TECHNICAL NOTE

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An ELISA Procedure for the Detection of Soluble ABH Blood Group Substance in Semen, Saliva, and Vaginal Samples

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ABSTRACT: Enzyme linked immunosorbent assay (ELISA) was used for the detection of ABH blood group substances in body fluids. The original procedure was modified to provide maximum sensitivity and reliability. The modified ELISA method proved to be more sensitive and required much less sample than previously described techniques.

KEYWORDS: forensic science, criminalistics, ELISA, monoclonal antibodies, blood typing, and body fluid typing

Absorption-inhibition [*1,2*] and absorption-elution techniques [*3*] are currently used for ABH grouping of body fluids. As more advances in forensic serology occur through the development of monoclonal antibodies [*4–7*] these techniques [*8*] get modified. The forensic application of enzyme linked immunosorbent assay (ELISA) [*9*] has lead to modifications of absorption-inhibition [*10,11*]. It is more sensitive, easier to automate and requires much less sample than traditional techniques.

The source of monoclonal antibodies will determine if premixing, simultaneous assays or sequential addition in two steps can be used [*12*]. This paper describes an ELISA procedure for the detection of ABH blood group substance in body fluids using sequential addition of antibodies in two steps with modifications to allow for optimum results.

Materials and Methods

The microtitre plate is prepared by extracting a 2–5 mm^2 cutting of sample in 20 μL of coating buffer in a microcentrifuge tube. Coating buffer is prepared by adding .015 M sodium carbonate, .035 M sodium bicarbonate, .00025 M thimerosal (Sigma, P.O. Box 14508, St. Louis, MO, 63178), and adjusting the pH to 9.6 using concentrated sodium hydroxide. Extract for one hour at room temperature or overnight in the refrigerator. Remove the cutting from the extract and place in a 1–100 μL pipette tip. Place tip back in extract microcentrifuge tube and centrifuge for thirty seconds. Expel liquid in the end of the tip by placing finger over the larger opening and pushing. Put extract microcentrifuge tubes in a floating bubble rack (Whatman, 5285 N.E. Elam Young Parkway, Suite A-400, Hillsboro, Oregon, 97124) and place into a boiling water bath for 10–20 minutes then dilute the extract by adding 10 μL of neat extract into 90 μL of coating buffer. This gives 100 μL of a $^1/_{10}$ extract dilution.

The extract dilutions are placed in an Immulon® 4 flat bottomed microtitre plate made of rigid polystyrene. (Dynatech Laboratories, 14340 Sullyfield Circle, Chantilly, Virginia, 22021) as follows:

i) Add 200 μL of coating buffer to the 1/110 wells and 100 μL of coating buffer to the 1/220, 1/440, and 1/880 wells (Fig. 1).

ii) Add 20 μL of the 1/10 extract dilution to the 200 μL of coating buffer in the 1/110 wells.

iii) Mix by pumping pipette several times and transfer 100 μL of mixture from the 1/110 wells to the 1/220 wells (Fig. 1).

iv) Repeat part iii by transferring 100 μL of mixture from the 1/220 wells into the 1/440 wells.

v) Repeat above procedure until transfer to the 1/880 wells are complete. Discard the last 100 μL portion from the 1/880 wells.

vi) Each 1/110 well should now contain 120 μL. All other wells should now contain 100 μL.

100 μL or 120 μL of antigen coating solution is now in each well of microtiter plate. Incubate overnight at 4°C, or 3 hours at room temperature. Discard the coating solution, after incubation, by inverting the microtiter plate. Shake out any remaining liquid and tap on paper towel if necessary. Wash all wells with 200–300 μL washing buffer for 30 seconds to one minute. The eppendorf

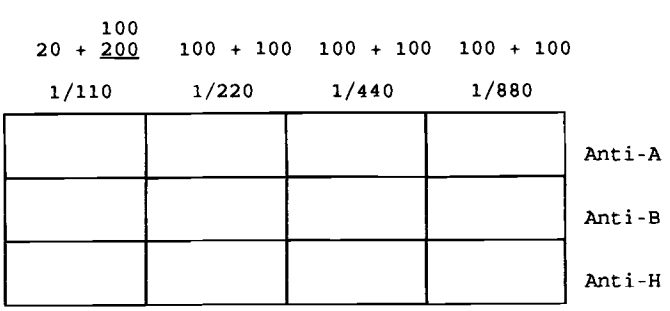

FIG. 1

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repeater plus/8 pipette, cartridge, and tips (Brinkman Instruments, Inc, Cantiague Road, Westbury, New York, 11590) with reagent reservoirs for multichannel pipettes (Cole-Parmer, 7425 North Oak Park, Chicago, Illinois, 60648) will make pipetting easier and faster. Repeat three times and discard as described above. Washing buffer is prepared by adding .144 M sodium chloride, .0015 M potassium phosphate monobasic, .003 M sodium phosphate, .0027 M potassium chloride, .05% (v/v) tween 20 (Sigma, P.O. Box 14508, St. Louis, MO, 63178), .00025 M thimerosal, and adjusting the pH to 9.8 using concentrated sodium hydroxide.

Add 300 μL of blocking buffer to each well. One 96 well microtitre plate requires 29 mL of coating buffer with 1 mL bovine serum albumin (21%). Incubate three hours at room temperature. Blocking buffer consist of coating buffer with addition of 0.5–2.0% bovine serum albumin (BSA). Wash three times with washing buffer and discard.

Add 100 μL of antibody dilutions (diluted in washing buffer) to the appropriate wells. One 96 well microtitre plate requires 3.8 mL of each antibody dilution. These are the dilutions being used. 1/60 dilution of Bioclone© Monoclonal anti-A murine monoclonal blend from hybridoma cell line Birma-1 (Ortho Diagnostic Systems, Raitan, New Jersey, 08869). 1/80 dilution of Bioclone© Monoclonal anti-B murine monoclonal blend from hygidoma cell line ES-4 (Ortho Diagnostic Systems, Raritan, New Jersey, 08869). 1/54 dilution of Mouse monoclonal antibody to blood group antigen H from hybridoma cell line 92FR A2 [*13,14*] (Dako, 6392 Via Real, Carpinteria, CA, 93013). Incubate two hours at room temperature then discard the solution and wash three times.

Add 100 μL of enzyme conjugated antibody. The antibody-enzyme conjugate used was anti-mouse IgM-horseradish peroxidase (Boehringer Mannheim, P.O. Box 50414, Indianapolis, IN, 46250). These are the dilutions being used. 1/18000 in the "A" wells. 1/12000 in the "B" wells. 1/11000 in the "H" wells. Incubate two hours at room temperature or overnight at 4°C and discard the solution and wash four times.

Dilute 2.2'Azino-di-[3-athyl-benzthiazolinsulfonate (6)] (ABTS) (Boehringer Mannheim, P.O. Box 50414, Indianapolis, IN, 46250) substrate solution 1/3 (5 mL in 10 mL water for one plate) and add appropriate amount (30 μL) of 30% hydrogen peroxide, then add 100 μL of ABTS enzyme substrate solution to each well. Blue-green color should develop within ten to sixty minutes. ABTS substrate solution for peroxidase is made of .1 M citric acid anhydrous, .001 M ABTS, and adjusting the pH to 4.2 with sodium hydroxide. ABTS solution is stable for approximately one week at 4°C or two months kept frozen. Immediately before use, add 2 μL 30% hydrogen peroxide per mL of ABTS solution. Discard any unused ABTS and hydrogen peroxide solution.

Stop the reaction with 100 μL stopping buffer when negative controls just start to turn color (within one hour). Stopping buffer consist of .05 M sodium dodecyl sulfate [SDS] (Mallinckrodt, Paris Overpass, Paris, KT, 40362). Optional, read absorbance at 415 nm. See Fig. 2 for ELISA flowchart.

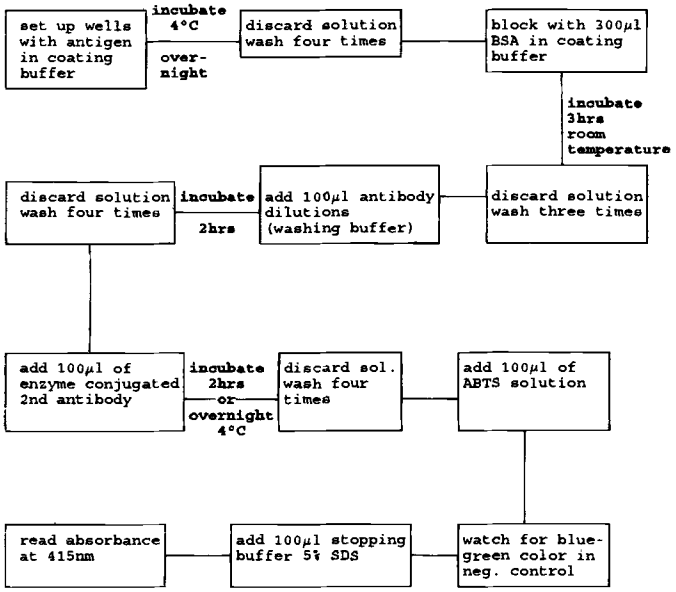

FIG. 2

Result and Discussion

Volunteer donors provided blood, semen, saliva, and vaginal samples and included A, B, O, and AB secretor and nonsecretor individuals. Data was collected from 288 saliva samples, 99 semen samples, 20 vaginal samples, and 78 semen/vaginal mixtures. These samples were obtained from volunteers and casework. The ELISA results were compared with the results from absorption inhibition and blood grouping. No conflicting results were obtained once the modifications were added to the procedure (see Table 2).

Serial dilutions of saliva from 1/5 to 1/320 and from neat to 1/2000 were tested to estimate the dilutions of antigen now being used. The antigen dilutions now being used are 1/110, 1/220, 1/440, and 1/880.

Initially polyclonal anti-A and Anti-B from Ortho Diagnostics were used but these reagents gave complete reaction across the board even with blank controls (see Table 1). Mouse monoclonal anti-A and Anti-B were used and gave excellent results (see Table 2). Mouse monoclonal anti-H purchased from Gamma Biologicals was titered against blood group O. It was determined to have a 1/8 titer compared with the 1/64 titer normally used with absorption inhibition. This reagent was found to be unacceptable after several attempts to type known blood group O secretors (see Table 1). Mouse monoclonal anti-H with a titer of 1/32 was purchased from Dako corporation in Carpinteria, California and reliable results were obtained (see Table 2). Antibody dilutions were tested against antigens to determine optimal dilutions. Varying concentrations of antibodies were used to help determine appropriate antibody concentrations to be used for this procedure. It was found that slight changes from the recommended dilutions has little or no effect on the overall procedure and results obtained. The antibody dilutions now being used are anti-A 1/60, anti-B 1/80, and anti-H 1/54.

Conjugated second antibody concentrations were initially tested at 1:15 000. Ranges from 1/6000 to 1/20 000 were used to determine the final concentrations used in this paper. These concentrations are A = 1/18 000, B = 1/12 000, H = 1/11 000.

The blue-green color that developed with ABTS solution occurred very quickly at full strength. A weaker dilution (one to three) was prepared to slow down the reaction. Results now are obtained within five to ten minutes after addition of ABTS solution.

Some known blood group ABO semen samples were diluted from 1/25 out to 1/250,000,000 and gave correct results at the 1/250,000,000 dilution. Initially, some known blood group B semen samples gave spurious results with anti-A. The other proteins and enzymes found in semen samples as compared to saliva may be

TABLE 1—*ELISA results before modifications.*

Sample	Blood Results	Corresponding Sample	Number Tested	Number ELISA Results Obtained	ELISA Results	Incorrect ELISA Results	Number A/I Results Obtained	A/I Results
Blood	A secretor	SALIVA	13	10/13	A	3/13	13/13	A
Blood	A nonsecretor	SALIVA	4	1/4	no activity	3/4	4/4	no activity
Blood	A nonsecretor	SALIVA	2	2/2	A	—	2/2	no activity
Blood	B secretor	SALIVA	13	10/13	B	3/13	13/13	B
Blood	B nonsecretor	SALIVA	5	5/5	no activity	0/5	5/5	no activity
Blood	O secretor	SALIVA	15	10/15	O	5/15[a]	15/15	O
Blood	O nonsecretor	SALIVA	3	3/3	no activity	0/3	3/3	no activity
Blood	AB secretor	SALIVA	7	5/7	AB	2/7	7/7	AB
		BLANK	6	6/6	AB	6/6	0/6	no activity

[a]Initial ELISA results (5/15) displayed no activity with blood group O secretors.

TABLE 2—*ELISA results after modifications.*

Sample	Blood Results	Corresponding Sample	Number Tested	Number ELISA Results Obtained	ELISA Results	Incorrect ELISA Results	Number A/I Results Obtained	A/I Results
Blood	A secretor	SALIVA	91	91/91	A	0/91	91/91	A
Blood	A nonsecretor	SALIVA	30	30/30	no activity	0/30	30/30	no activity
Blood	B secretor	SALIVA	53	53/53	B	0/53	53/53	B
Blood	B nonsecretor	SALIVA	13	13/13	no activity	0/13	13/13	no activity
Blood	O secretor	SALIVA	86	86/86	O	0/86	78/86	O
Blood	O nonsecretor	SALIVA	24	24/24	no activity	0/24	24/24	no activity
Blood	AB secretor	SALIVA	15	15/15	AB	0/15	15/15	AB
Blood	AB nonsecretor	SALIVA	2	2/2	no activity	0/2	2/2	no activity
Blood	A secretor	SEMEN	11	11/11	A	0/11	11/11	A
Blood	A nonsecretor	SEMEN	4	4/4	no activity	0/4	4/4	no activity
Blood	B secretor	SEMEN	10	10/10	B	0/10	10/10	B
Blood	O secretor	SEMEN	16	16/16	O	0/16	16/16	O
Blood	O nonsecretor	SEMEN	4	4/4	no activity	0/4	4/4	no activity
Blood	AB secretor	SEMEN	10	10/10	AB	0/10	10/10	AB
Blood	A secretor	VAGINAL	5	5/5	A	0/5	5/5	A
Blood	A nonsecretor	VAGINAL	1	1/1	no activity	0/1	1/1	no activity
Blood	B secretor	VAGINAL	3	3/3	B	0/3	3/3	B
Blood	B nonsecretor	VAGINAL	3	3/3	no activity	0/3	3/3	no activity
Blood	O secretor	VAGINAL	8	8/8	O	0/8	6/8	O
Blood	O nonsecretor	VAGINAL	2	2/2	no activity	0/2	2/2	no activity
Blood	AB nonsecretor	VAGINAL	4	4/4	no activity	0/4	4/4	no activity

ELISA corresponded with A/I results from 78 semen/vaginal mixtures obtained from casework.

NOTE: Modifications include using monoclonal anti-A & anti-B instead of polyclonal anti-A & anti-B and also using monoclonal anti-H from Dako Corp. instead of anti-H from Gamma Biologicals.

a possible reason for this spurious activity. Boiling the samples alleviated this problem and is now part of the regular procedure.

Experimentation with combined techniques was also tried. Combination of antibody and enzyme conjugate steps into one step is possible. However, some loss of B activity was observed when using the combined method. Therefore, the procedure previously outlined does not provide for this alternative.

Over 150 known saliva samples received in casework were typed using ELISA and the results were compared with results from traditional absorption inhibition techniques. None of these samples were incorrectly typed using ELISA. Older stains stored in the freezer and at room temperature were resolved using ELISA where traditional absorption inhibition techniques could not detect some of the A, B, or H antigens original present in the stains. Weak secretors could be detected using this ELISA method.

Summary

The ELISA method described is capable of reliably identifying the ABH blood types of body fluids. Maximum sensitivity is achieved by coating the plates with antigen overnight (15 hours) at 4°C. This is followed by coating the plates with blocking buffer for three hours at room temperature. Monoclonal anti-A, anti-B, and anti-H antibodies are incubated two hours at room temperature. Antimouse IgM horseradish peroxidase conjugate is incubated one hour at room temperature then overnight at 4°C, if possible.

Automation of the entire system is possible. The Biomek™ 1000 Automated Laboratory Workstation from Beckman or other suppliers can accommodate these needs.

The antigen-antibody reaction rate can be increased by making the concentration of both antibody and enzyme conjugate higher.

The titers given in this paper have been found to be optimal for overall sensitivity and reliability.

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