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DETECTION OF HUMAN BLOODSTAINS BY ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)*

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SUMMARY

The microplate method of enzyme-linked immunosorbent assay(ELISA), using alkaline phosphatase anti-human IgG conjugate, was modified and applied in the detection of the human blood specimens among the blood samples prepared from human and laboratory animals in the form of small dried bloodstains on filter paper. The modified ELISA technique was compared with the agar double diffusion method of precipitin test.

The results of this primary study showed that the ELISA in compare to gel diffusion test is sensitive and specific.It is also enough reproducible and practical to be consider as a competent technique for identification of human bloodstains in medicolegal laboratories.

Introduction

The precipitin test was applied as a medicolegal procedure for detection of bloodstains by Uhlenhuth in 1901 (1). He found that anti-human serum prepared in rabbit

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gave a positive reaction only with human blood among 19 blood samples collected from different animal species. However,Nuttall,1904(2),who examined collected serum samples from many more animals of the world by the precipitin test,found that a common property exists in the blood of certain groups of animals. Thus he placed man and apes in one group.

Based on the precipitin test Ascoli,1902(3) developed the ring test for species identification of bloodstains. In addition to the ring test the other methods that specific antisera (antiglobulins) are used such as the anti-human globulin inhibition test introduced by Wiener et al.,1949(4),gel diffusion test specially the immuno-electrophoresis technique developed by Culliford,1964(5)and the latex particle agglutination test by Cayzer and Whitehead, 1973(6) have also used more or less with satisfactory results (7).

The microplate method of enzyme-linked immunosorbent assay (ELISA), based on the technique described by Engvall and perlmann, 1972(8),was established as a new microserological test by Voller et al. in 1974(9). The ELISA has been used widely in recent years with satisfactory results in diagnostic medicine (10). It seemed to be a possible useful test, also,for identification of human blood samples. This present communication describes the modification of the microplate method of ELISA for detection of the human blood stains and comparison of this technique with agar double-diffusion method of precipitin test.

Materials and Methods

Blood samples form human and available laboratory

animals (mouse,rat,guinea pig and rabbit) were collected in heparinized capillary tubes and a small amount(approximately 1 μ l)was placed on to filter paper. The dried bloodstains were kept in a desciccator containing calcium chloride at the room temperature (around 20°C)for two to six weeks before examination. The fresh dried bloodstains on filter paper as well as fresh blood (about 1μ l) from the samples collected in heparinized capillary tubes from human and laboratory animals were also used for examination. The human blood samples were prepared from different individual subjects.

For comparison of the ELISA and agar double-diffusion method of precipitin test, in addition of the blood stains each containing 1 μ l of the blood samples prepared from human and laboratory animals (series A),bloodstains of 0.1 μ l from the same blood samples were also prepared by putting 1 μ l of 1:10 diluted blood in phosphate buffer saline(PBS) on filter paper(series B).

The samples for both test were chosen in random sampling method from the bloodstains of series A and B. and the selected samples were numbered and tested as unknown origin blood samples.

Principle

The ELISA is an enzyme immunoassay test. The globulins eluted from the human bloodstains are passively absorbed to a plastic surface as antigens. The added anti-human immunoglobulins conjugated with the enzyme,alkaline phosphatase, attach to the human globulins as antibodies. Excess non attached materials are washed away.When

the substrate,paranitro-phenyl. phosphate is added to the globulin-antiglobulin complexes,the enzyme linked with the antiglobulins portion hydrolyzes the substrate and produces yellow colour which indicates positive reaction.

But,in the cases of non-human blood samples,as their globulins are heterologous to the human globulins, the antihuman immunoglobulins conjugate are not attached to the nonhuman globulins and are washed away.Therefore, there is no enzyme to hydrolyse the substrate and the solution remains colourless which indicates negative reaction.

THE ELISA technique

The microplate indirect method of ELISA,described in detail by Voller et al.1976(11),was modified for this purpose and applied as follows:

Dried bloodstains on filter paper were cut out on small paper discs 2 to 3 mm in diameter. Each disc was put in a well of the "Dynatech" ELISA microtitration plastic plate.

The elution of dried blood from filter paper was carried out by 50 µl of distilled water in two hours at room temparature

Then,50 µl of coating buffer i.e.carbonate bicarbonate buffer,PH 9.6(11) was added and each moisted disc of filter paper was stirred inside the well and then discarded by a pin.

For coating the plate was either left inside a humid box at $4^{o}C$ overnight or at the room temperature for two hours.

After coating, the plate was washed by empting and refil ling with PBS Tween PH 7.4(11) three times,each time three minutes.Then the plate was shaken dry.

The conjugate,anti-human IgG prepared in goat and linked to alkaline phosphatase (made by Miles Laboratories and received through World Health Organization grant)was diluted to 1:1000 in PBS Tween and 100 µl was added to each well. The plate was incubated two hours in a humid box at room temperature and then was washed as before.

Then,100 µl of substrate solution i.e.1mg/ml para-nitro phenyl phosphate, Sigma 104,in diethanolamine buffer(11) was added to each well.

The positive result,which is indicated by appearance of yellow colour in the solution of the wells after addition of substrate solution,was subjectively assessed by naked eye. For manipulation of the test in eight various experiments,in each experiment a series of blood samples prepared from human and laboratory animals,each sample in two wells, were tested in one plate and two wells without blood were used as the blank in the same plate.

Precipitin test

For evaluation of the modified ELISA technique in detection of human bloodstains,the samples prepared from human and laboratory animals(series A and B) were also tested against antihuman precipitin serum by agar double-diffusion method of precipitin test (12),in Medicolegal Laboratory,Ministry of Justice, Iran,where the slide gel diffusion test is currently used for identification of

blood species.

Elution of dried blood from the filter paper was done in the possible minimum volume of saline(approximately 20 µl) in the sealed plate in overnight period at 4^{o}C.

The results of the test were read after 24 and also 48 hours.

Results

Using the modified ELISA technique described above for the examination of the blood samples,prepared from human and laboratory animals in the form of bloodstains on filter paper(tested freshly or after 2 to 6 weeks storing) of fresh blood samples in capillary tubes, the following results were obtained:

a, The human blood samples showed a clear positive reaction by 30 minutes. The weak yellow colour sometimes produced, with samples of animal blood or the very weak yellow colour which occasionally appeared in the blank wells, was not at the level to be confused with the rather strong yellow colour that always was observed in the case of the human blood samples much rapidly.

b, The intensity of the yellow colour,which indicates the positivity of the test, in the case of fresh human blood samples were rather more than the colour produced in the testing of the same samples in the form of dried bloodstains.

c,There was not a considerable difference between the ELISA results of human bloodstains prepared from different individuals and also, the human dried blood samples tested freshly or after storing,in a desciccator at room temperature, from 2 to 6 weeks.

d, The blood samples coated in two hours at room temperature gave more or less the same results as the samples coated over night at 4^{o}C.

e, The results of comparison of the ELISA with slide gel diffusion precipitin test in identification of human blodstains,using anti-human ELISA conjugated and anti-human precipitin serum, are summarized in Table 1.

In the ELISA all human bloodstains in series A(1 μ l) and series B (0.1 μ l) were clearly detected and no considerable false positive reaction was observed in laboratory animals bloodstains. However in slide gel diffusion precipitin test,the reaction with anti-human precipitin serum in one case from six human bloodstains of series B (0.1 μ l) was negative and in one case of two rat bloodstains of series A(1 μ l) and two cases of three guinea-pig bloodstains of series B(0.1 μ l) were weakly positive.

Discussion

This primary investigation showed that the ELISA is quite sensitive in detection of a small bloodstain on filter paper,stored at least six weeks in the laboratory conditions.

As far as the limited blood samples prepared from man and available laboratory animals and tested in this study it is also specific for identification of human bloodstains. Comparison of the ELISA with slide gel diffusion precipitin test in this study showed that the ELISA is more sensitive and also more specific in detection of human bloodstains.

As the IgG fraction of alkaline phosphatase anti-human conjugate was used in the ELISA, it is expected

that this test be more specific for detection of human blood samples than the precipitin test in which the whole anti-human globulins were used.

Although the technique used in this study is simple to perform and the end-point results is quite easy to read and generally, the test is practical and reproducible for most medicolegal laboratories, suitable automated systems for ELISA,such as those developed in the recent years(13,14)could be helpful for the large scale screening of the blood samples.

Consequently, the ELISA technique might be considered as a potentially useful test for identification of human bloodstains in medicolegal laboratories.

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Table 1, The results of the ELISA and slide gel diffusion tests in identification of human bloodstains.

Human and laboratory animals blood stains	ELISA				Slide gel Diffusion			
	Series A		Series B		Series A		Series B	
	No. Examined	No. Positive	No. Examined	No. Positive	No. Examined	No. Positive	No. Examined	No. Positive
Human	5	5	6	6	5	5	6	5
Rabbit	3	-	2	-	2	-	2	-
Guinea pig	3	-	2	-	2	-	3	(2)
Rat	3	-	3	-	2	(1)	3	
Mouse	3	-	2	-	2	-	2	-

, Weakly positive

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