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Detection of Prostate Specific Antigen by ELISA

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ABSTRACT: A method for the detection of prostate specific antigen (PSA or P30) by enzyme linked immunosorbent assay (ELISA) has been developed. The method was evaluated for sensitivity and specificity and compared to other methods of semen identification on numerous casework samples. A sensitivity of less than 1 ng/mL PSA is routinely achieved. Numerous domestic contaminants and body fluids were tested and none were found to give a false-positive result. However, false-negative results did occur in samples contaminated with detergents. Additionally, some poorly preserved items of evidence did not give positive results where expected. In spite of these minor limitations, this method was found to be highly sensitive and specific and an efficient method for identifying semen in forensic casework.

KEYWORDS: pathology and biology, prostate specific antigen, P30, ELISA

Prostate specific antigen (PSA or P30) is a protein produced only in the prostate and thus can be used to identify semen [*1–4*]. Detected by crossover electrophoresis, rocket electrophoresis, or ELISA, it has been used for semen identification for years (*5–11*). Detection by ELISA offers several advantages: ELISA is more sensitive than standard immunoelectrophoretic methods and more cost effective than sperm search when the cost of labor is considered. The assay parameters were optimized with consideration for reagent costs and assay time. A number of experiments were conducted to validate the procedure, to determine the best threshold for casework analysis and to determine the sample dilution factor most appropriate for casework.

Materials and Methods

Detection of PSA by ELISA

PSA was detected using a modification of the two-site (double antibody sandwich) ELISA methods of Baechtel and Fletcher [*12,13*]. Immulon II microtiter plates (Dynatech, cat# 011-010-3450) were coated with mouse monoclonal anti-human prostate specific antigen (INCStar, cat# 22516) and IgG kappa chain mouse myeloma protein (Sigma, cat# M9269). After overnight incubation at 4°C each plate was blocked with a solution of Hammerstein casein (0.02%) in phosphate buffered saline (0.01 M, pH 7.4, 0.98%

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NaCl). Stain extracts (0.5 cm^2 in 100 μL water) were diluted 1/50 or 1/250 in PBS-casein. The dilute extracts along with PSA standards of 1, 5, and 10 ng/mL were added to each plate, 100 μL per well, and incubated for 1 h at room temperature. (PSA standard was kindly provided by the Federal Bureau of Investigation.) Each sample or standard occupied four wells: two coated with monoclonal anti-PSA and two coated with mouse myeloma protein. Following sample incubation, each plate was washed three times with PBS-casein. All washes were done with an Ultrawash II microtiter plate washer (Dynatech). The plates were then incubated with rabbit polyclonal anti-PSA (Dakopatts, cat# A562), 100 μL per well, for 1 h at room temperature. Plates were washed as above and incubated with goat anti-rabbit IgG-alkaline phosphatase conjugate (Sigma, cat# A7778), 100 μL per well, for 1 h at room temperature. Plates were washed as above for the final time and incubated with 4 mM p-nitrophenyl phosphate (Sigma, cat# 104-105) in 1 M diethanolamine buffer, pH 9.8, 100 μL per well, at 37°C for 1 h. Plates were read for absorbance at 405 nm on a Molecular Devices microtiter plate reader and the data exported to a Microsoft Excel spreadsheet. All reagents used were added to the plates with a Titertek dispenser (Flow, Inc.).

Data Analysis and Threshold

The Excel spreadsheet calculates a final absorbance value for each standard and sample. The first column on the microtiter plate is a substrate blank. The average of the eight wells in this column is subtracted from all other readings on the plate. For each standard or sample the average of the corresponding mouse myeloma wells is subtracted from the average of the monoclonal anti-PSA wells. This difference is then compared to the plate threshold. A value greater than the threshold indicates the presence of PSA. The threshold is the higher of the calculated threshold or the default threshold. The calculated threshold is twice the average plus three standard deviations of the eight wells in column 2, the reagent blank column. The default threshold is 100 milliabsorbance units (mau). Data used to determine this threshold were obtained from samples known to be negative, control areas expected to be negative and the calculated thresholds of 120 plates.

Sensitivity and Dilution Factor

The sensitivity of the assay was determined both by comparison of results to the known standards obtained from the FBI and from Scripps Institute (Cat# P0724) and by direct comparison to crossover and rocket immunoelectrophoresis. Liquid semen from one individual was diluted by doubling dilution from 1/2 to 1/128 000. For PSA ELISA, the dilutions were treated as stain extracts and further diluted 1/50 and 1/250. For crossover and rocket immunoelectrophoresis the dilutions were treated as stain extracts and used without further dilution. Crossover immunoelectrophoresis was performed for 20 min in 1% EEO 0.25 agarose in TBE buffer, pH 9.1, against polyclonal anti-P30 (Seri, cat# A404) [*15*,*16*]. Precipitin bands were visualized by staining with Coomassie Blue. Rocket electrophoresis was performed overnight in 1% EEO 0.07 agarose, TBE buffer, pH 9.1, containing 1.5% polyclonal anti-P30 (Seri, Cat# A404) [*15*,*16*]. Rockets were visualized by staining with Coomassie Blue.

Fresh extracts from casework material were tested for PSA by ELISA at dilution factors of both 1/250 and 1/50. While a lower dilution factor will increase the possibility of detection for weak samples it will also increase the background reaction of some very strong samples. The number of samples that were positive at both dilutions was compared to the number of samples positive at only the 1/50 dilution and the effect of the lower dilution factor on background was examined.

Specificity: Domestic Contaminants and Body Fluids

Pairs of stains were made on cotton cloth of six human body fluids (urine, saliva, blood, milk, vaginal fluid, sweat) and 27 various household products (cosmetics, cleaning agents, foods, lotions, contraceptives, etc.). One stain of each pair was then stained with a 1/10 dilution of liquid semen. All stains were allowed to dry for 72 h at room temperature. Both stains in each pair were tested for acid phosphatase by a spot test on a small cutting using Fast Blue B and alpha-napthylphosphate [*14*] and PSA by ELISA using extracts diluted to both 1/50 and 1/250.

Stain extracts from ten bloodstains, 15 urine stains, 31 vaginal swabs, and 25 saliva stains were diluted 1/20 and tested for PSA by ELISA.

Casework Samples: Comparison of AP, Sperm Search, and ELISA

Stains and swabs from casework material were examined for acid phosphatase, sperm and PSA by ELISA using a 1/250 dilution factor. At the time that these samples were processed no decision had been made as to the dilution factor that was best. Relevant control areas, negative for acid phosphatase, were examined for PSA by ELISA and in some instances for sperm. When sperm were found in a "control" area it was counted as an acid phosphatase negative, sperm positive sample rather than as a control area. Acid phosphatase was detected by spot test as described as above. The test was done either on small cuttings taken from the stain or on damp filter paper blotted on the stain. Sperm were observed by staining smears on microscope slides prepared from each stain with Nuclear Fast Red and Indigocarmine. PSA ELISA was run on the same extract prepared for ABO/Lewis testing by ELISA. Due to logistical constraints many of the extracts were frozen, from one week to six months, until PSA ELISA testing could be conducted.

Results and Discussion

Data Analysis and Threshold

Known negatives—Nearly 200 samples known not to contain PSA were examined. These consisted of body fluids and domestic contaminants as well as simply blank samples. The distribution of the test values for these samples is shown in Fig. 1.

Control areas—Over 200 control areas from casework samples were evaluated. The distribution of the test values for these samples is shown in Fig. 2.

Calculated thresholds—The distribution of the calculated thresholds from 120 plates is shown in Fig. 3.

The threshold is calculated using twice the average of the reagent blank column (background) plus three standard deviations rather than simply the average plus three standard deviations. The average is doubled to make the threshold more conservative. Even with this safety margin there are occasionally plates with very low thresholds. Approximately 13% of the plates tested had thresholds below 100 mau. Evaluation of the known negative samples and the control area samples indicates that these samples can produce test values greater than zero, with the observed maxima being 30 mau and 43 mau, respectively. To avoid the theoretical combination of a very low threshold with a very high test value for a negative sample, a potential false positive, a default threshold of 100 mau has been incorporated. However, in our experience this situation is not likely to occur. We find that plates with low thresholds, that is, very low background, tend to have low background values across the plate.

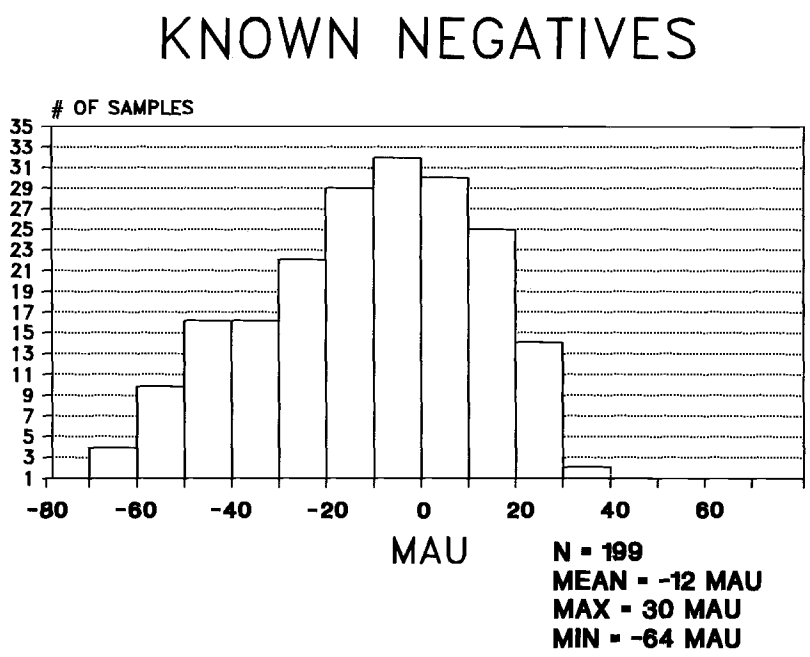

FIG. 1—*The distribution of 199 samples known to be negative. MAU = milliabsorbance units.*

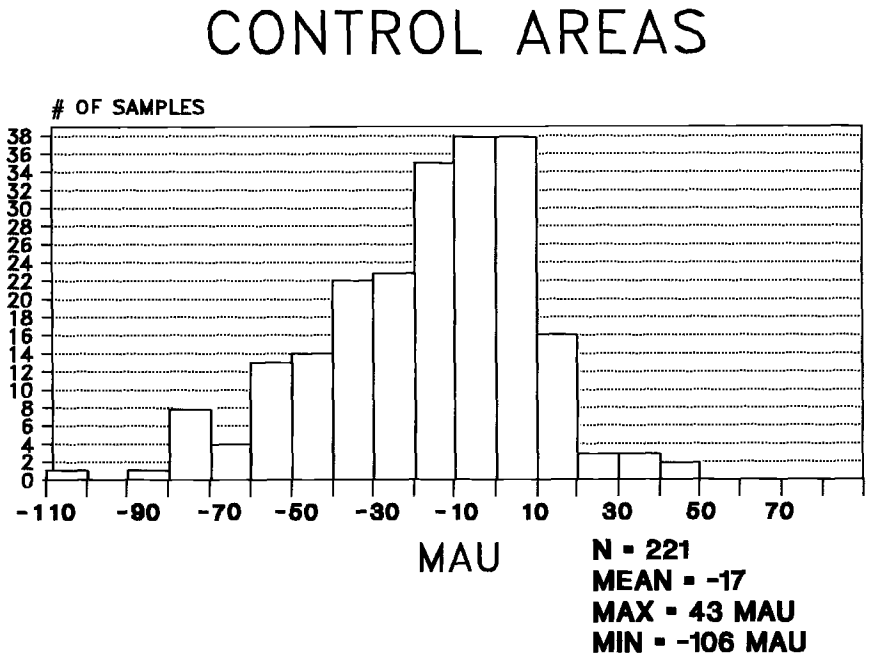

FIG. 2—*The distribution of 221 control areas from casework material. MAU = milliabsorbance units.*

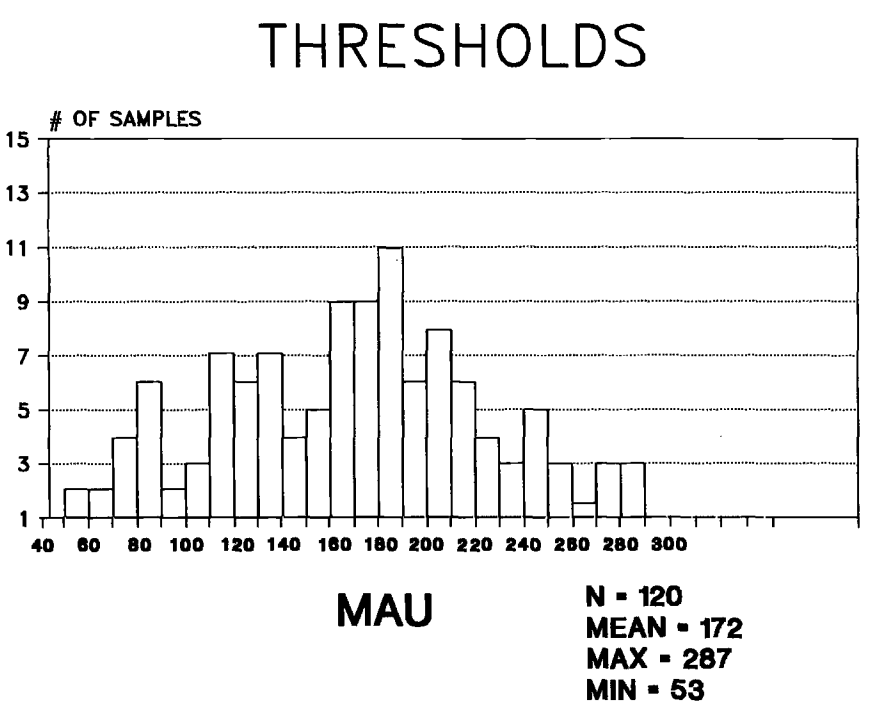

FIG. 3—*The distribution of calculated thresholds from 120 plates. MAU = milliabsorbance units.*

Sensitivity and Dilution Factor

This assay is easily capable of detecting 1 ng/mL PSA as determined both by the standard from the FBI and the PSA standard produced by Scripps.

The results of the direct comparison are shown in Table 1. Crossover and rocket immunoelectrophoresis gave positive results through the $1/512$ dilution of semen. ELISA gave positive results though the $1/2000$ dilution and the $1/8000$ dilution depending on whether the extract was diluted $1/250$ or $1/50$. This ELISA is more sensitive than either of the two immunoelectrophoresis methods used. This comparison does not suggest the semen dilution that may be expected to give a positive reaction with any of these methods; PSA levels in semen have been shown to vary through the population [*1*]. It is simply a comparison of three methods used on the same samples.

Of the casework samples run at both $1/250$ and $1/50$, 80 were positive at at least one dilution. The majority of stains were detected at both the $1/250$ and $1/50$ dilutions (88%). About one in nine stains (12%) were positive only at the $1/50$ dilution. The $1/250$ dilution worked well for most stains and gave less background reaction with very strong stains. However, the background from very strong stains with the $1/50$ dilution was not unacceptable and the advantage of detecting weak samples that might otherwise be missed makes the $1/50$ dilution the better choice for casework. The effect of the lower dilution factor on contaminated samples is addressed below.

Specificity: Domestic Contaminants and Body Fluids

The results of acid phosphatase and PSA testing on the paired stains are shown in Table 2.

TABLE 1—*Sensitivity: direct comparison.*

Dilution of semen	ELISA 1/250 dilution	ELISA 1/50 dilution	Crossover	Rocket
1/2	+	+	+	+
1/4	+	+	+	+
1/8	+	+	+	+
1/16	+	+	+	+
1/32	+	+	+	+
1/64	+	+	+	+
1/128	+	+	+	+
1/256	+	+	+	+
1/512	+	+	+	+
1/1000	+	+	−	−
1/2000	+	+	−	−
1/4000	−	+	−	−
1/8000	−	+	−	−
1/16 000	−	−	−	−

− = Negative reaction.
+ = Positive reaction.

None of the household contaminants or body fluids tested gave a false positive result for acid phosphatase or PSA with either the 1/50 or the 1/250 dilution. Several of the combined semen and contaminant stains did produce false negative results. False negatives for acid phosphatase were found with toothpaste, face cleanser, shampoo, laundry detergent (liquid and powder), dishwashing liquid, and automatic dishwasher liquid. False negatives for PSA were found with face cleanser, shampoo, laundry detergent (liquid and powder), dishwashing liquid, and horseradish. With powdered laundry detergent and horseradish the false negatives for PSA in the 1/250 dilution became weak positives in the 1/50 dilutions. Using the lower dilution factor does not appear to be detrimental and seems to be beneficial with some substances. While the majority of contaminants tested posed no problem, it appears that those containing detergents did interfere. The contaminant stains used in this experiment were neat stains on cloth. It is possible that when the substance is diluted, as is more likely to occur on evidence, the interfering effects will also be diluted. None of the human body fluids was found to interfere with the detection of PSA.

Body fluid stains and swabs were evaluated for crossreactivity at a lower dilution than that anticipated for use in casework. None of the blood, urine, vaginal fluid, or saliva stain extracts that were diluted 1/20 gave a positive result when tested for PSA.

Casework Samples: Comparison of AP, Sperm Search and ELISA

The results of casework samples tested for acid phosphatase, sperm and PSA are shown in Table 3. The results in this section may be viewed as worst-case. The extracts used were frozen; freezing can cause loss of PSA in some dilute solutions. (Data not presented here.) The extracts were diluted for use 1/250. Results in casework may be expected to be better than the results found here.

PSA was detected by ELISA in 83% of samples where it was expected based on the presence of acid phosphatase and sperm. Of the 158 samples in this group, 145 were stains and 13 were vaginal swabs. PSA was not detected in 33 samples (17%) found to be positive for both acid phosphatase and sperm. Some of the 17% may have been weak samples missed because of the dilution factor or freezing. (Our findings suggest that 12%

TABLE 2—*Paired stain study.*

Substance	AP A	AP B	PSA 1/250 dilution A	PSA 1/250 dilution B	PSA 1/50 dilution A	PSA 1/50 dilution B
Cheese	−	+	−	+	−	+
Suntan lotion	−	+	−	+*	−	+*
Eye shadow	−	+	−	+	−	+
Toothpaste	−	−	−	+	−	+
Face cream	−	+	−	+	−	+
Hand lotion	−	+	−	+*	−	+
Face cleanser	−	−	−	−	−	−
Shampoo	−	−	−	−	−	−
Yogurt	−	+	−	+	−	+
Lip balm	−	+	−	+	−	+
Contraceptive gel	−	+	−	+	−	+
Contraceptive cream	−	+	−	+*	−	+
Diet soda	−	+	−	+	−	+
Soda	−	+	−	+	−	+
Hair spray	−	+	−	+*	−	+
Coffee	−	+	−	+	−	+
Laundry detergent, liquid	−	−	−	−	−	−
Dishwashing liquid	−	−	−	−	−	−
Lipstick	−	+	−	+	−	+
Vaseline	−	+	−	+	−	+
Tea	−	+	−	+	−	+
KY jelly	−	+	−	+	−	+
Dishwasher liquid	−	−	−	+*	−	+
Cow's milk	−	+	−	+	−	+
Laundry detergent, powder	−	−	−	−	−	+*
Deodorant	−	+	−	+	−	+
Horseradish	−	+	−	−	−	+*
Urine (3)	−	+	−	+	−	+
Sweat (2)	−	+	−	+	−	+
Human milk (1)	−	+	−	+	−	+
Vaginal swab (1)	+/−	+	−	+	−	+
Saliva (4)	−	+	−	+	−	+
Blood (4)	−	+	−	+	−	+

A = substance alone.
B = substance with semen.
AP = acid phosphatase.
− = negative reaction.
+ = positive reaction.
* = weaker than expected.
The number following each body fluid indicates the number of different individuals from whom that fluid was tested.

of the total samples may be negative due to the dilution factor.) Some samples may not have had very much PSA initially because of a low level of PSA in the semen source. However, it is of interest to note the items from which these samples were taken. Six of the 33 samples are from a single case in which the evidence was packaged poorly. Four of the six items were very wet swabs. While these swabs and two stains had very strong acid phosphatase and many sperm there was no detectable PSA. Another six samples were from a single item, a comforter, that was rolled and partially wrapped in plastic. Of the remaining 21 samples, eight were swabs (seven vaginal, one rectal) and the rest were stains from various items of clothing. It appears that inadequate evidence preservation may contribute to the loss of PSA. Because of this finding, samples, swabs in

TABLE 3—*Casework samples: comparison of AP, sperm search, and ELISA.*

Acid phosphatase and sperm results	PSA by ELISA	
AP +, sperm +	+	−
191 AP +, sperm −	158 (83%) +	33 (17%) −
42 AP −, sperm +	30 (72%) +	12 (28%) −
17 Control areas and salivas total = 246	2 (12%) +	15 (88%) −
	0	246
Total number of samples = 496		

particular, that are positive for acid phosphatase but negative for PSA should be checked for sperm.

A total of 42 samples were positive for acid phosphatase but negative for sperm. Nearly three-quarters of these samples were identified as semen by PSA ELISA. These 30 samples may be azoospermic semen or simply samples negative due to the limitations of the sperm search procedure.

It is of interest that almost the same number of samples were missed by sperm search as were missed by PSA ELISA, 30 versus 33. Both sperm search and detection of PSA have limitations and if either test is used exclusively some semen samples will not be identified.

A small number of samples, 17, fell into the final category: acid phosphatase negative but positive for sperm. PSA was identified in two of these. Of the remaining 15 samples, seven were penile swabs and four were control areas. In all of these only a small number of sperm heads were found.

Summary

Detection of PSA by ELISA is a sensitive and efficient method for identifying semen. The test is sensitive to less than 1 ng/mL PSA. None of the domestic contaminants or body fluids tested produced a false-positive result. A few of the domestic contaminants, those containing detergents, did produce false-negative results. The test was very successful in identifying semen in casework samples although inadequately preserved evidence seems to be a problem. It is necessary to search for sperm in samples that are acid phosphatase positive but PSA negative. The opposite problem, semen stains without sperm, also occurred in the casework samples tested, demonstrating the benefit of routine testing for PSA.

Certain limitations were present when these samples were evaluated (extracts frozen, $\frac{1}{250}$ dilution) that would not be present in routine casework, that is, better results in routine casework may be expected. The method allows anywhere from a few samples to hundreds of samples to be tested simultaneously. When a sufficient number of samples are processed together this PSA ELISA is more cost effective than sperm search because less labor per sample is required. Our practice is to test for PSA by ELISA extracts being

tested for ABO and Lewis factors because of the time saved by greatly reducing the number of sperm searches needed.

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