TECHNICAL NOTE

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A Modification of the Microplate Method for Reverse ABO Typing of Bloodstains and Additional Validation Studies

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ABSTRACT: The results of additional validation studies of a sensitive microplate hemagglutination assay for ABO reverse grouping of bloodstains are presented. The results of the validation study demonstrate the reliability of the microplate assay for use in routine serological casework. Based on these studies, the microplate assay has now replaced the Lattes crust test for ABO reverse grouping of bloodstains in the FBI Laboratory.

KEYWORDS: pathology and biology, serology, genetic typing, blood

A microplate method for the reverse ABO grouping of bloodstains has been placed into operation by the Serology Unit of the FBI Laboratory. This microplate technique replaces the Lattes crust procedure [*1*] that was developed in the early 1900s. This paper reports on additional validation studies that were conducted during the implementation of the microplate assay, as well as minor modifications to the original procedure [*2*] that facilitated its implementation.

Materials and Methods

Nonprobative case samples and bloodstains of known ABO phenotype, prepared on washed cotton cloth from liquid blood samples, were obtained from the Serology Unit of the Federal Bureau of Investigation (FBI) Laboratory. Absorption elution and Lattes crust (LC) grouping tests were conducted as previously described [*2*]. The microplate (MP) reverse grouping procedure [*2*] was modified as follows: two 4- by 4-mm bloodstains were extracted in 200 μL of 1% (w/v) bovine serum albumin (BSA) in HEPES buffered saline (HBS), pH 7.2. Indicator red blood cells were prepared by mixing 400 μL of stock 3% red cells with 400 μL of 1% ficin and incubating at 37°C for 15 min. Following incubation, the cells were immediately washed 3 times with HBS. After the final wash was decanted, the cell pellet was resuspended in 2.4 mL of HBS. This volume of cells was sufficient to test approximately 100 samples.

The elimination of Tween-20 from the extract enabled the same bloodstain extract to

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be used in more than one assay. The preparation of ficin-treated red cells was modified to simplify the enzyme treatment process. Neither modification had an effect on the quantitative or qualitative performance of the MP assay.

Results

The performance of the LC and MP assays was compared by analyzing 136 bloodstains of known ABO phenotype. The results are summarized in Table 1. As shown in this table, all Group O (n = 56) and Group B (n = 19) bloodstains were grouped correctly (both anti-A and anti-B detected for Group O; only anti-A detected for Group B), and no discrepancies were observed between the two assays.

Of the 54 Group A bloodstains tested, 51 and 53 stains, were identified as Group A by the LC and MP procedures (only anti-B detected), respectively. For one stain, both the LC and MP procedures failed to detect the presence of anti-B. Two stains, which tested positive for anti-B by LC, also produced false positive agglutination by LC: one false positive for anti-A (suggesting Group O) and the other producing nonspecific agglutination of the Group O indicator cells (inconclusive).

Of seven known group AB stains tested, no false positive MP results were observed (neither anti-A or anti-B detected). However, one stain resulted in a false positive for anti-B when tested by LC.

A blind study comprised of stains prepared from 94 liquid blood specimens was also performed. These stains were grouped using the absorption elution, LC and MP techniques. Conclusive grouping results were obtained for those stains where the forward (elution) and reverse (LC or MP) results were in agreement. These bloodstain grouping results were then compared with the results initially obtained for the liquid blood specimens. The results of this study are presented in Table 2.

Liquid blood grouping results of the 94 specimens identified 40 as Group O, 32 as Group A, 11 as Group B, and 11 as Group AB. For those specimens grouped as A, B, and O, the same blood types were identified from the corresponding dried stains following testing by elution and MP. However, when the elution results were matched with those from the LC test, 3 of the 40 Group O specimens produced inconclusive results due to the failure to detect anti-A.

Out of the eleven Group AB specimens initially identified, the elution and LC test results showed agreement for only ten specimens. One specimen was incorrectly identified as Group B due to a false negative elution result for the A antigen and a false positive

TABLE 1—*Comparison of MP and LC tests using bloodstains of known ABO phenotype.*

Blood Group	No. Tested, %	Microplate		Lattes Crust	
		Anti-A	Anti-B	Anti-A	Anti-B
O[a]	56 (41)	56	56	56	56
A[b]	54 (40)	0	53	1	51
B[c]	19 (14)	19	0	19	0
AB[d]	7 (5)	0	0	0	1

[a]Group O has anti-A and anti-B; all specimens correctly typed.
[b]Group A has anti-B only; 1 as LC false positive for anti-A and grouped as O; 1 as LC false positive O cells (inconclusive).
[c]Group B has anti-A only; all specimens correctly typed.
[d]Group AB has neither anti-A or anti-B; 1 as LC false positive for anti-B.

TABLE 2—*Elution, MP, and LC results from blind study using laboratory prepared bloodstains of known phenotype.*

Blood Group	No. Tested, %	Elution	Microplate		Lattes Crust	
			Anti-A	Anti-B	Anti-A	Anti-B
O[a]	40 (43)	40	40	40	37	38
A[b]	32 (34)	32	0	32	0	32
B[c]	11 (12)	11	11	0	11	0
AB[d]	11 (12)	10	0	0	1	0

[a]All specimens correctly typed by elution and MP; 37 specimens correctly grouped by LC.
[b]All specimens correctly typed by elution, MP, and LC.
[c]All specimens correctly typed by elution, MP, and LC.
[d]One as Group B (false negative A-antigen; false LC positive anti-A).

LC result for anti-A. When tested by MP, however, the specimen was negative for both anti-A and anti-B, and the final grouping result would be reported as inconclusive due to conflicting elution (Group B) and MP (Group AB) results.

To compare the performance of the LC and MP procedures with actual case work material, we grouped 431 question stains from 47 different cases by elution, LC, and MP. The results of this study are presented in Table 3.

Blood group antigens were detected by absorption-elution in 418 (97%) of the questioned stains: 200, H antigen, consistent with Group O; 116, A antigen, consistent with Group A; 85, B antigen, consistent with Group B; and 17, A and B antigens, consistent with Group AB.

Of 401 stains initially grouped as A, B, or O, the LC results for 276 (69%) stains were in agreement with those of elution. When these same stains were tested using the MP procedure, 311 (78%) of the stains produced results that were consistent with those of elution.

Of 17 stains initially grouped by elution as AB, 8 stains produced LC results consistent with those of elution, while only 5 produced MP results that were consistent with those obtained by elution.

A comparison of the time required to perform the LC and MP procedures was conducted using 50 bloodstains. Excluding incubation, the total "hands-on" time for both

TABLE 3—*Grouping results from 431 nonprobative case specimens as determined by elution, MP, and LC.*

Blood Group Identified[a]	Elution[b]	Reverse Grouping Procedure	
		MP	LC
O	200	165	147
A	116	83	68
B	85	63	61
AB	17	5	8

[a]Identification of blood group based upon elution test results.
[b]Of 431 bloodstains, 13 could not be typed by elution.

set up and scoring the results for each assay was approximately 2 h. Although the MP assay required an additional 40 min to set up, relative to the LC procedure, the time necessary to score the agglutination for the MP assay was reduced by more than 50 min. Thus, the additional time necessary to set up the MP assay is offset by the reduction in scoring time.

Discussion

The analysis of the 230 bloodstains of known phenotype used in the known and blind studies (Tables 1 and 2), as well as the 431 question bloodstains from Table 3, provides an opportunity to evaluate the performance of the LC and MP assays (as well as the elution assay).

No false positive results were obtained using the MP procedure for any of the 230 bloodstains of known phenotype tested in Tables 1 and 2. However, 3 of the stains (2 group A and 1 Group AB, Table 1) produced false positive results using the LC procedure which resulted in an inconclusive grouping call. A fourth stain (Group AB, Table 2) also produced a false positive result by LC, but was incorrectly identified as a Group B due to the false positive LC result for anti-A and a false negative result by elution for the A antigen.

The occurrence of pseudoagglutination (rouleaux formation) is known to occur in the presence of a high concentration of serum proteins [*3*] or of a concentrated stain extract [*2,4*]. However, if the serum or stain extract is diluted with saline, the rouleaux can be dispersed while true agglutination is not disturbed. Although rouleaux formation occurs with both the LC and MP procedures, it can be readily identified using the MP procedure but difficult to identify with the LC procedure.

The MP procedure uses a single homogenous stain extract, and the extract-to-red-cell ratio is constant in each well. If the extract is too concentrated, rouleaux formation occurs equally in all three wells of the microplate and the indicator red cells remain as a pellet in the bottom of the well. If this occurs, the remaining extract can be further diluted to eliminate rouleaux formation.

On the other hand, the LC procedure uses a separate cutting for each slide, and the amount of stained material added to each slide can vary. Consequently, the extract-to-red-cell ratio is uncontrolled and varies between each slide for each stain tested. Because of this, rouleaux formation may occur on any number of the three slides and may result in the interpretation of weak rouleaux formation as positive agglutination. Accordingly, the occurrence of false positive LC results may be attributed to the difficulty in microscopically differentiating weak agglutination from nonspecific aggregation of the indicator cells because of rouleaux formation.

In addition, the presence in dried bloodstains of non-ABO antibodies, such as anti-D, may also contribute to the occurrence of the false positive results observed with the LC test [*5*]. While some bloodstains, as noted above, produced false positive LC results, none of these bloodstains produced any false positive MP results.

Whatever the cause, the occurrence of false positive results with bloodstains of known phenotype, as observed using the LC method, are of critical importance, particularly when they occur in the presence of a false negative result by elution (Table 2, Group AB).

Bloodstain evidence is often subjected to various and uncontrolled environmental insults which may affect the stability of the blood group antigens and antibodies. Additionally, anti-A and anti-B antibodies may be actually absent from the blood itself. Consequently, the occurrence of false negative results by either LC or MP (as well as elution) were neither surprising nor unexpected. For this reason, the conclusive identification of the group AB phenotype from bloodstain evidence may at times prove difficult.

The difficulty of conclusively identifying the group AB phenotype in bloodstains of unknown origin is further compounded by the potential that more than one person may have contributed blood to the stain. For these reasons, the exact cause for the differences observed between the LC and MP results for Group AB case stains in Table 3 cannot be determined.

The use of the MP method provides the serologist with a more reliable and easily interpretable assay for reverse ABO grouping. Although the overall time required to conduct the MP assay is approximately the same as that required for the LC test, the MP method eliminates the tedious and time-consuming microscopic examination required for the LC test. In addition, the interpretation of agglutination results is much simpler and more objective, and the variation in agglutination strength is better defined using the MP assay [*2*].

Further, the MP assay uses a single, homogeneous bloodstain extract instead of a separate cutting of varying size for each microscope slide in the LC test. Consequently, the MP assay provides greater reproducibility and reduces intraassay variation by maintaining a constant extract to red cell ratio within each well.

The MP assay uses approximately a third of the total extract volume per assay. Therefore, sufficient extract remains if dilution and retesting of the extract are required. In the LC assay, however, complete recutting of the specimens is required for retesting.

Based on the results of this validation study, as well as the advantages of the MP assay mentioned above, the MP procedure has replaced the LC test for reverse ABO grouping of bloodstains in the FBI Laboratory.

References

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