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Research Article

Study Prozone Phenomenon in Sexual Assault Evidence

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ABSTRACT

Globally, the incidence of sexual assault cases, especially rape is increasing, sexual aggression is a serious social and public health issue that requires an urgent forensic medical examination. The ability to detect seminal fluid is vital in forensic cases involving sexual assaults and Sodomy crimes. Prostate Specific Antigen (PSA, also known as P30) is a glycoprotein produced by the prostatic gland and secreted into seminal plasma, is now accepted as a marker for detecting semen in criminal cases where detecting of sperms is of challenge such as in vasectomized or azoospermia males. It is important to note that when the PSA concentration is too high it may overwhelm this very sensitive test. The prozone or high-dose hook effect phenomenon, documented to cause false-negative assay results still remains a problem in one-step chromatographic sandwich immunoassay, immunoturbidimetric assays, and immunonephelometric assays. To detect the prozone effect, samples are often tested undiluted and after dilution, If the result on dilution is higher than for the undiluted sample, then the undiluted sample most likely exhibited the prozone effect.

Objectives: This study was design to measure the results from using three different procedure of one-step chromatographic sandwich immunoassay; short, long and dilution on forensic evidence to detect sexual assault at laboratory of forensic serology division.

Materials and methods: The material and methods were used ABA card® kits for detection prostate specific protein by use three procedures, the principle of test is one-step chromatographic sandwich immunoassay. Total sample (2870) 1910 swab (vagina, rectum, penis, Labia, perineum and mouth) and 960 different clothing were investigated for semen detection.

Results: The results show that the one-step chromatographic sandwich immunoassay method (dilution procedure) for P30 detection is useful for the identification of seminal fluid (Plasma) in sexual assault because it is evidence saved, highly sensitivity (98.8%), specificity (100%) for human semen detection with Negative Predictive Value (0.99) and eliminate high dose hook effect Phenomena. There is uneven distribution of positive results for clothes and swabs (P =0.0005) where it is evident that it was mainly underwear for cloths and vaginal, rectal swabs were more positive.

Conclusion: The rapid membrane test (chromatographic sandwich immunoassay short method, long method) easy to implement into routine casework protocols and provides identifying seminal fluid from vasectomized and azoospermia individuals but this procedure not efficient to prevent High Dose Hook Effect Phenomena therefore this study emphasis to use dilution procedure as first choice to maintain time and evidence and elemental High Dose Hook Effect Phenomena (False Negative results).

Keywords: Forensic serology science; Prozone or high-dose hook effect Phenomena; Prostate specific antigen (PSA)

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INTRODUCTION

Biological evidence in most cases is the only one to prove the occurrence of sexual contact and to identify the perpetrator and is most important for legal proof in courts of law now a days. Contrary to other offenses, where major effort is invested upon investigating the crime scene, in sexual assault cases the victim his/herself constitutes the crime scene [1] Sperm production and hence detection is a good forensic marker of sexual assaults; however it is affected by many medical conditions like aspermia, vasectomy, psychiatric conditions, environmental factors and the age of the male.

Forensic scientists have recognized the need for other diagnostic tests which do not rely upon presence of sperm cell. P30 is a 30,000 Dalton semen glycoprotein of prostatic origin has detected to be a better surrogate for sperm detection to be considered as an evidence for sexual assault. The ratio of seminal fluid to sperm cells is very high therefore it is more preferable in forensic work [2].

In Iraq, the laboratory of Medico-Legal Directorate (MLD) was using the conventional methods like UV light for flavin detection (as a source of illumination) or direct microscopically detection of head or tail of the sperm using special stains. However, since 2011 the laboratory of the MLD has switched to use one-step chromatographic sandwich immunoassay as efficient technique for seminal fluid investigation [3].

There are four methods for forensic semen detection ,firstly Choline is important in cellular membrane composition protein when it found, this might mean sperm contamination, this protein is low concentration in seminal fluid and have same chemical structure with many other non-human proteins [4].

The second are enzymes (such as Acid Phosphatase ,ACP) also was used and detected in high concentrations in semen, it can also be detected in other body fluids, It degrades at a much faster rate than sperm cells also increase in many pathologic diseases, Acid phosphatase test is commonly used only as a screening test for semen because it is not only present in semen and prostate tissue, but also in normal vaginal secretions [5].

The third is Seminal Vesicle Specific Antigen (SVSA), also known as Semenogelins (Sg) can be employed to detect the presence of semen as a source of seminal vesical but it has low concentration in seminal fluid; unfortunately all old methods are not absolutely specific and may give false positive or false negative [6].

Fourth technique have time consuming process of the crossover electrophoresis technique of measuring PSA and ELISA based measurements of PSA also unpractical [7]. Compared to the time consuming process of the crossover electrophoresis technique of measuring PSA and ELISA based measurements of PSA, rapid membrane tests offer the same sensitivity within 10 minutes using 200 µl of the extract [8].

PSA Rapid Test chromatographic sandwich immunoassay is very specific and sensitive to detect PSA in seminal fluid, the high sensitivity and specificity of PSA rapid test is suitable for seminal fluid screening test [9].

Therefore, this device is suggested for forensic use in sexual assault cases [10]. Spermatozoa are usually found in the vagina up to 3 days after intercourse and occasionally up to 6 days later. Tail are frequently found attached to spermatozoa on swabs taken within one hour of intercourse [11]. They are commonly found up to 16 hours and rarely up to 72 hours [12].

Various antigen specific membrane tests are currently used in clinical setting to screen a patients serum for the presence of PSA in levels >4 ng/ml indicating either benign prostatic hyperplasia or prostatic cancer [13].

The P30 can be detected in seminal fluid without spermatozoa (e.g. seminal fluid of vasectomized or sterile man) it shows high stability and could be detected in 30 years old semen stain (dried) , it is possible to detect PSA from vomit samples at least up to 4 hours using simulated gastric juice also its shows PSA is a more specific marker than acidic phosphatase , many studies of PSA in vaginal swabs is more reliable than the detection of seminogeline [14].

Table 1 represents PSA against numerous biological fluids of men and women, since no cross reactivity has been reported to date, this supports the hypothesis that P30 is a male-specific protein. For this reason, the detection of the P30 antigen in a forensic stain is strong evidence that the stain is seminal in nature [14].

Table 1: Represents PSA in different body fluids.

Fluid	Concentrations of PSA (ng/ml)	References
		Sensabaugh, et al.1978 Lovgren, et al. 1999
Semen	200,000 to 5.5 million	
	820,000 (mean)	
Amniotic fluid	0.60 ng; 8.98 ng	Lovgren, et al.1999
		Lovgren, et al.1999

Breast milk	1 ng -210 ng	Filella, et al. 1996
		Yu and Diamandis,1995
Saliva	Non	Lovgren, et al.1999
		Breul, et al. 1994
		Breul, et al. 1997, Schimidt, et al. 2001
Female urine	<0.01 ng -3.72 ng	Yu and Diamandis, 1995, Diamandis and Yu,1997

The prozone or high-dose hook effect phenomenon , documented to cause false-negative assay results still remains a problem in one-step immunometric assays, immunoturbidimetric assays, and immunonephelometric [15]. To detect the prozone effect, samples are often tested undiluted and after dilution. If the result on dilution is higher than for the undiluted sample, then the undiluted sample most likely exhibited the prozone effect [16].

MATERIALS AND METHODS

Setting and study design: This is cross sectional study conducted in Iraqi Medical Legal Directorate/DNA Fingerprint department/forensic Serology Division, which serves as the referral center for all Iraqi provinces sexual assault victims and Sodomy crimes. From first of June 2014 to 31 of May 2015 about 1910 swab (vagina, rectum, penis, Labia, perineum and mouth) and 960 different clothing and evidences were investigated for semen detection.

Ethical consideration: This study was approved form scientific and ethical council in the Iraq medical legal directorate/ Ministry of Health and Environment (MOH). This research was conducted based on Article 2 of the Iraqi Forensic Medicine Law of 2013.

Case definition inclusion and exclusion criteria: Any evidence referred officially from police offices and investigation bureaus to our department for seminal detection as part of investigating a sexual assault during the period of the study were included in this study. The evidence was either a direct swab from vagina, rectum, penis, labia, perineum and mouth or pieces of clothes. Evidence that was improperly packaged was excluded from the study.

Sampling: During the one year prior to the implementation totally (2870) forensic evidence were evaluated for sexual assault and Sodomy crimes. The 1910 different swabs were investigated while 960 different clothes evidence.

Outcome: ABA P30 card® PSA kits was used; Positive and Negative results are recorded for all forensic evidence. The kit contains 25 test cards and one transfer pipette (sealed and desiccated in a foil pouch) and 25 tubes containing 2 ml of extraction buffer.

Note: Each new lot number of kits must be validated using a positive and negative control before using it in casework.

The instruction and procedures given by the manufacturer revealed that pink lines in the test and control areas resulted in a positive test and indicated that the PSA level was at or above 4 ng/ml .If there was only one pink line in the control area, the test result was negative. This indicated the absence of PSA or that there was less than 4 ng/ml of PSA present. A negative result may also have been due to the high dose hook effect due to over concentration of PSA on the test strip; high dose hook effect term means that PSA concentration is too high it overwhelms this very sensitive test.

Membrane test assay (short method)

- If this procedure gives negative result, then confirmatory long procedure must be done.
- All samples are allowed to warm to room temperature if they had been refrigerated.
- A small section of the swab approximately (1/4) or stain (1–2 CM) was cut and extract with 4 to 6 drops (approximately 300 µl) of specific buffer at room temperature for 15 minutes.
- The device and the dropper from the sealed pouch were removed.
- ABA card® was labeled with case and item, exhibit numbers, data and initials.
- 200 µl of sample was added to the sample well (S) of the test device.
- Result at 10 minutes was read, positive results could be seen as early as 1 minute depending on the p30 concentration. for negative results, one might wait for the full 10 minutes.

Membrane test assay (long procedure)

- If this procedure gives negative results then to prevent phenomena called [High Dose Hook Effect], the dilution (1/10000) must be done and repeat the test.
- Samples were allowed to warm to room temperature if they had been refrigerated.
- Small section of the swab approximately (1/4) or stain (1–2 CM) was cut and extract with 6 to 8 drops (approximately 800 ul) of specific buffer for 2 hours at 4°C.
- The sample was allowed to warm at room temperature for 5 minutes.
- Samples at 3000 RPM (Round per Minute) for 3 minutes were centrifuged and 300 µl of supernatant were removed and 200 µl were used. This aliquot may be stored at 2–8°C if not used immediately. Immediately before use, with ABA card® p30

test, the sample should be brought back to room temperature. Remaining sample may be used for further DNA analysis (DNA fingerprint, DNA sequencing) without affecting the DNA yield.

- The device and the dropper from the sealed pouch were removed.
- 200 µl of sample was added to the sample well (S) of the test device.
- Result at 10 minutes were, positive results could be seen as early as 1 minute depending on the p30 concentration, for negative results, one might wait for the full 10 minutes.

Dilution procedure to prevent high dose hook effect phenomena prepared dilution (1/10000) as follow

- When results give negative by long methods from first aliquot we get 100 µl.
- 9900 µl of DDW (sterile double distill water) was added to 100 µl of aliquot sample the dilution became 1/100.
- 100 µl from step 2 dilution add to 9900µl of DDW (sterile double distill water) the dilution became 1/10000.
- 200 µl from step 3 was added to the sample well (S) of new test device. 5- Results at 10 minutes were read.

Statistical analysis

The database was examined for errors using range and logical data cleaning methods, and inconsistencies were remedied. An expert statistical advice was sought for.

Statistical analyses were done using SPSS version 21 computer software (statistical package for social sciences) in association with Microsoft Excel 2013.

Sensitivity

Sensitivity of a clinical test represents test ability to correctly identify people with illness within all people with illness. It is a proportion of people with disease who positive, expressed in percentages. Sensitivity as a fixed test characteristic provides a true positive rate.

$$\text{Sensitivity} = \frac{\text{True positive}}{\text{True positive + False negative}}$$

If we apply screening test to our hypothetical population and receive that 80 of the 100 people with disease X test positive, than the sensitivity of this test is 80/100 or 80%. A test with 80% of sensitivity, while 20% (false negative) will not be detected.

Specificity

The specificity of a clinical test represents test ability to correctly identify people without illness within all people free from illness. It is a proportion of people without disease who test negative. Specificity is also a fixed characteristic of the test and represents true negative rate.

$$\text{Specificity} = \frac{\text{True Negative}}{\text{True Negative + False Positive}}$$

Predictive values

The real questions to be answered are the following: "What is the probability that a person with a positive test results will have the disease? Also if a person has a negative test, what is the likelihood that he is healthy? " These questions refer to what's called the "predictive values". Therefore, the mission of the clinician is to determine the likelihood of a disease present given a positive test (positive predictive value – PPV),or the likelihood of a disease absent given a negative test (negative predictive value – NPV) [17].

Calculation Sensitivity, Specificity, Negative Predictive Value (NPV) (Tables 2 -5).

Table 2: Represent calculation of sensitivity and specificity for swabs evidence.

TP (250)	FP (0)
FN (3)	TN (1657)
TP=True Positive, FN=False Negative, FP=False Positive, TN=True Negative	
Sensitivity=TP/TP+FN 250/(250 +3) = 0.98=98.8 % is the sensitivity of test for swabs evidence.	Specificity=TN/TN+FP 1657/1657+0 = 1=100%

Table 3: Represent the sensitivity and specificity of test for clothes evidence.

TP (410)	FP (0)
FN (5)	TN (545)
Sensitivity=410/410+5 = 0.98=98.7	Specificity=545/545+0 1=100%

Table 4: Represent NPV for swabs evidence.

TP (250)	FP (0)
FN (3)	TN (1657)
NPV=1657/1657+3 = 0.99	

Table 5: Represent NPV for clothes evidence.

TP (410)	FP (0)
FN (5)	TN (545)
NPV=545/545+5 = 0.99	

RESULTS

During the one year prior to the implementation of the study, 2870 forensic samples were evaluated for sexual assault and Sodomy crimes. Total 1910 different swabs were investigated 250 (13%) of them had a positive test for semen while 960 different clothes evidence 410 (43%) had positive test for this evidences. There is a high significant relation with positive finding in the clothes; the results in this study are summarized in t-Table 6.

Table 6: Total results for P30 test by three procedures (Swab and Clothes).

	Evidence	Positive P30 (%)	Negative P30 (%)	Total	P value
Short method	Swabs	250 (13%)	1660 (87%)	1910 (100%)	P=0.0005
	Clothes	410 (43%)	550 (57%)	960 (100%)	
Long method	Swabs	250 (13%)	1660 (87%)	1910 (100%)	P=0.0005
	Clothes	410 (43%)	550 (57%)	960 (100%)	
Delusion method	Swabs	3 (0.2)	1657 (99.8)	1660	P = 0.005
	Clothes	5 (0.9)	545 (99.1)	550	

There are highly percentage for positive evidences (clothes) 43 % and less in swabs 13% as shown in table-6 this may be possibly because of loss of samples in toilet washing done by victims and may be due to improper methods of sample collection, preservation , packaging and transporting of swab by authorized person.

About 410 (61%) out off 960 of evidences clothes (Underwear) were positive as show in table- 7 this confirms sex assaults and rape while, vaginal swab 66 (26%) and rectal swab 57 (23%) are most common evidences because sperm and seminal fluid can be viable up to three days but, fresh swab (within up to 6 hours) has been found to produce good results may due to its high concentration of semen containing it while in rectal swab may be semen loss were happened during enter toilet.

All negative evidences (swabs and clothes) by short procedure confirmed by long and dilution procedure , in this study 2210 negative evidences retested or confirmed by long procedure and the results still negative but when retested by dilution procedure about (0.18% swabs and 0.90% clothes) from them give positive results (it's were negative or false negative) as shown in table- 6 therefore, we emphasis applying the dilution technique in semen investigation to save time, cost , evidence for DNA investigation and eliminate High Dose Hook Effect phenomena.

Table 7: Represents the total and percentage of positive evidences in front each kind of samples.

No	Evidences	Number	Percentage	Total
1	Underwear	250	61%	410
2	Bra	100	24.50%	
3	Trousers	50	12.50%	
4	T-Shirt	10	2%	
5	Vaginal swab	66	26%	250
6	Rectal swab	57	23%	
7	Labia swab	30	12%	
8	Penile swab	23	9%	
9	Dried stain	29	12%	
10	Feminine pad	15	6%	
11	Condom	5	2%	
12	Perineum swab	25	10%	
Total		660	100%	

DISCUSSION

First stage in DNA typing is the identification of biological fluid on collected evidence material. In most of the crime, laboratories serological tests are used to screen evidence material for the presence of biological fluid of human origin. The key issue in serological analysis is the human specificity and sensitivity [18]. As PSA is protein this may be degraded by many physical, chemical and with extreme environmental factors lead to lose their three-dimensional conformation. It is possible that the monoclonal antibodies used in the kits lose their ability to bind the partially degraded PSA [19-25]. It is important to note that when the PSA concentration is too high it may overwhelm this very sensitive test. The mechanism behind this, is that huge amounts of human PSA bind both to the antibodies to form an antigen- antibody complex but also free PSA migrates toward the test area 'T'. The antibody in the test area 'T' is blocked by this free PSA this interpretation is agreeing with our results [26-30].

The mobile antigen-antibody complex with the pink color cannot bind to the antibody. When this false negative occurs, a 1:100 or 1:10,000 dilution of the remaining extract should be retested. This rapid membrane test is easy to implement into routine casework protocols and provides the forensic community with a very sensitive, reliable, and expeditious way of identifying seminal fluid from vasectomized individuals [31-33].

If the result on dilution is higher than for the undiluted sample, then the undiluted sample most likely exhibited the prozone effect [33-35]. Unfortunately, this approach increases labor and reagent costs for assays that may only rarely encounter extremely high analytic concentrations [36].

CONCLUSION

The present study concludes that confirmatory procedure [Dilution procedure to prevent High Dose Hook Effect Phenomena] for semen detection is the best and can recovers approximately 99 % of the extractable p30 on the swabs and clothe stains and can prevent false negative results, the study revealed about (0.2% and 0.9%) of false negative occurs from swabs and clothes consecutively ,there is however highly sensitivity (98.8%),specificity (100%) with (0.99) Negative Predictive Value , and with significant p-value 0.05 therefore, dilution procedures must be the first choice in routine work.

Iraq's weather is very hot the summer continue for eight months, therefore P30 proteins is very affected (denaturant) especially when far provinces referred their evidences to medical legal directorate/Baghdad the center for all Iraqi provinces also condition and circumstance of each case (crime) must be known to conclude right decision about it.

RECOMMENDATION

Finally we enhance manufactures to produce tow new kits for P30 one with sensitivity less than 4 ng/ml because some forensic evidences may contains very low concentration the second with high monoclonal antibodies concentration to react with huge amount of p30 found in some cases evidence, we emphases the Autopsy physician in living and died investigation departments for collect more than two swab form each part of body to get enough amount of biological materials.

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