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A Microplate Method for Reverse ABO Typing of Bloodstains

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ABSTRACT: A sensitive and reliable hemagglutination assay, using V-bottom microplates, is described for the detection of the ABO blood group alloantibodies in bloodstained material. When used in conjunction with an absorption-elution procedure, the microplate assay resulted in a 300% increase in the number of conclusive grouping results when compared to the Lattes crust test. The use of the microplate reverse grouping assay permits 24 specimens to be assayed conveniently on a single plate and eliminates the tedious and time-consuming microscopic examination required for the Lattes crust test.

KEYWORDS: criminalistics, genetic typing, blood, alloantibodies, blood grouping, blood groups, hemagglutination, immunologic technics, and isoantibodies

In the early 1900s, Lattes began his studies of blood group alloantibodies and their usefulness for typing bloodstained material in criminal cases. His studies culminated in the development of what is now known as the Lattes crust (LC) test, which is used by some forensic science laboratories to confirm the results obtained by forward grouping. A thorough review of the development of this procedure has been given by Gaensslen [*1*]. While reverse typing of well preserved bloodstains is usually possible using the Lattes crust method, results are obtained only after a tedious and time-consuming microscopic examination. Further, since naturally occurring anti-A and anti-B require centrifugation to achieve complete red cell agglutination [*2*], the use of microscope slides in the LC reverse grouping test does not afford optimal sensitivity. Consequently, the microscopic interpretation of agglutination results may at times prove difficult, particularly with aged bloodstains [*3*].

In 1966, Wegmann and Smithies [*4*] introduced a microplate (MP) hemagglutination assay for use in blood group determinations. Since that time, various microplate methods have been described in both the clinical and forensic science literature [*5-10*]. Consequently, a microplate hemagglutination assay was evaluated for its applicability to the detection of anti-A and anti-B alloantibodies in bloodstained material.

Materials

The 96-well V-bottom microplates were obtained from Dynatech Laboratories, Alexandria, VA.

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Affirmagen Group A and B red blood cells (RBC) and Selectogen Group O red cells (Ortho Diagnostics, Raritan, NJ) were used to prepare the indicator cells for both the MP and LC assays.

Buffered saline (HBS), used as a RBC and enzyme diluent, consisted of 0.144*M* sodium chloride and 0.01*M* HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) (Sigma, St. Louis, MO) at pH 7.2. Bloodstain extracts and serum dilutions were prepared using HBS containing 1% (w/v) bovine serum albumin containing 1 to 3% globulins (Sigma) and 1% (v/v) EIA grade Tween-20 (Biorad, Richmond, CA) at pH 7.2 (THBS). Crude ficin (Sigma) was prepared as a 1% (w/v) solution in HBS. The enzyme solution was filtered through Whatman No. 1 paper, divided into 1.0-mL aliquots and stored at −20°C until used. Unless otherwise stated all other reagents were stored at 4°C.

Serum specimens used in this study were obtained from the Rh Typing Laboratory, Baltimore, MD. Bloodstains of known ABO phenotype, prepared on washed cotton cloth, were obtained by finger puncture from volunteer donors at the FBI Academy. Nonprobative case samples were obtained from the Serology Unit, FBI Laboratory.

Methods

Ficin-treated indicator RBCs, used in the MP assay, were prepared by taking 340-μL aliquots of Type A, B, and O stock (3%) red cells and washing once with HBS. The HBS supernatant was decanted after centrifugation and 200 μL of 1% ficin added to the cell pellet. The RBCs were resuspended and incubated at 37°C for 15 min. Following incubation, the cells were immediately washed three times with HBS. The supernatant from the last wash was decanted and the RBCs resuspended in 2.0 mL of HBS which produced a final red cell concentration of 0.5%. This volume of cells was sufficient to conduct approximately 100 assays. Ficin treated RBCs were prepared daily and stored at 4°C.

Two bloodstain cuttings, 16mm^2 (4 by 4 mm) each, were placed into a 1.5-mL conical polypropylene tube and extracted in 100 μL of THBS for 1 h at room temperature. If necessary, extraction also can be accomplished overnight at 4°C. Following incubation, the cloth material was transferred to a smaller, 0.5-mL conical polypropylene tube, the bottom of which had previously been punctured with a heated dissection needle. The smaller tube was then inserted in the larger tube and centrifuged for 1 min. This method permitted almost complete recovery of the fluid from the cloth as well as providing a clean supernatant for MP analysis.

The MP hemagglutination assay was a modification of the MP procedure described by Baechtel [*10*]. Briefly, the procedure was as follows: 20-μL aliquots of bloodstain extract or diluted serum were added to triplicate wells in Columns 1 to 12 of the microplate. This arrangement permitted 24 specimens to be assayed per plate. Twenty-microlitre aliquots of appropriately diluted Group A, B, and O indicator RBCs were then added to the corresponding wells and the contents mixed by gently agitating the plate. The plate was covered and incubated undisturbed for 30 min at room temperature. Following incubation, the plate was centrifuged at 1500 rpm for 5 min and then tilted at an approximately 60° angle for a minimum of 30 min. The amount of time required before the agglutination results can be read varied with each specimen. This interval, usually 50 to 60 min, was based upon the length of time necessary for the RBCs in the O well to produce a smooth run to the bottom edge of the well.

Anti-A and anti-B alloantibody titers in serum were determined using serial doubling dilutions and the titer recorded as the reciprocal of the last dilution producing 4+ agglutination. When comparing titers, a difference of two doubling dilutions was necessary before the titers were considered significantly different [*2*]. Mean titer values were calculated using the $\log_2$ of the individual titers.

To conduct the LC assay, three cuttings (4 by 4 mm each) were taken from the bloodstained material. The specimens were placed on three separate microscope slides labeled A, B, and O and covered with a 18- by 18-mm glass coverslip. Corresponding indicator cells, prepared as a

0.015% (v/v) suspension in 0.85% sodium chloride were allowed to flow in and completely fill the area under the coverslip. The slides were incubated for 6 h at room temperature in a moist chamber and the agglutination results, recorded as positive or negative, were read microscopically.

The tube and thread methods of absorption-elution, previously described by Lee [3], were used with the following modification: indicator red cells were prepared as a 0.25% (v/v) suspension in 0.3% (w/v) BSA in HBS. Bloodstain elution was carried out at 60°C in 0.3% BSA in HBS.

Results

The typical agglutination patterns, obtained using the MP reverse grouping assay for the different ABO phenotypes, are shown in Fig. 1. Complete agglutination (4+) was demonstrated in those wells where the red cells remained in a tight pellet following tilting of the plate. The absence of blood group alloantibodies was exhibited by a smooth run of cells extending from the pellet to the bottom edge of the well. Specimens having low antibody titers can produce agglutinations of intermediate strengths: 3+, 2+, or 1+ as illustrated in Fig. 2. The rate at which the cells stream will depend upon the protein concentration of the extract. In most cases, this was associated with the color intensity of the extract; the darker the extract, the slower the rate at which the cells will run. As a result, the length of time that the plate was tilted before the agglutination results could be evaluated varied with each specimen. This interval

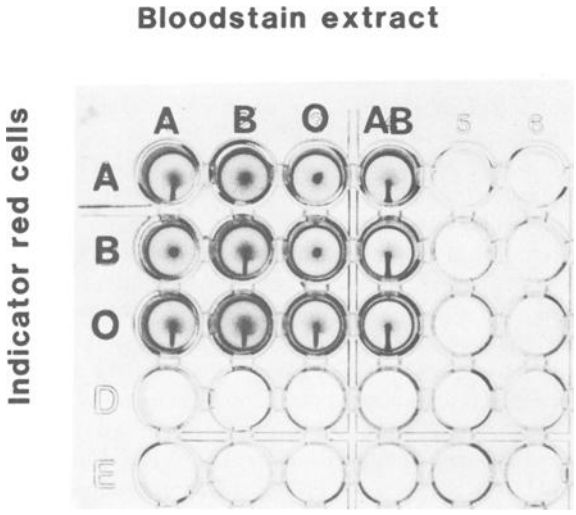

FIG. 1—*Agglutination patterns obtained using the microplate reverse grouping assay with bloodstain extracts prepared from Group A, B, O, and AB donors.*

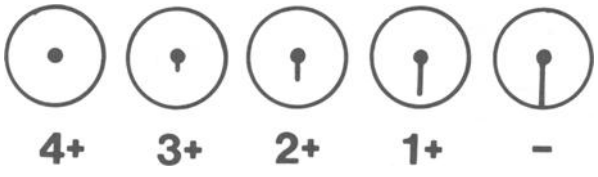

FIG. 2—*Variation in agglutination strength observed in the microplate assay.*

was based upon the time that was required for the cells in the O well to produce a smooth run extending to the bottom edge of the well. In this assay, only agglutination scores of 2+ or greater were considered as a positive result. Those wells which exhibited a red cell run extending more than half way down the well were recorded as a negative result.

The results of a study, which contrasts the effectiveness of the MP and LC assays in detecting anti-A and anti-B from Group O bloodstains stored at various temperatures, are shown in Table 1. In this study, triplicate bloodstains were prepared from each of 20 Group O donors. After air-drying, the bloodstains were stored at −20°C, room temperature (RT), and 37°C, and were assayed by both techniques after storage for 4, 8, and 18 weeks.

When those stains stored at −20°C were tested by the LC method, both anti-A and anti-B were detected in 17 of the 20 stains after 4 weeks; 18 of 20 after 8 weeks; and in 16 of 20 after 18 weeks. Of the stains stored at RT, anti-A and anti-B were detected in 18 of 20 after 4 weeks; 16 of 20 after 8 weeks; and 9 of 20 after 18 weeks when tested using the LC procedure. In contrast, when the above bloodstains were tested using the MP technique, both anti-A and anti-B were detected in all 20 stains regardless of the temperature or length of storage.

When the stains stored at 37°C were tested using the LC procedure, both anti-A and anti-B were detected in 15 of 20 stains after 4 weeks and in 7 of 20 after 8 weeks. Analysis of these same stains using the MP technique revealed that both anti-A and anti-B could be detected in all 20 stains.

Using the LC procedure, neither anti-A nor anti-B were detected in any of the bloodstains storaged for 18 weeks at 37°C. However, when these stains were tested using the MP technique, both anti-A and anti-B could still be detected in 4 of the 20 stains.

Eighty-one bloodstains prepared from individuals of known ABO phenotype (twenty-seven O; forty-three A; ten B, and one AB) were grouped by both the MP and LC assay. Both assays produced results which were consistent with the phenotype of the known bloodstains.

To validate further the microplate procedure for use in the examination of forensic science evidence, 213 nonprobative bloodstains from 70 cases submitted to the FBI Laboratory were forward grouped by absorption-elution (AE) and reverse grouped by both the MP and LC assay. For the bloodstains to be conclusively grouped, both the forward and reverse grouping test must have produced corroboratory results. Of the 213 bloodstains tested, only 27 produced LC results that were consistent with those obtained by AE. However, when these same stains were grouped by the MP procedure, 81 specimens produced results that were consistent with those obtained by AE.

To study the topographical variation of the alloantibody within a bloodstain, four stains from each of two Type O donors were prepared using known volumes of whole blood. After the area of each stain was determined, a section equivalent to one half the total area was removed

TABLE 1—*Comparison of the microplate and Lattes crust tests for the detection of both anti-A and anti-B in bloodstains from Group O donors stored at various temperatures.*

Test	Age, weeks	Storage Temperature, °C		
		−20	22	37
Microplate	4	20[a]/20[b]	20/20	20/20
	8	20/20	20/20	20/20
	18	20/20	20/20	4/20
Lattes	4	17/20	18/20	15/20
	8	18/20	16/20	7/20
	18	16/20	9/20	0/20

[a]Number of stains where both anti-A and anti-B were detected.
[b]Number of stains tested.

from the center of each stain. The center and outer portion from each bloodstain were then extracted and the titers of anti-A and anti-B determined. The results, presented in Table 2, show no significant difference between the titers obtained from the center and outer portions of each bloodstain.

The levels of the ABO blood group alloantibodies obtained for serum specimens from 100 white and 100 black individuals are shown in Table 3. In the white population, the mean titer for anti-B, in 46 Group A individuals, was 733 with titers ranging from 32 to 10 240. The mean anti-A titer, in 9 Group B donors, was 871 with titers ranging from 320 to 2560. For 42 Group O individuals, the mean anti-A titer was 2207 with titers from 160 to 327 680, while the anti-B titer ranged from 160 to 20 480 with a mean of 2280. The remaining three individuals were classified as Type AB based on the absence of both anti-A and anti-B in the specimens.

For the black population, 24 specimens were grouped as Type A having a mean anti-B titer of 879 with a range of titers from 40 to 40 960. The mean titer for anti-A in 25 Group B individuals was 1114 with titers ranging from 160 to 5120. In 44 Group O specimens, the mean anti-A titer was 3045 with a range of titers from 1660 to 40 960, while the anti-B titers ranged from 160 to 10 240 with a mean of 2221. The remaining specimens were grouped as Type AB.

Differences in mean titers for anti-A and/or anti-B, observed between the white and black populations for Group A, B, and O individuals, were not significant. Within each population, however, differences in the mean anti-A titers, observed between Group B and O indi-

TABLE 2—*Topographical distribution of serum alloantibodies in dried bloodstains.*

	Titers[a]			
	Anti-A		Anti-B	
Blood Type	Center	Outer	Center	Outer
O	640	640	1280	1280
O	80	160	5120	5120

[a]Reciprocal of last dilution producing 4+ agglutination.

TABLE 3—*Distributions of ABO blood group alloantibody titers in serum specimens from 100 black and 100 white individuals.*

		Alloantibody Titer[a]			
		Blacks		Whites	
Blood Type		Anti-A	Anti-B	Anti-A	Anti-B
A	mean		879 (N = 24)		733 (N = 46)
	range		40-40 960		32-10 240
B	mean	1114 (N = 25)		871 (N = 9)	
	range	160-5120		320-2560	
O	mean	3045 (N = 44)	2221	2207 (N = 42)	2280
	range	160-40 960	160-10 240	160-327 680	160-20 480

[a]Reciprocal of last dilution giving 4+ agglutination.

viduals, and in the mean anti-B titers observed between Group A and O individuals, appears significant.

To determine if a correlation existed between anti-A and anti-B titers in Group O individuals, a least-squares linear regression analysis was performed for both Group O populations. A plot of anti-A versus anti-B titers obtained for these specimens is shown in Fig. 3. The correlation coefficient determined for anti-A and anti-B in whites was $r = 0.30$ and 0.36 for blacks, indicating a lack of correlation.

Discussion

Since anomalies may sometimes occur in either the forward or reverse grouping test, particularly when dealing with bloodstained evidence [*1,3,11-14*], it is essential that both tests produce results that are consistent with one another if an accurate and reliable determination of the blood group is to be made.

The results of this study clearly demonstrated that the MP assay was superior to the LC assay for the reverse typing of bloodstained material. The MP assay provides the forensic serologist with a method by which agglutination strengths can be easily standardized and interpreted. Further, this assay eliminates the time-consuming microscopic examination necessary with the LC procedure. But most importantly, the increased sensitivity of the MP assay permits the detection of anti-A and anti-B in aged bloodstains beyond what was possible using the LC assay.

The agglutination results shown in Fig. 1 are typical of those normally seen with the MP assay and demonstrate the ease with which the agglutination patterns can be scored. Further, this assay provides the laboratory with a standardized method for scoring red cell agglutina-

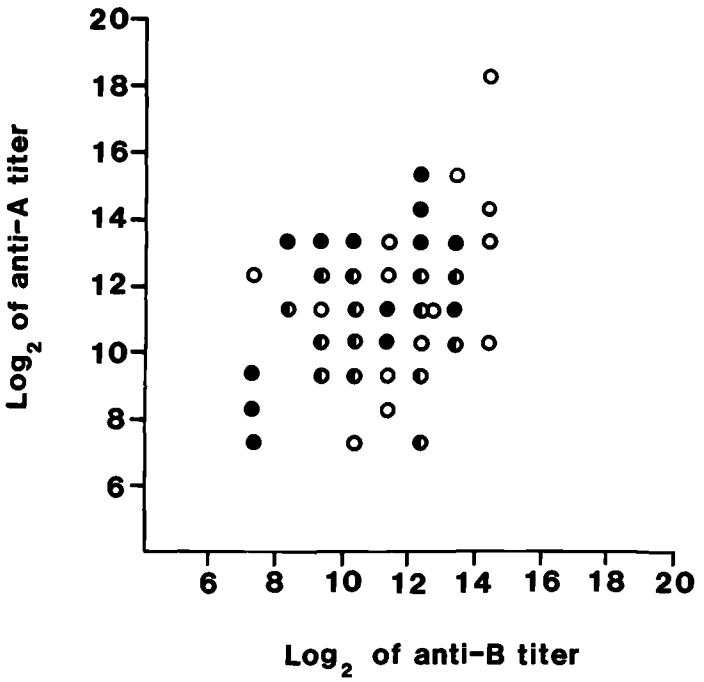

FIG. 3—*Least squares linear regression analysis of anti-A and anti-B titers from Group O black (●) and white (○) populations.*

tion. Although the strength of the agglutination can vary, depending upon the concentration of the antibody in the extract (Fig. 2), 3+ or 4+ agglutinations were usually obtained with bloodstain extracts using the MP assay. As in the LC test, the cells in the O wells should not exhibit any agglutination and thereby act as internal negative controls. Consequently, any well in the O row that fails to produce a smooth run extending to the bottom of the well would invalidate the reverse grouping result for the specimen in question.

The increased sensitivity of the MP assay over the LC assay was apparent following the analysis of 20 Group O bloodstains that had been stored at various temperatures. These results, obtained at different times during 18 weeks of storage, were presented in Table 1 and show that at no time were both anti-A and anti-B detected in all 20 stains using the LC procedure. When the stains were assayed by the MP method, however, both antibodies were detected in all 20 stains after 8 weeks, regardless of the storage condition, and after 18 weeks storage at −20°C and RT. It should be noted that the agglutination obtained for these bloodstains, using the LC procedure, was at times very weak. On the other hand, when the bloodstains were tested by the MP method, only 4+ agglutination was obtained for any specimen where the presence of either antibody was detected.

The analysis of 81 known, laboratory prepared bloodstains by both the MP and LC assay revealed no inconsistencies in the results obtained by either assay. The LC assay required the microscopic examination of over 240 slides to determine the presence or absence of agglutination and took approximately 2 h to complete. In contrast, these same specimens were conveniently assayed in four microplates and the presence or absence of agglutination determined visually in less than 10 min.

More than 200 nonprobative bloodstains from 70 cases submitted to the FBI Laboratory were subjected to analysis by both the LC and MP method. These specimens were subsequently grouped by an absorption-elution assay (forward typing) and a determination of blood type was made for those bloodstains where the forward and reverse grouping results were consistent. Of 213 bloodstains tested, only 27 gave results that were consistent based on the elution and LC test, while 81 specimens produced grouping results that were consistent using the elution and MP assays. This represents a threefold (300%) increase in the number of conclusive blood group determinations that could be made when the MP assay was used in conjunction with the absorption-elution assay. These results do not include those bloodstains which grouped as Type AB since the absence of detectable antibody in a bloodstain does not always indicate an AB phenotype.

A study of the topographical distribution of blood group alloantibodies in bloodstains (Table 2) showed that no significant differences were observed in their distribution within the stains. Since there appears to be a uniform distribution of antibody in the bloodstain, one would not expect the area of the bloodstain from which the specimen was obtained to influence the quantitative recovery of these antibodies in the MP assay.

The titers obtained for anti-A and anti-B in serum specimens from 100 black and 100 white individuals (Table 3) varied over a wide range for the different blood groups within each population. This variation in titer is important to remember, particularly when dealing with Group O individuals. In these individuals, both anti-A and anti-B are present, but their titers in the serum vary independently of each other. Consequently, where the initial titer of one of the antibodies is much lower than the other, it would be possible for the stains prepared from these individuals to be reversed grouped as Type A or B. This was particularly true when using the LC assay, as illustrated in Table 1, where either anti-A or anti-B or both were undetected in 3 of the 20 laboratory prepared Group O bloodstains that were stored at −20°C for 4 weeks. Although the titers for anti-A or anti-B or both varied over a wide range within the different blood groups, no significant differences in the mean titers were observed between the two populations. Within each population, however, significant differences in titers were observed for anti-A between Group B and O individuals and for anti-B between Group A and O individuals. Similar trends have been observed by others [*15*].

The microplate reverse grouping procedure described in this paper was shown to be superior to the Lattes crust assay in sensitivity. This technique significantly reduces assay time through elimination of a time-consuming microscopic examination. Further, the microplate reverse grouping procedure, when used in conjunction with a standard forward grouping test, will result in an increase in the number of conclusive blood determinations that can be made from bloodstained material.

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