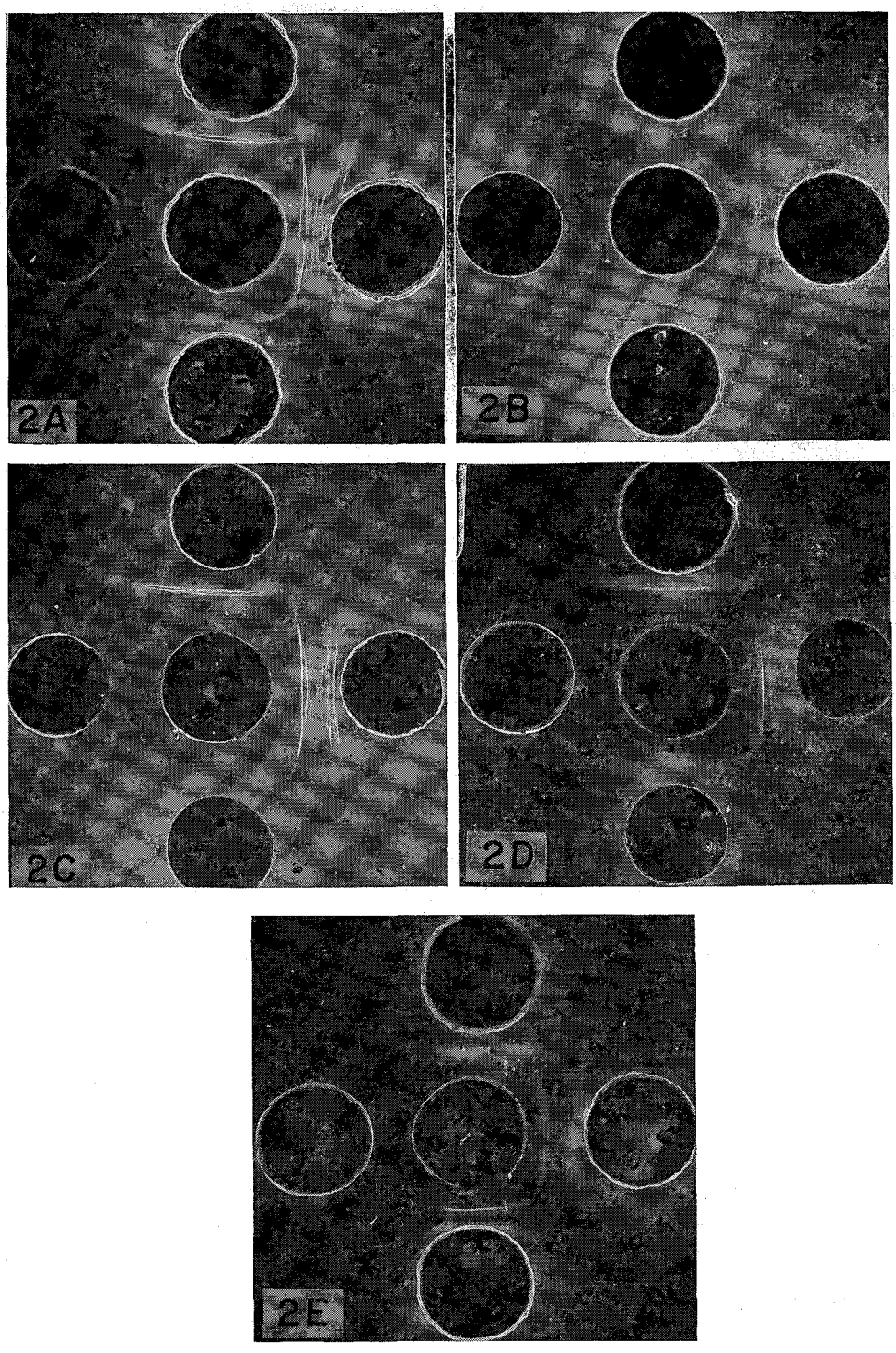

FIGURE 2

In each case 0.05 ml. of semen was dried on cloth and extracts were subjected to double diffusion according to the pattern shown in Figure 1.

A. Normal stain
B. Stain exposed to 40° C for 30 minutes
C. Stain exposed to 62° C for 30 minutes
D. Stain exposed to 68° C, moist heat, for 30 minutes
E. Stain washed with detergent prior to extraction

Note that in each case precipitation lines occurred with all 3 antiserums, and in particular, that the organ and

could be found in all extracted stains. A reasonable lower limit of semen, 0.03 ml., that could be found was established, but as this amount always reacted strongly it is believed that smaller amounts could be detected. Similar precipitin patterns and number of precipitation lines within and between conditions showed the specificity of the results.

The conditions imposed upon the seminal stains demonstrated that the proteins remained immunologically reactive under a variety of conditions. This is seen by the fact that not only did the normal and 42° C stains retain their reactivity; but stains subjected to temperatures normally in the range of protein denaturation, either dry heat (62° C stains) or moist heat (68° C humidified stain), also remained immunologically reactive. Finally, washing the stained cloth in soapy tap water demonstrated not only the ability of the proteins to retain their reactivity under adverse conditions, but also their rather firm fixation to the cloth.

This research shows that, within the conditions imposed on the stains and with the amounts of semen originally present, the seminal proteins will remain immunologically reactive. Therefore, it is believed that human seminal stain proteins will retain their reactivity over a great variety of environmental conditions and amounts of semen. The results of this research indicate that the immunological double diffusion technique, along with proper controls, can be used to positively identify human seminal stains. A lack of a positive reaction is a good indication that the stain is not human semen.

Parallel investigations were conducted on the properties of human semen using cellulose acetate electrophoresis, disc electrophoresis, immunoelectrophoresis, and immunological double diffusion. This work will be published elsewhere. Double diffusion was chosen to identify human seminal stains for these reasons: (1) It is highly organ- and species-specific for human semen; (2) Reproducible results are easily obtained and observed; (3) A permanent record of results is obtained.

Summary

The proteins of human seminal stains were investigated by using immunological double diffusion. The purpose was to more fully characterize these proteins with regard to the conditions under which they remain immunologically reactive. Human semen in three graduated amounts (0.1 ml., 0.05 ml., and 0.03 ml.) was pipetted onto pieces of cloth. These stains were subjected to a variety of experimental conditions, which consisted of dry heat at 42° C and 62° C for 30 minutes, moist heat at 68° C for 30 minutes, and washing in soapy water. It was possible to extract the proteins and identify them by means of an organ and species specific antiserum to human semen. This research demonstrates that human seminal stain proteins remain immunologically reactive over a variety of environmental conditions and that this technique is sensitive to small semen volumes. The results of this research show that, since seminal proteins are immunologically stable, the technique presented in this paper could be used to identify human seminal stains.

Acknowledgments

The authors wish to thank Drs. James S. Feurig and Raymond Kinzel for their aid in procuring the human semen. We greatly appreciate the advice of Drs. Lloyd C. Ferguson and Harold D. Hafs in planning this research. Finally, we want to thank Professor Ralph F. Turner for his helpful suggestions with regard to the preparation of this paper.

The work reported herein was supported in part by National Institutes of Health Training Grant GM 1121-02.

REFERENCES

Coombs, R. A. A., Richards, C. B., and Dodd, B. 1963. Serological identification of seminal stains. Med. Sci. and Law, *3:* 65.

Farnum, C. G. 1901. The biologic test for semen. J. A. M. A., *37:* 1721.

Muller, M., Fontaine, G., Muller, P., and Donazzan, M. 1958a. Application medico-legale de la reaction antigene-anticorps en milieu gelifie. Lille. Med., *3:* 218.

Muller, M., Fontaine, G., and Muller, P. H. 1958b. Identification medico-legale de differents liquides biologiques pour les reactions immunologiques en milien gelifie. Lille. Med. *3:* 773.

Muller, M., Fontaine, G., and Muller, P. H. 1959. Interet medico-legal de l'etude immuno-chimique du sang animal. Lille. Med., *4:* 150.

Ouchterlony, O. 1958. Diffusion-in-gel methods. Prog. Allergy, *5:* 1.

Weil, A. J., Wilson, L., and Finkler, A. E. 1959. Immunological test for semen on female genitalia as evidence of intercourse. J. For. Sci., *4:* 373.

Weil, A. J. 1961. Antigens of the adnexal glands of the male genital tract. Fertil. and Steril., *12:* 538.