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Evaluation of Hepatitis-B Surface Antigen As a Forensic Marker

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HEPATİTİS B YÜZEY ANTİJENİNİN ADLİ İŞARET OLARAK KULLANILABİLİRLİĞİNİN İNCELENMESİ

Özet

Kanda yabancı antijenlerin bulunması ikizleri ve yakın akrabaları birbirinden ayırmada adli açıdan çok büyük önem taşıyabilir. Bu görüşten yola çıkılarak hepatit yüzey antijeninin (HbsAg) kan lekelerinin kaynağını belirlemede adli marker (işaret) olarak kullanılabileceği düşünüldü. Çift antikor sandviç prensibine dayanan ELISA yöntemi kullanılarak kan lekelerinde HbsAg varlığı araştırıldı. Kan lekelerinde HbsAg'nin dayanıklılığı ve Haydarabad popülasyonundaki frekansı incelendi. Normal koşullarda 5 hafta bekletilen kan lekelerinde dahi HbsAg'nin saptanabildiği görüldü. Taranan 2150 kişinin %11.4'ünde HbsAg'ye rastlandı. Frekansı 0.08 olarak hesaplanan HbsAg'nin incelenen popülasyonda amibli dizanteri, influenza ve tüberküloz kadar yaygın olmaması sayesinde, bu antijenin adli işaret olarak kullanılabileceği, ikizleri ve yakın akrabaları birbirinden ayırmada yararlanılabileceği sonucuna varıldı.

Summary

The presence of extraneous antigens in blood may be of unique forensic importance in discriminating source among identical twins and genetically close related individuals. Hepatitis surface antigen (HBsAg) as a forensic marker in establishing the source of blood stains has been evaluated by studying the stability of HBsAg in stains and its frequency in Hyderabad populaiion. Microstrip based double antibody sandwich ELISA is employed to detect HBsAg in blood stains. HBsAg was found to be stable up to 5 weeks in blood stains exposed to ambient conditions.

Key words: *HBsAg - ELISA - Storage - Stability - Frequency*

INTRODUCTION

Human blood contains several components which are highly useful in forensic analysis (1). Species specific antigens (2), blood group substances (3), polymorphic enzymes (4), and human leucocyte antigens (5), which constitute the expressed part of genome are being extensively studied to find out the source and to individualize blood and its stains.

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Analysis of highly variable tandem repeats (HVTR) of DNA by using multilocus probes (MLPs) and single locus probes (SLPs) with or without polymerase chain reaction (PCR) is a new advancement being applied as a final test for individualization or sexing of bodyfluid stains and tissues (6). Blood may also harbour antigens derived from pathogenic bacteria, virus, protozoa or other micro-organisms in free or as circulating immune complexes (7) which could be highly informative as forensic marker in individualization of source of blood stains especially where genetic markers fail to distinguish biological samples derived from two related individuals. An elaborate study of extraneous antigens and their potential in forensic investigation therefore assumes unique importance.

In this study we have evaluated the stability of hepatitis surface antigens (HbsAg) as an extraneous marker by examining (a) the stability of HBsAg antigens in stains stored for various lengths of time at room temperature and (b) prevalence of HBsAg in population at Hyderabad. Microstrip based double antibody sandwich and highly sensitive ELISA is employed to detect HBsAg.

MATERIALS and METHODS

Microstrips (16 wells) coated with antibodies to HBsAg were purchased from Hoechst India Ltd (India). Wells were blocked with 2 % BSA. Second antibody to HBsAg labelled with horse radish peroxidase enzyme was also obtained from the same source. All incubations were carried out in phosphate-buffered saline (PBS), pH 7.2 and washings at the end of each binding step were carried out in PBS, pH 7.2 with 0.1 % Tween 20 (8).

Blood stains: Hepatitis positive blood samples were obtained from Gastroenterology Department of Osmania General Hospital, Hyderabad. Sixty blood stains, each approximately 1.0 cm^2, were prepared on sterile cotton cloth by pouring sufficient volume of blood. Stains were divided into 6 groups, each comprising of 12 stains and left at room temperature in petridishes. On the first day each group was screened for the presence of HBsAg and subsequently testing was done at the end of every week up to 5 weeks. Two stains from each group were used as representatives of group during each testing.

Stain extracts: Stains were cut into fine pieces and extracted in 500 µl of normal saline for one hour, in separate microfuge tubes (1.0 ml). Later the bottom of the microfuge tube was punctured, placed in 1.5 ml tube and centrifuged for 15 mins at 1000 rpm. Stain extract was collected in the outer tube. 100 µl of extract of each stain was used to detect the presence of HBsAg.

ELISA procedure: Anti-HBsAg antibody coated microstrip was taken out from the refrigerator and acclimatised at room temperature.

First two wells of microstrips were charged with 100 µl of positive controls. Extracts of stains of 6 experimental groups (Figure 1) were tested in remaining 12 wells. From each group two stain extracts were tested side by side in serial order. Wells after loading with extracts were covered with adhesive foil and incubated at 37°C for 4 hours. Thereafter, wells were washed 3 times with washing buffer. 100 µl of second antibody (1:1000 dil) were added in each well and incubated for 30 min at 37°C. Wells were again washed three times with washing buffer and 100 µl of 0.2 % o-phenyldiamine (OPD) in citrate buffer, pH 5.5 was added to each well, covered and kept for 30 min at room temperature in dark.

The reaction was terminated by adding 100 µl of 2 N HCl. The intensity of the colour was read visually against positive and negative controls (Figures 1 and 2 ; Table I).

Population Study: 2150 individuals were screened for the presence of HBsAg in their blood by using the ELISA method.

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1	2	3	4	5	6	7	8
+ve	+ve	-ve	-ve	I	I	II	II
III	III	IV	IV	V	V	VI	VI
9	10	11	12	13	14	15	16

Figure 1. Schematic diagram showing the controls (wells 1 to 4) and test samples in wells 5-16 of microstrip.

Table I. Stability of HBsAg in blood stains.

	Reaction intensities in various groups*					
	I	II	III	IV	V	VI
'0' day	4^+	4^+	4^+	4^+	4^+	4^+
1st week	4^+	4^+	4^+	4^+	4^+	4^+
2nd week	3^+	3^+	3^+	3^+	3^+	3^+
3rd week	2^+	3^+	3^+	3^+	1^+	3^+
4th week	2^+	2^+	2^+	2^+	1^+	1^+
5th week	1^+	1^+	1^+	1^+	2^+	1^+

* 4^+ = very strong, 3^+ = very strong, 2^+ strong, 1^+ = weak

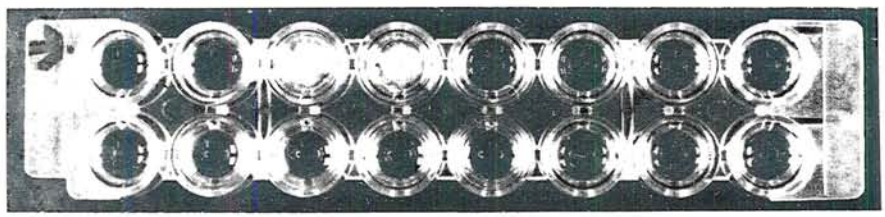

Figure 2. Typical 16 well Microstrip used in the study.
Wells no. 1-2 contain positive control, 3-4 negative control and the rest are used for the blood stain extracts. Positive reactions are clearly seen in comparison with the negative controls.

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RESULTS AND DISCUSSION

The stability of a biological marker for forensic analysis depends upon the amount of information it can provide and it's stability in body fluids and their stains besides somatic and germline stability. Many endogenous genetic systems present in blood and other body fluids are highly polymorphic, stable in the stains for longer periods (9) and follow Mendelian fashion of inheritance (10). Hence endogenous markers are highly informative and routinely analysed for forensic purpose, an extraneous marker can be of forensic utility provided it is stable in stains and it's frequency distribution in a given population is known.

Hepatitis is a very important disease and the hepatitis surface antigens are easily detected in the body fluids of infected persons. We have therefore opted HBsAg as a useful marker for forensic study. We could successfully detect HBsAg in blood stains stored up to 5 weeks. Stains of one day and one week old gave very strong positive reactions and thereafter the intensity of the reaction gradually declined with the length of storage period. In five-week old stains the reaction was relatively weak. HbsAg positive stains could clearly be detected from negative controls. HbsAg are spherical and high molecular weight proteins measuring 22 nm in diameter (11). High molecular weight and strong antigenicity of HBsAg are responsible for its extreme stability. Isolated HBsAg was found to be stable for more than 20 years when stored at -20°C and it is not destroyed by UV radiation (12). It is reported that HBsAg retains its antigenicity in conditions in which the infectious virus is rapidly inactivated (13). Eventhough temporal disintegration of HBsAg has been observed in stains exposed to ambient climatic conditions, the rate of deterioration is significantly low (Figure 3) and their presence was unambiguously detected even after five weeks.

Table II. Incidence of HBsAg in Hyderabad population.

S. No.	Population group	No. of subjects tested	% of positive cases of HBsAg
1.	Hospital staff	314	10.0
2.	Professional and voluntary blood donors	1300	7.2
3.	Antinatal	300	3.0
4.	Mentally retarded	86	26.7
5.	Prison children	150	22.0
Total		2150	11.4 %

$$\text{Frequency of HbsAg} = 1 \times \frac{1}{11.4} = 0.08$$

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Several authors reported the successful detection of HBsAg in fresh tissues and body fluids (14,15). But no reports were available on the stability of HBsAg in stains and their detectability. From the present study it is clear that the HBsAg present in blood stains are highly stable, that they could withstand the stress of normal climatic conditions up to 5 weeks and could be detected by using a suitable method.

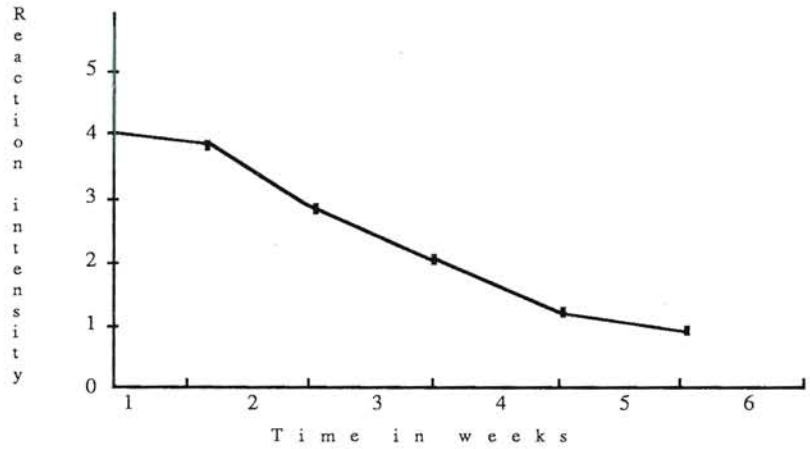

Figure 3. Temporal disintegration of HBsAg in blood stains stored for different lengths of time.

Disease marker of high prevalence is not satisfactory for forensic considerations. In this respect HBsAg marker is highly suitable for forensic investigation as its distribution is not very high in the population unlike other diseases such as amoebiasis, influenza and tuberculosis. However, meaningful conclusions could only be drawn for extraneous marker analysis, when other genetic systems are also simultaneously examined. Hepatitis is not endemic to India and its prevalence is moderate. The frequency of HBsAg in Hyderabad population was found to be 0.08 (Table II). The probability of finding another stain with HBsAg depends upon the frequency of the marker in that population. Lower the frequency of a marker, lower will be the probability and it is highly suitable for the forensic analysis.

The presence of HBsAg in the stains found at crime scene and also in the blood of suspect could be of immense help as additional marker to supplement the findings obtained by typing the other genetic systems. Merits of HBsAg in forensic analysis supersedes the inherent demerits of lack of germline and somatic stability and infective risk posed by antigen, especially in cases requiring discrimination between identical twins and genetically very close related individuals.

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