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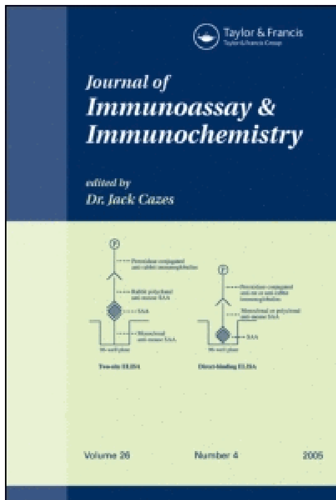

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An Enzyme Linked Immunosorbent Assay (ELISA) For Detection of Seminal Fluid Using a Monoclonal Antibody to Prostatic Acid Phosphatase

Stewart M. Allen[a]
[a] Forensic Analyst Biology Unit, Forensic Science Laboratory, Pretoria, RSA

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AN ENZYME LINKED IMMUNOSORBENT ASSAY (ELISA) FOR DETECTION OF SEMINAL FLUID USING A MONOCLONAL ANTIBODY TO PROSTATIC ACID PHOSPHATASE

Stewart M. Allen
Forensic Analyst, Biology Unit, Forensic Science Laboratory, Pretoria, RSA.

ABSTRACT

A microtitre plate format enzyme linked immunosorbent assay (ELISA), employing commercially available PASE/4LJ mouse monoclonal hybridoma antibody is described. The technique is a solid phase indirect ELISA for prostatic acid phosphatase, applicable to specific detection of semen. Maximal detectability was found to be one hundred thousand fold dilution of pooled seminal plasma. No cross reactivities with human vaginal fluid, blood, saliva, female urine, nasal discharge, earwax, sweat or faeces have been found (Key Words: ELISA, PASE/4LJ, prostatic acid phosphatase, semen, seminal fluid, forensic).

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INTRODUCTION

Human seminal fluid exhibits exceptionally high acid phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) activity [1]. This led to the development of many methods for the detection

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of seminal fluid, employing acid phosphatase activity [1-3].

Quantitative enzyme activity determination methods have been proposed [4-7] which discriminate prostatic acid phosphatase (PAP) activity based on a predetermined cutoff point. Although there may be merit in the simplicity of such procedures, specificity can be lacking [5,8]. Attempts have also been made using L-tartrate and various substrates to increase the specificity of these methods but unfortunately with limited success [9,10].

Electrophoretic techniques can also provide a possible discriminative method in the variability of acid phosphatase mobility patterns from different origins [11-13]. Due to the extreme charge heterogeneity that has been found for PAP and other acid phosphatases, interpretation of these results can though be difficult [14,15].

This prompted the development of an ELISA technique that will specifically identify prostatic acid phosphatase. A microtitre plate format was chosen as several samples can be analysed simultaneously. Development of the indirect solid phase ELISA was further based on PASE/4LJ, a commercially available mouse monoclonal hybridoma antibody to PAP [16].

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MATERIALS AND METHODS

Pooled Seminal Plasma (PSP)

Individual samples of whole frozen human semen were obtained from the Centre for Fertility Studies, Department of Urology, University of Pretoria. Equal volumes from 100 samples were combined. After centrifugation 1ml aliquots of pooled seminal plasma (PSP) were stored at -10°C.

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Samples

The following semen negative body fluid samples, donated by laboratory staff, were collected on sterile cotton swabs: Vaginal fluid (n=54), saliva (n=26), human blood (n=12), female urine (n=4), nasal discharge (n=3), earwax (n=7), sweat (n=3). Non-human primate semen samples were obtained from Roodeplaat Research Laboratories, originating from vervet monkeys (*Cercopithecus aethiops*) and chacma baboons (*Papio ursinus*). Domestic animal samples included goat, dog, bovine, horse, pig and sheep semen, obtained from Onderstepoort Veterinary Research Institute.

Extractions were made by addition of 500µl 0.8% saline, incubation for 30 minutes at 37°C and centrifugation to remove debris. Supernatants were either tested immediately or stored at -10°C.

STEP 1

Add Sample + Binding promoter

Incubate 1hr 37°C/60°C

Wash 2X

STEP 2

Add PASE/4LJ + GtαMo IgG Peroxidase

Incubate $1^1/_2$hr

Wash 3X

STEP 3

Add Substrate

Develop for 15min

Add H_2SO_4 and read at 492nm

Figure 1. Outline of ELISA procedure used

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Reagents

Monoclonal PASE/4LJ and polyclonal peroxidase-conjugated, affinity isolated goat anti-mouse immunoglobulins (GαMPerox) were purchased from Dako A/S Denmark, code numbers M792 and P447 respectively. All other reagents were general analytical or synthesis grade from Merck, Sigma, and Boerhinger Mannheim GmbH.

ELISA

Binding promoters were all tested according to the illustrated procedure (fig. 1). To 50µl sample extract, or PSP dilution, dispensed in the microtitre plate (Flow Laboratories, Linbro cat. no. 76-307-05), was added 50µl binding promoter. Duplicates of each of the above were made and one incubated at 37°C and the other at 60°C for 1 hour. Wells were then washed 2 times, using an automated Dynatech Multi Reagent Washer, with 400µl volumes of Tris buffer (pH 7.4, 20mM Tris base, 0.8% NaCl, 0.02% KCl) containing 0.05% Tween 20 (TBS-T). After 1.5 hours incubation at 37°C with 50µl of antibody solution (TBS-T containing 0.5% casein, 1:1000 dilution of PASE/4LJ and GαMPerox) wells were again washed as given above.

Substrate solution (6mg hydrogen peroxide urea and 10mg orthophenylenediamine per 10ml 0.1M citrate buffer, pH 5) was freshly prepared and added at 50µl/well. Plates were then incubated for 15 minutes at room temperature and the enzymatic reaction stopped by addition of 50µl 0.5M H_2SO_4. Absorbances were read at 450nm with a Titertek Multiskan MCC/340 microtitre plate reader.

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RESULTS

The most suitable binding conditions were found with 0.1M NaOH as binding promotor incubated at 60°C, which suggests surprisingly robust antigenicity for PAP (fig. 2(a)). No significant binding was observed with incubation at 37°C using saline (results not shown). Overnight drying of samples notably improved detectability when using saline (fig. 2(a)). The higher temperature step was significant with all promoters except 0.1M NaOH. Use of NaOH results in a uniform curve with both temperature conditions, the only difference being a slight drop in detectability at 37°C. This drop in detectability is primarily due to a higher background occurrence when using the milder conditions (with all promotors). The high concentration inflection apparent with saline and carbonate buffer use is also eliminated when using NaOH. High temperature incubation with sodium hydroxide appears to facilitate a rough alkaline hydrolysis purification process, with no noticeable effect on PAP antigenicity.

No PAP positive results were found with tested human body fluids other than primate semen (table 1). No reactivity was found with pure undiluted vaginal fluid and blood samples, the latter being from male and female origin.

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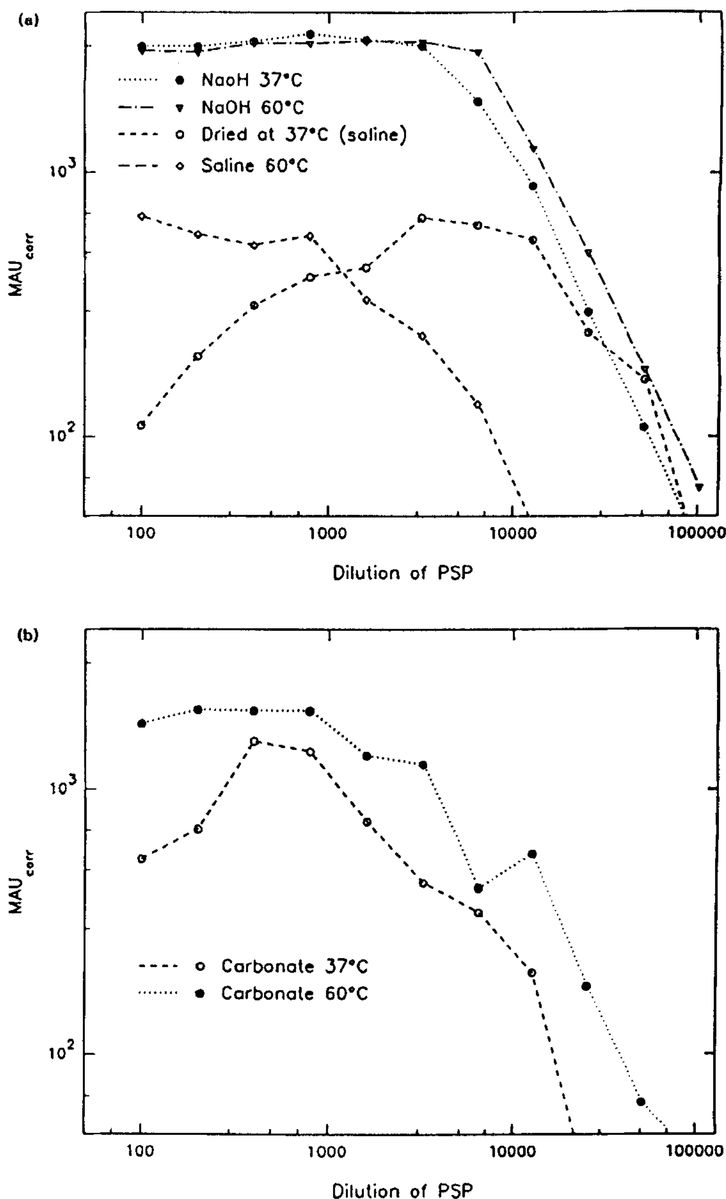

Figure 2. Binding patterns for PAP from pooled seminal plasma (PSP) utilising various promoters and conditions.
NaOH = 100mM NaOH; Carbonate = 100mM Carbonate buffer pH 9.6;
Saline = 0.8% Saline

$$MAU_{corr}=1000(Abs_{492}^{sample}-(2XAbs_{492}^{Control^{neg}}))$$

Mau_{corr} = Corrected milliabsorbance units
$Control^{neg}$ = No GαMoPerox negative control

TABLE 1. Results from selected samples

	Sample		Mau_{corr}
Primate Semen	Human - 1:10 dilution	n=80	2699 ±220
	Human - 1:50 000 dilution	n=80	189 ±34
	Vervet monkey - concentrated	n=4	420 ±210
	Chacma baboon - concentrated	n=4	590 ±340
Human Body Fluids	Vaginal fluid - concentrated	n=54	-95 ±36
	Saliva - concentrated	n=26	-55 ±22
	Earwax - 1:5 extract dilution	n=7	-120 ±43
	Human Blood - 1:5 diliution	n=12	-192 ±58
	Nasal discharge - 1:5 dilution	n=3	-110 ±18
	Sweat - concentrated	n=3	-180 ±26
Domestic Animal Semen	Goat - concentrated	n=2	-92 ±25
	Dog - concentrated	n=1	-115 ±15
	Bovine - concentrated	n=3	-98 ±31
	Horse - concentrated	n=2	-108 ±22
	Pig - concentrated	n=3	-112 ±24
	Sheep - concentrated	n=2	-124 ±18

Values are the mean of independant triplicate tests done on different days over a period of about six months. The n indicates the number of unique donors.

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All the non-human primate semen samples developed positive results for PAP presence. The concentration of the PAP determinant in these samples is markedly lower than in human semen. The highest titre value being at 200 fold dilution, as compared to 100 000 fold for human semen. No PAP activity was detectable for goat, dog, bovine, horse, pig and sheep semen.

Results given in figure 2 and table 1 are expressed as corrected milliabsorbace units. The

correction assumes a 100% fault tolerance on the background colour development. This is a conservative approach due to the forensic application of this test. All positive results are taken as positive for PAP.

DISCUSSION

No measurable phosphatase activity was found on plates fixated with PAP, using any of the given conditions. PAP has however been noted to be rather labile [12,17], according to activity assays and electrophoretic methods, even at room temperature [8,13,18]. Foti *et al.* noted a marked reduction in both acid phosphatase activity and radioimmunoassay PAP detectability with increased temperature incubation. Repeated freezing and thawing did not affect either enzyme activity or immunological activity [17]. Derechin *et al.* postulated that tertiary structure unfolding and hydrophobic bonding led to observed aggregation of PAP subjected to alkaline conditions [19]. This hydrophobic bonding process can be responsible for the strong affinity for hydrophobic polystyrene. The described ELISA was also attempted using Nunc-Immuno Maxisorp microtitre plates, which have a hydrophylic surface nature. Almost no binding was found, further suggesting that

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PAP solid phase bonding is primarily hydrophobic. If PAP has a strong tendency towards hydrophobic solid phase attachment, then this will adversely affect the extraction and therefore detection of low concentrations of PAP samples collected in certain vials and subjected to drying or even mild heating.

No false positive reactions have been found with other human body fluids, therefore a positive result is indicative of semen presence. Detectability was found to be in the region of 100 000 fold dilution of seminal plasma

Prostatic acid phosphatase has a strong affinity for polystyrene and possibly other hydrophobic surfaces. This affinity can be enhanced by increased temperature incubation and alkaline conditions.

This technique is suitable for rapid simultaneous testing of numerous samples in forensic investigations of sexual assault.

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