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Immunologic Determination of Seminal Secretions by a Latex Microtiter Technique

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ABSTRACT: The presence of human seminal plasma proteins in biological stains was demonstrated by an absorption test using anti-human seminal protein rabbit serum. A decreased degree of antibody activity was titrated on a microtiter plate by the agglutination of latex particles coated with human seminal plasma proteins. This method of test was sensitive and highly specific. The latex reagent for this test could be preserved in a refrigerator for over one year without the loss of reactivity.

KEYWORDS: pathology and biology, semen, human identification, human seminal plasma, latex particles, microtiter method

Seminal stains are important in forensic medical laboratory tests. Immunologic methods for determining the presence of seminal secretions have been reported by some workers [*1-6*]. These methods of test, however, are rarely performed in practice, probably because of the difficulty of antiserum production.

Some authors [*7-10*] have studied the immunochemical properties of seminal plasma specific proteins and found that ring or in gel precipitation tests using specific antisera were available to the forensic science laboratory. Others participated in the invention of stable latex particles for immunologic reactions [*11*] and reported a method for species identification from bloodstains by the absorption test using latex particles [*12*]. This report deals with a product obtained as a result of our cooperative research and a new method for determining human seminal secretions, which may be more practically applicable owing to its specificity and sensitivity.

Materials and Methods

Human Seminal Plasma

Fresh semen from eight volunteers, 20 to 25 years old, was pooled and stored overnight at 5°C, and the supernatant was collected after centrifugation for 30 min at 10 000 rpm. The

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protein concentration of undiluted seminal plasma was 68 mg/mL and was stored at −75°C until use.

Antisera

Rabbits were immunized five times at intervals of six to seven days with an emulsified mixture of 1 mL of Freund's complete adjuvant and an equal volume of human seminal plasma. Two weeks after final immunization, blood was obtained from the rabbits and separated antiserum was inactivated by heating for 30 min at 56°C. The antisera formed eight to nine precipitation lines by immunoelectrophoresis against human seminal plasma. For the purification of antibody activity, one part of the antiserum was absorbed with ⅓ part of human serum, ⅓ part of clarified colostrum, and 1/20 part of saliva concentrated 20 times. Additional absorptions were repeated, if necessary. In the ring precipitation test, an absorbed antiserum used in this study reacted to human seminal plasma diluted 2000 to 3000 times, and the antiserum could be diluted up to eight times and still obtain positive reactions. Immunoelectrophoretic analysis of the absorbed antiserum showed a single precipitation line produced by the human seminal specific antibody, which was called anti-γ-seminoprotein in previous reports [*7,8,10*] and is probably identical to the antibodies to E1 reported by Li and Shulmann [*13*] or p30 described by Sensabaugh [*14*]. Titers of the antiserum determined by the agglutination of latex particles coated with human seminal plasma were 1:160 (slide technique) and 1:1280 (microtiter technique). The seminal-specific protein was purified by column chromatography followed by electrofocusing as in earlier reports [*8,10*], and antisera to this substance were also provided by a method similar to that described above. This group of antisera was absorbed with small quantities of human serum, colostrum, and concentrated saliva. Since some antisera to the seminal plasma nonspecifically agglutinated uncoated latex particles even after dilution to 100 times, they were mixed with an equal quantity of 0.01*M* dithiothreitol, as described by Su [*15*], for obtaining specific reactivity. Then 1/100 volume of 10% sodium azide was added to the antiserum and it was stored at 5°C until use.

Latex Particles Coated with Human Seminal Plasma

Stock human seminal plasma was thawed and diluted with sodium azide and added to glycine-buffered saline to reach a protein concentration of 4 mg/mL. This diluted solution was added to an equal quantity of 1% latex suspension (SDL-59-10, supplied by Takeda Chemical Ind., Osaka). After the mixture stood for 30 min at room temperature, 30% bovine serum albumin was added to reach a concentration of 1% and stored for two or three days at 5°C. This mixture was then centrifuged for 10 min at 5000 rpm to precipitate the latex particles, which were resuspended into a freshly prepared 0.1% bovine serum albumin in the buffered saline to provide a 0.5% stock latex suspension. Three lots of the stock suspension were prepared by using a pool of the seminal plasma, and these were stored at 5°C and diluted ten times with 0.5% bovine serum albumin in the buffered saline immediately before use.

Biological Stains

Clean gauze or cotton strings were stained with various body fluids, completely dried, and stored at room temperature. Vaginal secretions were tested before the staining process to show that no spermatozoa could be observed by microscope.

Microtiter Latex Technique

An appropriate size of experimentally prepared test specimen was immersed in methanol for 5 min and followed by complete drying on a glass slide at room temperature. The specimen was then dipped into 75 μL of diluted antiserum to the seminal plasma, titer of 1:8 to 1:16 by the microtiter latex agglutination technique. After a 2-h incubation at room temperature with 20 to 30 shakings every 20 to 30 min, the specimen was removed and the diluted antiserum was serially diluted with the buffered saline on a disposable U-type microtiter plate. To each well of the plate was added one drop of 0.05% suspension of the seminal-plasma-coated latex, which had been freshly prepared by the dilution of stock 0.5% latex suspension with 0.5% bovine serum albumin in the buffered saline. The plate was vibrated by a microtiter mixer (Tomy Seiko, Tokyo) for 2 min and allowed to stand overnight at room temperature in a moist chamber. Results were read by the settling patterns of latex particles. When no agglutination occurred, the particles completely precipitated to the deepest portion of the microtiter well and formed small dots of pure white. Agglutination patterns were thin, broadly distributed precipitations of aggregated particles. A slight increase in the diameter of the precipitated area, 1.5 times or less compared to that of the no-antibody-added control, was considered to be no agglutination. Test results were determined to be positive for human seminal secretions when complete inhibition occurred through all dilutions of the antiserum. Procedures for the slide technique were similar to those in an earlier report [*12*].

Results

Preliminary Studies

Biological stains were extracted with the glycine-buffered saline and 25 μL of the extracts were mixed with an equal quantity of 0.05% seminal-plasma-coated latex on a microtiter plate. Two in seven extracts from the stains of vaginal secretions nonspecifically agglutinated the seminal-plasma-coated latex particles, whereas none of the extracts from the stains of other body fluids caused such an undesirable phenomenon. Since methanol fixation of the vaginal secretion stains prevented occurrence of the nonspecific agglutination, all specimens for this study were thereafter fixed with methanol before testing.

As a part of preliminary studies, a rapid and simple slide technique was used. However, the slide technique was insufficiently sensitive because ten times as much test material was required to obtain positive results as compared to the microtiter latex technique.

Determination of Human Seminal Secretion

After absorption with 0.15- by 0.15-cm gauze stained with various human body fluids, anti-human seminal plasma was titrated to determine the degree of decreased agglutinating capacity by the use of the seminal-plasma-coated latex. As shown in Fig. 1, human seminal stains completely inhibited the antiserum, whereas no inhibition occurred in tests of other human body fluids, such as vaginal secretions, meconium, colostrum, saliva, blood, and urine. Another experiment showed no inhibition of the antiserum by the stains of amniotic fluids, synovial fluids, bronchial secretions, or pleural exudates. Human and animal bloodstains partially inhibited the anti-human seminal plasma, as shown in Fig. 1 and Table 1. However, this phenomenon did not lead to misinterpretation of results because it was so weak as to cause complete inhibition of the antiserum.

Different quantities of various body fluids were tested by this method to prove its sensitivity and specificity. The minimum quantity of material needed for positive results was 25 μL of pooled human seminal plasma diluted 10 000 times or seminal stain on a 0.3-cm-long thin cotton string. The largest unstained fabric tested was a 1.5-cm^2 sheet of cotton containing 75-μL diluted antiserum, which was the limit for obtaining absorbed antiserum by aspiration

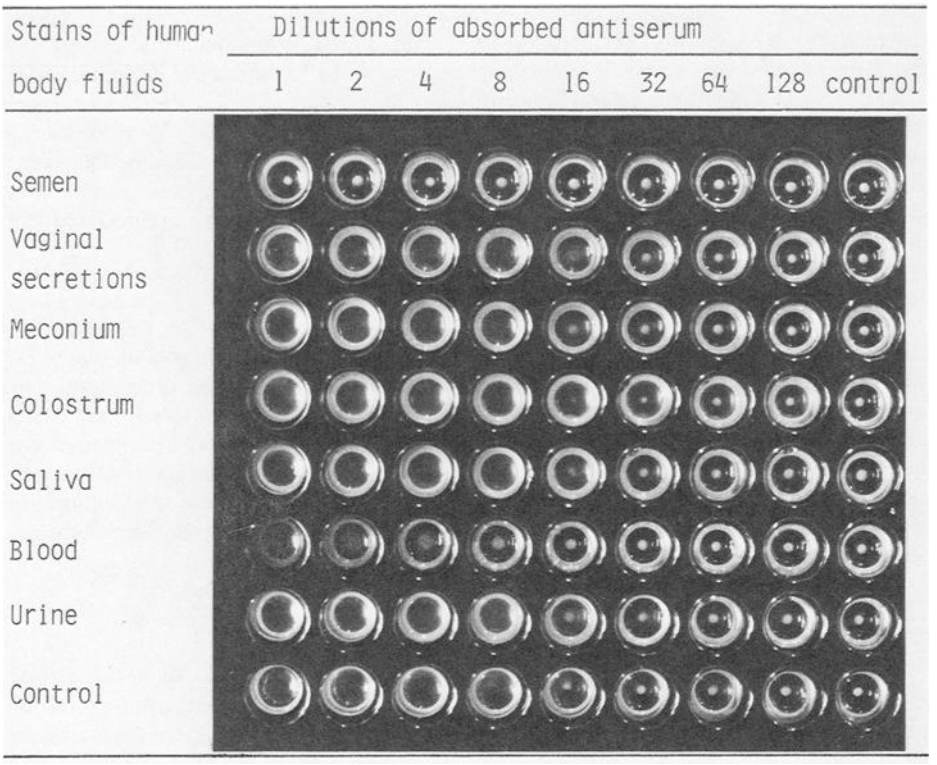

FIG. 1—*Patterns of anti-human seminal plasma absorption test by the latex microtiter technique. The controls at right had no antibody added. Glycine-buffered saline was added to the controls in the lowest row.*

TABLE 1—*Decrease of anti-human seminal plasma after absorption by animal body fluids.*

Stains		Dilutions of Absorbed Antiserum					
Kinds of Body Fluids	Species	1	2	4	8	16	Control (Buffer)
Semen	human	−	−	−	−	−	−
	dog	+	+	+	weak	−	−
	cattle	+	+	+	weak	−	−
	rat	+	+	+	weak	−	−
Saliva	dog	+	+	+	weak	−	−
	rabbit	+	+	+	−	−	−
Blood	monkey	+	+	+	−	−	−
	dog	+	+	+	weak	−	−
	cat	+	+	+	−	−	−
	pig	+	+	+	−	−	−
	goat	+	+	+	−	−	−
	rabbit	+	+	+	weak	−	−
	rat	+	+	+	weak	−	−
	chicken	+	+	+	weak	−	−
Control (unstained fabrics)	...	+	+	+	weak	−	−

using a pipet; our results indicated no occurrence of nonspecific inhibition of the antibody activities. These results were obtained by using stains stored for one month after preparation. Two to four times as much seminal stain was required to inhibit the antiserum after storage for twelve months at room temperature.

This test was carried out with an antiserum whose activity was purified by previous absorption with body fluids other than semen. However, tests with three unabsorbed antisera, cross-reactive to human serum and colostrum by the immunodiffusion test, also showed good specificity for human semen. Both kinds of rabbit antisera to purified seminal specific antigens and crude seminal plasma showed similar sensitivity and specificity.

Preservation of Stock Latex Reagents

In the case of stock latex Reagent 3 in Fig. 2, the reactivities of seminal-plasma-coated latex stored at 5°C sometimes decreased during the two to three weeks after preparation, but the activities partially recovered at the fifth to seventh week and were stable for one year. Other reagents showed constant reactivities through test periods. As an experiment to secure the durabilities of the reagents, an antiserum to human seminal plasma and seminal-plasma-coated latex suspension (two months after preparation) were sent by mail without refrigeration in summer, and these showed good reactivities after a three-day transportation period.

Discussion

The anti-seminal plasma absorption test using the microtiter latex technique is evidently suitable for the immunologic identification of seminal stains. The principal advantage of this test is excellent sensitivity. Antisera for this test must contain anti-seminal protein specific antibodies, but purification of antibody activity is unnecessary. The presence of human seminal secretions is shown by the complete neutralization of the antibodies in an antiserum to human seminal plasma proteins; such results were obtained only with seminal secretions. In the case of absorption by other body fluids, antibodies to human seminal specific antigens

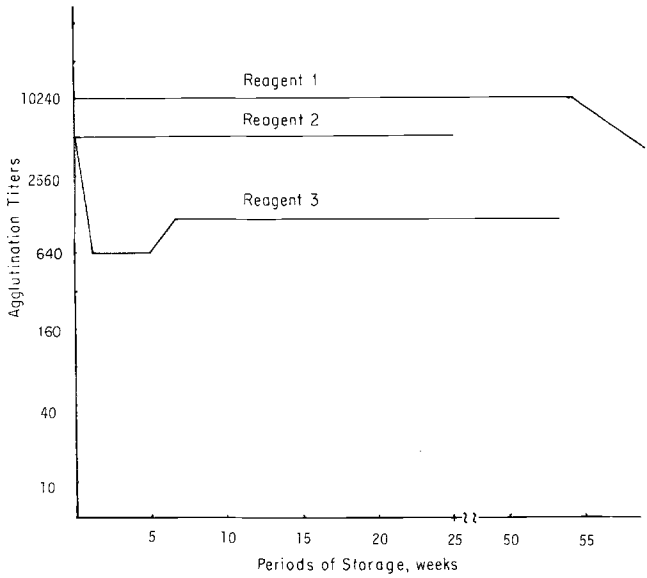

FIG. 2—*Stability after storage of seminal-plasma-coated latex reagents.*

remained unabsorbed, irrespective of the neutralization of cross-reacting antibodies, and this result was concluded to be negative for the presence of human seminal proteins.

Two days are needed to conduct the test; however, working time is short and many specimens can be tested at a time. If a more rapid test is required, the slide technique used in the preliminary study of this report may be a good choice.

Partial neutralization patterns shown by the tests of bloodstains are probably a result of the nonspecific agglutination inhibition caused by hemoglobin. Immunologic absorption by trace antigens in human serum is negligible, since animal bloodstains showed similar partial neutralization patterns.

Methanol fixation of the stains was thought to be an adequate process for obtaining clear test results. Although fixation of the stains lowered the sensitivity by decreasing the solubility of proteins, nonspecific agglutination by vaginal secretions did not occur. Some parts of contaminated substances, if present, could also be removed by this process.

Preparation of the seminal-plasma-coated latex suspension was laborious, partly because of the difficulty of obtaining fresh, clean semen suitable for this purpose. However, this reagent could be preserved for a long time after preparation, and each test consumed a very small quantity of the reagent. Rabbit antisera appropriate to this test could be provided without much effort because antisera with low-titered antibody could be used. Consumption of the antisera was also low.

Some data obtained from actual case work will be reported elsewhere.

References

[1] Farnum, C. G., "The Biologic Test for Semen," *Journal of the American Medical Association,* Vol. 37, No. 26, Dec. 1901, p. 1721.

[2] Kusumoto, T., "On the Specificity of Anti-Human Semen Precipitin," *Nagasaki Igakkwai Zassi,* Vol. 14, No. 4, April 1936, pp. 524–548 (in Japanese).

[3] Weil, A. J., Wilson, L., and Finkler, A. E., "Immunological Test for Semen on Female Genitalia as Evidence of Intercourse," *Journal of Forensic Sciences,* Vol. 4, No. 3, July 1959, pp. 372–377.

[4] Coombs, R. A. A., Richards, C. B., and Dodd, B., "Serological Identification of Seminal Stains," *Medicine, Science and the Law,* Vol. 3, No. 2, Jan. 1963, pp. 65–70.

[5] Mischler, T. W. and Reineke, E. P., "Immunological Identification of Human Seminal Stains," *Journal of Criminal Law, Criminology and Police Science,* Vol. 57, No. 1, March 1966, pp. 107–111.

[6] Fukuyama, T. and Iki, H., "A New Method of Blood Group Determination of Seminal Fluid Mixed with Vaginal Secretion, Menstrual Blood or Blood," *Reports of the National Research Institute of Police Science,* Vol. 22, No. 4, Dec. 1969, pp. 243–248 (in Japanese).

[7] Hara, M., Inoue, T., Koyanagi, Y., Yamasaki, H., Fukuyama, T., and Iki, H., "Immunoelectrophoretic Studies of the Protein Components in Human Seminal Plasma," *Japanese Journal of Legal Medicine,* Vol. 23, No. 2, March 1969, pp. 117–122 (in Japanese).

[8] Koyanagi, Y., Hara, M., Inoue, T., and Gohara, K., "Isolation of Antigenic Component Specific for Human Seminal Plasma 'γ-Seminoprotein (γ-Sm)' by Electrofocusing," *Japanese Journal of Legal Medicine,* Vol. 26, No. 2, March 1972, pp. 78–80 (in Japanese).

[9] Hara, M., "Forensic Immunological Studies on Human Body Fluids," *Japanese Journal of Legal Medicine,* Vol. 28, No. 3, May 1974, pp. 164–176 (in Japanese).

[10] Koyanagi, Y., "On the Preparation, Chemical Analysis and Some Physico-Chemical Characterization of 'γ-Seminoprotein,' an Antigenic Component Specific for Human Seminal Plasma," *Igaku Kenkyu (Acta Medica),* Vol. 44, No. 5, Aug. 1974, pp. 529–548 (in Japanese).

[11] Matsuzawa, S., Kimura, H., Itoh, Y., and Yeh, C. W., "Studies on Latex Agglutination Reactions. (1) Compared Results of 69 Polystyrene Latex Preparations," *Japanese Journal of Legal Medicine,* Vol. 29, No. 2, March 1975, pp. 103–109 (in Japanese).

[12] Itoh, Y., "Absorption Test Using Latex Particles as the Indicator System for the Species Identification of Bloodstains and Muscles," *Journal of Forensic Sciences,* Vol. 24, No. 3, July 1979, pp. 561–568.

[13] Li, T. S. and Shulmann, S., "Immunoelectrophoretic Analysis of Human Seminal Plasma Fractions after Fractionation by Various Methods," *International Journal of Fertility,* Vol. 16, No. 2, April–June 1971, pp. 87–100.

[14] Sensabaugh, G. F., "Isolation and Characterization of a Semen-Specific Protein from Human

Seminal Plasma: A Potential New Marker for Semen Identification," *Journal of Forensic Sciences,* Vol. 23, No. 1, Jan. 1978, pp. 106–115.

[15] Su, L. T., "Studies on Nonspecific Agglutination of Latex Particles," *Japanese Journal of Legal Medicine,* Vol. 32, No. 3, Aug. 1978, pp. 152–161 (in Japanese).

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