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The Rapid Determination of the ABO Group from Body Fluids (or Stains) by Dot Enzyme-Linked Immunosorbent Assay (Dot-ELISA) Using Enzyme-Labeled Monoclonal Antibodies

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ABSTRACT: Using ABH enzyme-labeled monoclonal antibodies, the authors could rapidly detect the ABO group from body fluids and body fluid stains by the dot enzyme-linked immunosorbent assay (dot-ELISA). In this test, the antigen was immobilized on nitrocellulose paper; the entire piece of paper was coated with an appropriate dilution of enzyme-labeled McAb directly against the antigen of interest; and, finally, 3,3′-diaminobenzidine (DAB) substrate solution was added. The site of a positive reaction is clearly visible as a brown spot. We analyzed 521 samples and got satisfactory results. We also analyzed 99 practical case samples by this method and achieved the same results as those obtained by other researchers using other methods. This method is accurate, simple, direct, rapid, and sensitive; it also produces easily observed results, requires no equipment, and can be completed in 30 min. The test proved to be clearly more sensitive for the detection of the ABO blood group in secretor saliva than the conventional hemagglutination inhibition test. Also saliva diluted 10^{-4} to 10^{-5} and the ABO group of nonsecretor saliva and urine could be easily detected by this method.

KEYWORDS: forensic science, genetic typing, ABO blood group, body fluids, ABH-enzyme-labeled McAb, dot-ELISA, body fluid stains

For blood group determination of samples of body fluids and body fluid stains, the conventional methods are the absorption inhibition test (neutralization), absorption elution test, and mixed agglutination test. All these methods are time-consuming, require special skills, and need fresh red blood cells of known type as the indicators. To overcome the inadequacies of these methods for blood group determination, the authors of this paper employed the dot enzyme-linked immunosorbent assay (dot-ELISA) to detect the blood group of body fluids and body fluid stains.

Dot-ELISA is a new immunologic detecting method developed in recent years. It is based on the ability of nitrocellulose paper to immobilize proteins and deoxyribonucleic acid. The procedure is one of immobilizing the antigen or antibody on the nitrocellulose

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paper and then adding enzyme-labeled antibody or antigen, which, through immunologic reaction, forms an antigen/antibody enzyme complex. At this point, adding the substrate corresponding to the enzyme will produce a visible color change which will indicate, by the color reaction, what the unknown antigen or antibody is.

Dot-ELISA has many advantages. It is simple, quick, sensitive, and specific and has been widely used in medical science. In forensic medicine, Takeshi and Satoshi [*1*] reported using dot-ELISA in examining human hemoglobin for identification. Akira et al. [*2*] reported using dot-ELISA to detect the Lewis blood group. Tetsuko and Yoshihiro [*3,4*] reported using dot-ELISA to detect the Gm antigen group.

In our test, we immobilize the sample extract on nitrocellulose paper, and then add anti-A, anti-B, and anti-H enzyme-labeled monoclonal antibodies to detect the corresponding antigen. The result is judged by the color change on the paper.

Materials and Methods

ABH Enzyme-Labeled Monoclonal Antibody

For the ABH enzyme-labeled monoclonal antibody test [*5*], the labeling procedure was as follows: 5 mL containing anti-A, anti-B, and anti-H monoclonal antibodies from ascitic fluid (with an antibody agglutination titer of 1:200 00) were purified using affinity chromatography on goat anti-mouse immunoglobulin G (IgG) with Sepharose 4B, and then eluted using 3*M* potassium thiocyanate (KCNS) solution to elute the purified antibodies. The elution reagent was discharged using Sephadex G-25, and the protein part was collected and concentrated to 3 mL using ultrafiltration. After that, the concentrated purified monoclonal antibodies were conjugated, using horseradish peroxidase (HRP), through modified periodate coupling method [*6*]. The enzyme-labeled monoclonal antibodies then were further purified; the free HRP was discharged using Sephadex G-200; and the labeling rate (LR) was evaluated (LR = λ 403/λ 280 nm; λ = wave length). Then the enzyme-labeled antibodies having immunoactivity and an LR of 0.5 were collected; an equal amount of bovine serum and glycerin was added; and the solution was stored at −30°C. The enzyme-labeled monoclonal antibody red blood cell agglutination titers were 1:256 for anti-A and 1:128 for anti-B and anti-H. In the dot-ELISA test, anti-A was diluted 30 times; anti-B and anti-H were diluted 15 times in the working concentrations.

Nitrocellulose Paper

Nitrocellulose paper was purchased from Zhejiang Huangyian People's Chemical Works, People's Republic of China, and Sigma Chemical Co., St. Louis, Missouri.

The Collection, Treatment, and Storage of the Samples

1. *Saliva*—Saliva specimens were obtained from faculty members and students of our department whose blood groups were known. Among these, there were 35 samples belonging to blood group A, 29 to group B, 30 to group O, and 15 to group AB. The saliva specimens were boiled in a boiling water bath for 5 min and stored at −30°C.

The preparation of the saliva stains was as follows—fresh saliva was drawn, dripping it on clean filter paper, dried at room temperature, and stored in envelopes.

2. *Semen*—Semen specimens were obtained from the First Affiliated Hospital, Sun Yat-Sen University of Medical Sciences, Guangzhou, China. All the specimens were typed by neutralization test. The semen specimens were boiled in a boiling water bath for 5 min and stored at −30°C. There were 14 samples belonging to blood group A, 14 to group B, 7 to group O, and 8 to group AB.

The preparation of the semen stains was as follows—semen was drawn, dripping it on clean cotton gauze, dried at room temperature, and stored in envelopes.

3. *Vaginal secretions*—Vaginal secretion specimens were collected from the Gynecology and Obstetrics Department of the First Affiliated Hospital of Sun Yat-Sen University of Medical Sciences. All the samples were collected as vaginal swabs. The blood groups were determined by neutralization test. The swabs were dried at room temperature and stored in envelopes for use. There were 8 samples belonging to group A, 10 to group B, 5 to group O, and 3 to group AB.

The Treatment of the Body Fluid Stains

A small amount of the sample stain was cut into small pieces and put in a small test tube; 200 μL of the diluted antigen solution (0.05*M*, pH 9.6, carbonate buffer) were added; and the test tube was kept at room temperature for 5 min. It was then put in the microrevolving mixing machine and mixed for 5 min. The sample extract was then centrifuged at 3000 rpm for 3 min. The supernatant was pipetted for use.

The Procedure for the Dot-ELISA Test

1. Dilute the body fluid to 1:100 to 1:500 and apply it to three pieces of nitrocellulose paper. For the body fluid stains, add the stain extract to three pieces of nitrocellulose paper, 20 μL for each, and incubate them for 3 min at room temperature.
2. Soak the paper in 30% bovine serum/0.01*M*, pH 7.6 phosphate buffered saline (PBS) solution for 5 min.
3. Put the paper into the appropriate diluted (with 30% bovine serum/PBS) enzyme-labeled monoclonal antibody solution for 10 min. (Anti-A was diluted 30 times; anti-B and anti-H were diluted 15 times.)
4. Put the paper in the 0.05% Tween 20/0.01*M*, pH 7.6 PBS solution, washing it for 2 min to remove the unreacted antibody.
5. Pick the paper up, adding the 3,3'-diaminobenzidine (DAB) substrate solution.
6. Wash the paper with water.
7. Observe the results. If a visible dark brown color appears on the positive site of the paper, the Ag-Ab reaction has occurred. For negative samples, no color change will take place. The color on the paper can be stored permanently and a photograph also could be taken.

The Sensitivity Test

1. Blood group typing was performed on saliva at different dilutions. The saliva was drawn; diluted with the antigen dilution solution to 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}; and typed for blood group using dot-ELISA.
2. Nonsecretor saliva samples were typed using dot-ELISA. These samples included 3 of blood group A, 2 of group B, and 4 of group O.
3. Human urine samples were typed. The 20 samples were obtained from faculty members and students in our department whose blood groups were known. Among those samples, 4 samples were in blood group A, 5 in group B, 5 in group O, and 6 in group AB. The urine samples were directly applied to the nitrocellulose paper and typed by dot-ELISA.

The Blind Study

In the blind study, 40 samples of known blood group (the groups were not known to the person conducting the test) were tested using dot-ELISA. The age of the samples at testing varied from 1 day to 1 year from the date on which the sample was drawn.

Use of the Method in Practical Cases

Specimens from 99 practical cases were tested using the dot-ELISA method: these included 38 saliva stains and 61 semen specimens and semen stains. Of the 38 saliva specimens, 23 were on gauze, 7 on cotton cloth, and 8 on paper (cigarette endings). Of the 61 semen specimens and semen stains, 30 were semen specimens, 20 were on vaginal swabs, and 11 were on the underclothes.

Results

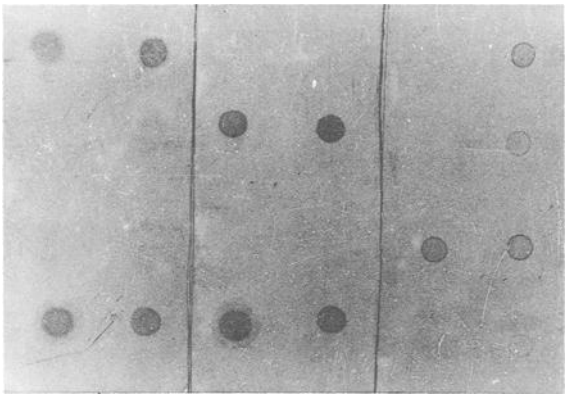
Typing Results of the Known Group Samples

Figure 1 shows the antigen-antibody reaction results using dot-ELISA. A visible dark-brown-colored dot appears on the antigen-antibody reaction site of the paper in a positive reaction. No visible color appears when the reaction is negative. We analyzed 521 samples at different periods from 178 persons in all, and all the results were correct.

The Sensitivity of Dot-ELISA as a Method for Typing Blood Groups

As is shown in Table 1, saliva diluted 10^{-4} to 10^{-5} could still be correctly typed by dot-ELISA; this amount corresponds to 0.0001 μL of fresh saliva. The color of the reaction becomes weaker and weaker as the saliva concentration decreases.

The nonsecretor samples of known group, which had already been determined by the



Blood Group	Anti-A		Anti-B		Anti-H	
	(1)	(2)	(1)	(2)	(1)	(2)
A	●	●			○	○
B			●	●		○
O					●	●
AB	●	●	●	●		○

FIG. 1—*Results of typing body fluids and body fluid stains by dot-ELISA (see the photograph):* (1) *body fluid stains;* (2) *body fluids.*

TABLE 1—*Results of the dot-ELISA sensitivity test.*

Blood Group	Anti-A						Anti-B						Anti-H					
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	Blank	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	Blank	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	Blank
A	+	+	+	−	−	−	−	−	−	−	−	−	+	+	−	−	−	−
B	−	−	−	−	−	−	+	+	+	−	−	−	+	+	+	−	−	−
O	−	−	−	−	−	−	−	−	−	−	−	−	+	+	+	+	−	−
AB	+	+	+	+	−	−	+	+	+	+	−	−	+	+	+	−	−	−

neutralization test, were also correctly typed by this method. The 20 urine samples were also correctly typed by this method.

The Results of the Blind Study

All 40 samples, which ranged in age from 1 day to 1 year, were correctly typed.

The Results for the Practical Cases

The samples for the 38 saliva stains were typed and the results were 13 samples in group A, 11 samples in group B, 11 samples in group O, and 3 samples in group AB. The samples of 61 semen specimens and semen stains (including stains of mixed semen and vaginal secretions) were typed, and the results detected 15 samples containing A antigen, 20 samples containing B antigen, 19 samples containing only H antigen, and 7 samples containing A and B antigen. These samples were also typed by others using other methods, and the results were the same as ours.

Discussion

We have described a simple, rapid typing method for body fluids and body fluid stains—the dot-ELISA method using enzyme-labeled monoclonal antibodies. The blood group was determined by the visible color change of the substrate of the enzyme binding onto the specific antibody.

Using this method, we could easily type the blood groups of body fluids and body fluid stains within 30 min. The results could be easily judged by the visible color changes. In this test, we directly labeled the enzyme onto the anti-A, anti-B, and anti-H McAb. This makes the whole reaction time much less than that for Cecka et al. [7] and the procedure becomes more simple. At the same time, it does not require the second antibody.

This method is extremely sensitive; when saliva was diluted 10^{-4} to 10^{-5}, a satisfactory result could still be obtained, which means this method can be used to test as little as 0.0001 μL of normal fresh saliva. The sensitivity is close to that found by Cecka et al. [7] and by Bolton and Thorpe [8]. The so-called nonsecretor samples tested by the neutralization test can also be satisfactorily typed by this method. In some practical cases, we could not determine the blood groups of the body fluids or body fluid stains by the neutralization test, which could be the result of two causes—one, that the samples belonged to nonsecretors and, two, that the samples did not contain body fluid. In these cases, dot-ELISA test could be used to determine the sample's blood group and whether the sample contained body fluid; however, the neutralization test does have an advantage in that it can distinguish between secretors and nonsecretors.

The blood group could be detected in the urine by the dot-ELISA; so that, if the underclothes contained urine stain, the blood group could be detected. However, because the dot-ELISA is very sensitive, in some cases, if a sample containing semen or vaginal secretion, or both, is contaminated by urine or sweat, its effect must be considered. Dot-ELISA is potentially quantitative; a rough quantitative determination is made based on the lighter or darker color of the spot [9]. For most blood grouping cases, the quantity of the substance is not an issue; instead, a positive or negative result is sufficient. If a semen or vaginal secretion sample is contaminated by urine or perspiration, it is necessary to distinguish the quantity of the soluble antigen, because the antigen content of semen and vaginal secretions is very different from that of urine and sweat; the former is much higher than the latter. Therefore, we can dilute the samples sufficiently to decrease the color of the spot for urine or perspiration so that it is very light or even colorless while the spot color for semen or vaginal secretion is still very dark. In this way, we can exclude the effect of urine or sweat.

In this method, we employed bovine serum to block the nonspecific absorption. This could decrease the base and background color [*10–12*]. We have also found, in this test, that a saliva dilution of 1:200 to 1:500 is suitable for the test.

In forensic science casework, we have found that some fresh negative saliva and semen samples can also produce a light brown color on the nitrocellulose paper, which may affect the interpretation of the results. After we boiled the samples in a boiling water bath for 5 min, the false positive reaction disappeared. We estimated that this phenomenon might be due to the endogenous peroxidase in fresh semen and saliva. Therefore, we suggest, when fresh body fluids are to be tested, that it is better to boil the samples for 5 min to destroy the endogenous peroxidase activity. If tested in a forensic science context, we suggest adding 1% hydrogen peroxide (H_2O_2) to the fresh samples before typing them [*13*].

During the testing, if all the anti-A, anti-B, and anti-H antibodies only produce a light color or if no color change occurs, the sample dilution should then be adjusted to 1:10 to 1:50; at this dilution, satisfactory results can be achieved. Most of these specimens are from nonsecretors.

Individuals who were group A or group B also showed different degrees of reactivity to anti-H. Some reactions were weaker, some stronger, and some even showed no reaction. However, all the group O individuals showed a positive reaction.

Conclusions

In conclusion, using dot-ELISA to type body fluids and body fluid stains has many advantages. It is simple, rapid, sensitive, and specific. The results can be easily observed. It requires no special equipment and does not need fresh red blood cells as indicators. The reagents needed for the test are easily carried and stored. There is no doubt that it will replace the conventional neutralization test, absorption test, elution test, and mixed agglutination reaction test. We are doing further work in this field.

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