

Use of low ionic strength saline for crossmatching and antibody screening

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SUMMARY A totally revised crossmatching and antibody screening procedure has been designed using low ionic strength saline as the suspending medium for red cells in the saline and enzyme phases as well as the antiglobulin test. The replacement of Pasteur pipettes with precision samplers for all serum and red cell dispensing has resulted in improvement in the reproducibility and standardisation of technique. The major advantages are improved sensitivity, shortened incubation time, and simplicity of technical performance.

In recent years, a number of workers have reported the advantages of using low ionic strength saline (LISS) suspended red cells for antiglobulin tests (AGT) (Austin, 1976; Löw and Messeter, 1974; Moore and Mollison, 1976; Moore and Sipes, 1976; Ross and Ducie, 1978; Wicker and Wallas, 1976). The increased sensitivity with reduced incubation time, which can be achieved with the LISS antiglobulin test, has been confirmed.

The feasibility of using LISS instead of 0.9% saline for the detection of agglutinating antibodies has also been established (Löw and Messeter, 1974; Moore and Mollison, 1976; Moore and Sipes, 1976; Ross and Ducie, 1978).

It was considered worthwhile to evaluate the use of LISS in all phases of the crossmatch, including the enzyme phase, with the further aims of simplifying the techniques and improving the efficiency of the procedures.

Because of the necessity of maintaining low ionic strength in the final reaction mixture for the LISS antiglobulin test, precision samplers were used instead of the usual Pasteur pipettes for all serum and red cell dispensing. This not only overcame the effect of drop size variation on the sensitivity of the LISS antiglobulin test but also resulted in standardisation of volumes and improved reproducibility.

Material and methods

CURRENT CROSSMATCH

(1) Room temperature phase:
Equal volumes of 2% saline (0.9%) suspension of

red cells and serum were mixed in 75 × 12 mm tubes, incubated for 30 minutes, and read microscopically without centrifugation.

(2) Enzyme technique:

The two-stage papain technique of Albrey and Simmons (1960) using a 20-25% suspension of red cells was performed on a tile.

(3) Antiglobulin technique:

Equal volumes of 50% saline suspension of red cells and serum were incubated for 30 minutes at 37°C; the cells were then washed four times and the test result was read on a tile.

LISS SOLUTION

This consisted of 0.3 M glycine in 0.03 M sodium chloride. No buffer was added. The pH of the solution was 6.0.

LISS CROSSMATCH

(1) Donor cells from a bag segment were washed once in 0.9% saline (in Sorvall CW-1 AF2 cell washer) followed by two washes with LISS. Cells were resuspended to give a 5% concentration in LISS.

(2) To each of three 75 × 12 mm plastic tubes were added 50 µl of cell suspension.

(3) To the first tube 50 µl patient's serum was added, and the tube contents were mixed and incubated at 37°C for 15 minutes. The cells were washed four times in a Sorvall CW-1 AF2 cell washer, and the AGT was performed by the tube method in the usual way.

(4) To the second tube was added 100 µl serum. It was mixed and incubated at room temperature for

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15 minutes and read macroscopically by the tube spin method.

(5) To the third tube 50 μ l of papain solution was added (one volume papain (CSL) to three volumes of phosphate buffered saline pH 7.4). It was mixed and incubated for 10 minutes at 37°C and washed once in the Sorvall cell washer; 100 μ l serum was added. After mixing and incubation for 5 minutes at 37°C it was read macroscopically by the tube spin technique.

The LISS crossmatching procedure is outlined in Table 1.

ANTIBODY SCREEN

The original screening procedure used was that outlined on the enclosures accompanying the Selectogen (Ortho Diagnostics) screen cells. In addition, the papain technique of Albrey and Simmons (1960) was carried out, concentrating the Selectogen cells to 20-25% by removing some of the supernatant preservative solution.

For the LISS procedure, the antibody screen was done by exactly the same techniques as the crossmatch. The Selectogen cells were centrifuged, the supernatant preservative removed, the cells washed once in LISS, and then resuspended to 5% concentration in LISS. This was done at the beginning of each day, preparing a sufficient volume for the day's work.

ANTIGLOBULIN TEST PROCEDURE COMPARISONS

An initial comparison was made between the LISS/AGT and the albumin/AGT (ALB/AGT) using 10-minute and 30-minute incubation times. The sensitivity of these procedures was also compared with the saline/AGT (SAL/AGT) performed in a tube using 5% red cell suspension and 60 minutes' incubation time. This comparison was performed using Pasteur pipettes for serum and red cell dispensing, the master dilution technique for titrations, and a 37°C waterbath for incubation.

In the later investigation, the LISS/AGT was compared with the ALB/AGT used in the previous screening procedure and the SAL/AGT used for the previous crossmatch. Dry incubation blocks were used for this study. Also, in this investigation all sera used were tested undiluted. The techniques were further standardised as far as possible by using precision samplers (Oxford Laboratories Inc) to dispense the required volumes of sera, red cells, and reagents.

For both series of investigations the same donor red cells of appropriate phenotype were used for any one antibody, and the comparisons for any one antibody were performed at the same time.

PAPAIN TECHNIQUES COMPARISON

For the purpose of comparing the Albrey and Simmons technique with the tube papain method, those antibodies reactive by enzyme technique only were compared separately from those reactive by AGT or saline also. This was done in an attempt to emphasise the significance of the papain technique as part of the overall crossmatch and antibody screening procedure.

ROOM TEMPERATURE PHASE TECHNIQUES COMPARISON

Twenty saline reactive antibodies were tested in parallel against LISS suspended cells of appropriate phenotype and against the same cells suspended in 0.9% saline. Two volumes of serum were added to one volume of red cell suspension in 75 \times 12 mm tubes using an Oxford sampler. The tubes were incubated at room temperature for 30 minutes, and the reaction strength was recorded.

A further comparison was made using the same antibodies between a 15-minute incubation using the tube-spin technique and a 30-minute incubation without centrifugation corresponding to the old crossmatch method. This was necessary to confirm that a shorter incubation time did not significantly reduce the sensitivity of this phase of the crossmatch.

Table 1 *LISS crossmatching procedure*

	1	2	3
Donor cells washed once in 0.9% saline, then twice with LISS—5% suspension	50 μ l	50 μ l	50 μ l
Papain solution	—	—	50 μ l
			Incubate 37°C 10 min, wash once with 0.9% saline
Patient's serum	50 μ l	100 μ l	100 μ l
Incubate	15 min 37°C	15 min room temp	5 min 37°C
	Perform AGT	Read by tube spin	Read by tube spin

For all method comparisons, any one reaction was deemed to be stronger than another only if there was at least a 1+ difference in reaction strength.

Results

Table 2 summarises the results of the comparison of LISS/AGT with the ALB/AGT and SAL/AGT performed by the tube-spin technique for different incubation times.

Table 2 *Antiglobulin test methods comparison*

Method comparison	Incubation time (min)	No. of antibodies tested	Titre		
			Higher	Equal	Lower
LISS antiglobulin ν saline antiglobulin	10	37	18	14	5
LISS antiglobulin ν albumin antiglobulin	60				
LISS antiglobulin ν albumin antiglobulin	10	37	9	16	12
LISS antiglobulin ν albumin antiglobulin	30				
LISS antiglobulin ν albumin antiglobulin	10	37	22	12	3
LISS antiglobulin ν albumin antiglobulin	10				

The LISS/AGT with 10 minutes' incubation in a waterbath can be seen to be more sensitive than the SAL/AGT with 60 minutes' incubation and equivalent to the ALB/AGT with 30 minutes' incubation. Shortening the ALB/AGT to 10 minutes' incubation time results in a relatively insensitive test.

This phase of the study was performed using Pasteur pipettes for all material dispensing, and it was interesting to note that in the comparison of LISS/AGT with 10 minutes' incubation and ALB/AGT with 30 minutes' incubation, of the 12 antibodies producing lower titres by LISS/AGT, nine were anti-K. With the other comparisons, all the antibodies resulting in lower titres were in the KELL system. Since introducing precision samplers for all serum and red cell dispensing, this anomaly has not been apparent. However, it appears that anti-K antibodies are not usually enhanced as much as antibodies of most other specificities.

Table 3 summarises the results of comparisons of the old SAL/AGT and ALB/AGT, and the LISS/AGT.

The 50 antibodies tested comprised (with number of examples in parentheses): D (4), D + C (2), K (Kell) (16), E (3), C + \bar{e} (2), \bar{c} + E (1), Fy^a (Duffy) (6), S (1), Wr^a (Wright) (6), \bar{c} (2), Jk^a (Kidd) (1), Jk^b (Kidd) (2), Le^a (Lewis) (2), Le^b (Lewis) (1), P₁ (1).

In the comparison LISS/AGT ν SAL/AGT, 11 antibodies reacted by the LISS/AGT but failed to react with the SAL/AGT procedure. The speci-

Table 3 *Antiglobulin test methods comparison*

Method comparison	No. of antibodies tested	LISS reactions		
		Stronger	Equivalent	Weaker
LISS antiglobulin test ν saline antiglobulin test	50	30	20	0
LISS antiglobulin test ν albumin antiglobulin test	50	17	31	2
Method comparison	No. of antibodies tested	Albumin reactions		
		Stronger	Equivalent	Weaker
Albumin antiglobulin test ν saline antiglobulin test	50	19	23	4

ficiencies were \bar{c} + E (1), C + \bar{e} (1), E (2), S (1), Wr^a (3), Jk^b (1), Le^b (1), P₁ (1).

In the comparison LISS/AGT ν ALB/AGT, six antibodies reacted by the LISS/AGT but not by the ALB/AGT. The specificities were E (2), S (1), Wr^a (1), D + C (1), Jk^b (1).

In the comparison ALB/AGT ν SAL/AGT, seven antibodies reacted by the ALB/AGT but not by the SAL/AGT. The specificities were \bar{c} + E (1), C + \bar{e} (1), Wr^a (3), Le^b (1), P₁ (1).

Two antibodies reacted by the SAL/AGT but not by the ALB/AGT. The specificities were Wr^a and D + C.

Four antibodies reacted only by the LISS/AGT. The specificities were S, E (2), and Jk^a. The anti-S was discovered on screening stored sera by the LISS/AGT method in a serum previously reported to contain only anti-E. The two anti-E antibodies were more readily detectable by the papain technique. The anti-Jk^b was from a sample collected in 1977 from a patient in whom the antibody had been originally identified in 1972. The antibody was significantly weaker in the recent serum.

Because of the comparatively short incubation times being recommended for the LISS/AGT, it was considered relevant to assess the comparative efficiency of a waterbath and dry incubation blocks to heat the tube contents. It was found that the time taken to heat the reaction mixture to 37°C from 24°C was 2½ minutes in a waterbath and 5 minutes in the dry block. As the latter were being used routinely, a 15-minute incubation time for the LISS/AGT was found to be the most suitable for routine use.

Table 4 summarises the results of the comparison between the Albrey and Simmons papain technique and the two-stage technique performed in a tube. The specificities tested were D (12), D + C (2),

Table 4 *Papain technique comparison*

Antibody type	No. of antibodies tested	Reactions by tube papain		
		Stronger	Equivalent	Weaker
Papain reactive only	21	10	10	1
Reactive by saline and/or antiglobulin methods also	35	20	14	2

K (7), E (16), \bar{e} (3), \bar{c} (5), Le^b (1), Wr^a (4), C^w (3), Le^{a+b} (1).

In the 'papain reactive only' group, three antibodies reacted only by the tube technique. The specificities were E, C^w , and \bar{c} . The anti- \bar{c} was originally detected when present at greater strength than in the serum used in this comparison. The anti- C^w was detected when investigating a serum sample by the tube technique as part of the investigation into the suitability of the method. The anti-E reacted only with homozygous E red cells by the tile method. Those used in this comparison were heterozygous for E.

In the second group, reactive by other methods, four antibodies reacted by tube papain only. The specificities were D, K, Le^b , and Wr^a . One antibody (K) reacted only by the tile method.

For the tube papain technique it was found necessary to dilute the papain in phosphate buffered saline (PBS) rather than the glucose citrate solution usually used with the Albrey and Simmons method. The ionic strength of the glucose citrate is also low, and it was found that in the presence of LISS suspended cells haemolysis often resulted on subsequent addition of serum. The PBS raised the ionic strength of the mixture sufficiently in the first stage to avoid this problem while maintaining sensitivity.

ROOM TEMPERATURE PHASE

Table 5 illustrates the suitability of LISS as a suspending medium for the room temperature phase of the tests and the adequacy of a 15-minutes' incubation time for detecting agglutinating antibodies.

The antibody specificities tested were: M (2), N (13), P_1 (2), Le^a (1), Le^b (2). Eleven of the anti-N antibodies were the haemodialysis-induced anti-N-like antibodies. The three antibodies producing stronger reactions in LISS than in 0.9% saline were an anti-M and two anti-N antibodies, one of these a haemodialysis-induced anti-N-like antibody.

In addition, anti-A and anti-B titres were determined in 12 group 0 sera using LISS and 0.9% saline suspended red cells. Overall, no significant differences in titre were obtained.

LISS suspended cells were adopted for use in routine blood grouping procedures also.

Table 5 *Saline agglutinating antibodies: saline versus LISS suspension*

No. of antibodies tested	Reactions in LISS cell suspension		
	Stronger	Equivalent	Weaker
20	3	15	2

15-minute v 30-minute incubation with LISS suspended red cells			
No. of antibodies tested	Reactions in LISS cell suspension		
	Stronger	Equivalent	Weaker
20	0	19	1

During parallel testing of the LISS procedure and the current procedure as part of the evaluation, several anomalies occurred:

- (1) An anti-K antibody was discovered and identified by the current antibody screening procedure. Subsequent testing by the LISS/AGT revealed anti-E also.
- (2) Two anti- Fy^a antibodies were detected weakly with the antibody screening procedure. Both could be readily identified only by the LISS/AGT. In one case four units of blood being crossmatched were all found to be Fy^{a+} but nevertheless appeared compatible with the current crossmatch procedure. Only the LISS/AGT gave positive results with heterozygous Fy^{a+} red cells.

Discussion

The incubation time used by Löw and Messeter for the LISS/AGT was 5 minutes. More recently, 10 minutes (Moore and Mollison, 1976) and 15 minutes (Wicker and Wallas, 1976) have been suggested as more appropriate. No mention, however, has been made of the means used to achieve 37°C incubation, and this factor is significant when short incubation times are being used. In addition, it has not been made clear whether the incubation times suggested represent the time the reaction mixture actually spends at 37°C or whether they represent the time from placing the tubes in the 37°C source to removing them from that source. Also the use of glass compared with plastic tubes has a significant influence on the time taken for tube contents to reach 37°C. Using plastic tubes, 10 minutes' incubation from the time of placing the tubes in the heat source was found to be adequate if a waterbath is used. A 5-minute incubation time could be satisfactory only if performed in glass tubes and using a waterbath as the heat source. Fifteen minutes' incubation was selected as the basis for the routine crossmatch procedure using plastic tubes and dry incubation blocks.

The necessity to maintain equal volumes of serum and LISS suspended red cells for maximum efficiency of the LISS antiglobulin test has been emphasised by Moore and Mollison (1976). They showed that a wide variation in drop sizes from 6 μ l to 14 μ l occurred when dispensing materials from Pasteur pipettes into plastic tubes. Although an excess of LISS of up to two to three volumes would not be likely to result in false-positive AGT results, the dilution factor incurred could reduce sensitivity. A small excess of serum markedly increases the ionic strength of the reaction mixture, which in turn reduces the rate of antibody uptake and thus sensitivity. The introduction of precision samplers not only met the needs of the LISS/AGT but also provided a level of standardisation not possible with the Pasteur pipette delivery methods usually used. The use of precision pipettes for all sampling and not just for the AGT provides obvious advantages when results from antibody identification panels are being interpreted. The initial problems encountered with anti-K antibodies while using Pasteur pipettes were overcome by changing to precision samplers. This level of precision was found necessary for the successful performance of the LISS/AGT.

False-positive results with the LISS/AGT were not a problem in this study. False-positive results may be due to serum:cell suspension ratio, cell suspension strength, cold agglutinin and complement activity in the serum, and the quality of the anti-human globulin reagent. The cell suspension strength for the LISS/AGT should be 5%. Lowering this to 2-3% tends to increase the incidence of false-positive results.

The quality of the polyspecific antiglobulin reagents used is an important factor in avoiding false results. These reagents need to be assessed with regard to their performance in the LISS/AGT system if this method is to be used. In particular, they should be checked for excessive anti-complement activity.

LISS does not appear to have any significant effect on the red cell enzyme digestion stage of the two-stage papain technique provided the papain solution is diluted with PBS. Similar results occurred whether 0.9% saline or LISS were used for this stage of the test. Red cells after enzyme treatment tend to form a tighter button than untreated cells after centrifugation. For this reason it was found necessary to spin the papain test tubes for 10 seconds instead of the usual 15 seconds at high speed in the Sorvall serofuge.

Suspending the enzyme-treated cells in LISS rather than 0.9% saline for the serum incubation phase results in marked enhancement of reactivity of some antibodies. However, problems with cold agglutinins were also increased, and it was not considered

practical for routine purposes to adopt this modification.

LISS has been found to have little, if any, enhancing ability for agglutinating antibodies with the exception of some anti-M antibodies (4, 8). As well as anti-M it was found in this study that some anti-N antibodies, including dialysis-induced anti-N-like antibodies, were more readily detected in the LISS medium.

The LISS solutions used in all studies reported so far have been buffered to pH 6.7 with phosphate buffer. It was found in initial experiments that buffering of the LISS made no noticeable difference in the antibody-detecting capacity of the medium for a variety of antibody specificities. For this reason the solution was not buffered and has consistently been found to have a pH of 6.0. This pH represents the lower limit of the optimal range for maximum strength of the D-anti-D bond established by Hughes-Jones *et al.* (1964).

No contamination problems have been encountered with LISS provided it is stored in a refrigerator. However, the solution was left in wash bottles on the bench for several days without showing signs of contamination.

It is necessary for the citrated whole blood from donor bag segments to be washed once with 0.9% saline before washing with LISS in order to avoid spontaneous agglutination of the red cells. This was done in a cell-washing centrifuge to avoid the confusion of having wash bottles on the bench containing 0.9% saline and LISS.

Apart from the initial outlay for precision samplers, the extra cost incurred through the increased use of plastic tubes and sampler tips was largely offset by savings made in the decreased use of albumin, papain, and commercial screening cells. The increase in work capacity achieved was regarded as a significant cost-benefit factor.

The advantages of using LISS as the suspending medium for red cells in the antiglobulin test can be extended to the use of the same red cell suspension for enzyme and room temperature phase tests, providing a sensitive, standardised, and rapid cross-matching and antibody-screening procedure compared with the conventional methods.

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