Antibody detection errors due to acidic or unbuffered saline

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Isotonic saline solutions, buffered with potassium phosphate or sodium phosphate salts, were evaluated in parallel with unbuffered saline to determine if they improved antibody detection by solid phase red cell adherence or hemagglutination methods. Saline buffered to a pH of 7.0 to 7.5, when used to suspend red cells or to wash sensitized red cells in preparation for the antiglobulin test, produced the best positive solid phase and hemagglutination results. The pH range of commercially prepared blood bank saline (unbuffered) was found to be 5.8 to 6.8, far lower than the desired pH for optimum antibody detection. In the case of solid phase assays employing intact, immobilized reagent red cells, saline with a pH of 7.0 to 7.5 also eliminated falsely positive results due to the dissociation of red cell monolayers from the solid support surface that occurred in the presence of unbuffered or acidic saline. These findings indicate that unbuffered isotonic saline should not be used in solid phase- or hemagglutination-based antibody detection tests. It is recommended that phosphate-buffered saline at a pH of 7.0 to 7.5 be employed. *Immunohematology* 1993;9:15.

Several factors affect the efficiency with which antibodies bind to antigens. One of these is the pH of the test environment. Antibodies can be encouraged to dissociate from antigens by making the test environment either too acidic or too alkaline.

Barnes[1] showed that binding of anti-D to red blood cells (RBCs) was significantly decreased when the pH of the test environment was below 6.4 or above 8.4. Other investigators have shown that selected examples of agglutinating antibodies to P or M antigens react better or best at a pH of 6.0 or 6.5.[2,3] Bruce et al.[4] showed that some examples of anti-D, -S, -s, -Fy^a, and -Jk^a failed to react, or did so only weakly, when an antiglobulin test for RBC antibody detection employed saline with a pH of 6.5 or lower. These authors suggested that saline at a pH of 7.0 to 7.2 be used for antibody detection tests.

The optimum pH for antibodies of most blood group systems has not been determined. However, it has been suggested that the optimum pH of saline used to suspend or wash red cells for routine work is 7.0.[5] For this purpose, most serologists in the United States purchase ready-made, unbuffered isotonic blood bank saline (sodium chloride, USP 0.9%). According to United States Pharmacopeia XXV, the allowable pH range for manufactured isotonic saline solutions is 5.0 to 7.0. We tested the pH of multiple lots of blood bank saline produced by five U.S. vendors and found the pH to range from 5.8 to 6.8. Most of the lots tested had a pH of 6.6 or lower. Thus, it is possible to purchase ready-made saline solutions that are below the desirable minimum pH. Furthermore, once unbuffered containers of saline are opened and exposed to the air, they become more acidic because of transfer of carbon dioxide from the atmosphere to the liquid.

Our study on the effects of isotonic blood bank saline had two purposes:

1. We wanted to determine if the pH of saline had any effect on the results obtained with solid phase red cell adherence assays.
2. We wanted to determine if we could replicate the findings of Bruce et al.,[4] using unbuffered saline and buffered saline at a neutral pH.

Methods

Antibodies

A total of 20 antibodies were tested by hemagglutination (HA) and Capture-R (Immucor, Inc., Norcross, GA) solid phase red cell adherence (SP). The samples were stored at 4°C or were frozen until use. Twofold master dilutions of the antibodies were prepared in inert group AB serum. Pretesting determined the number of dilutions of each antibody to test. The number selected included two to three dilutions that would produce definitive positive results. Tests included at least one dilution beyond the antibody endpoint that gave a negative result by the pretest method. Two examples of each antibody were tested. The antibodies were tested against RBCs carrying a single dose of the appropriate antigen.

Reagent red cells

Commercially prepared reagent RBCs (Immucor, Inc.) were used in HA or SP tests. The red cells were washed once and resuspended in the saline under test

to eliminate any buffering effect of the manufacturer's red cell diluent.

Saline solutions

Isotonic saline was prepared by adding sodium chloride salt to deionized water (final concentration 0.9%). The pH of this solution ranged from 6.4 to 6.6 at the time of manufacture. Aliquots of the unbuffered saline solution were buffered with monosodium and disodium or potassium phosphate salts to achieve a pH of 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0. The pH of the solutions was measured with a pH meter and, in the case of the buffered salines, checked before testing with pH paper. The washing equipment used in HA or SP procedures was completely primed before use with the saline under test.

Hemagglutination procedure

Two drops of each antibody dilution and one drop of the appropriate RBC suspension were added to 10 x 75 mm test tubes. The serum/cell mixtures were incubated at 37°C for 30 minutes, then washed four times in either unbuffered saline or buffered saline. Anti-IgG (Immucor, Inc.) was added to the tubes, and the results read after centrifugation. Reactions were graded and scored according to conventional protocols.[5]

Solid phase procedure

Capture-R (Immucor, Inc.) was used to perform SP evaluations. Monolayers were prepared in test wells according to the manufacturer's instructions. The density of each monolayer was inspected visually before proceeding with further testing. It was noted when the monolayer density was lighter than expected because of decreased binding of red cells to the test wells. One drop of each antibody dilution and two drops of Capture LISS were added to each test well. The tests were incubated for 20 minutes at 37°C, then washed with the saline under test. One drop of anti-IgG-coated indicator RBCs was added to each well, and the tests were centrifuged and the results read. Positive reactions were assigned a score value.[6]

Evaluating the results

Titration values for each antibody were calculated by adding the scores of the positive reactions of each dilution.[5,6] These were then added to produce the overall score value for each saline solution. A difference of 10 score units was considered significant when comparing titration results of the same antibodies in different salines. Antibody scores obtained with buffered salines were compared to those obtained with unbuffered salines, using the *t* test.

Results

The data obtained in parallel SP tests are given in Table 1. Differences in results between buffered and unbuffered saline, particularly at pH 7.0 and 7.5, were significant. Titration score values obtained with buffered salines of pH 5.5, 6.0, and 8.0 were artificially high since, with some samples, negative endpoints were not observed. It was noted in SP tests using saline at a pH of 5.5 and 6.0 that loss of reagent red cell monolayers occurred before the test conclusion. This situation leads to falsely positive results and artificially high titration endpoints. Two examples of anti-Jk^b went undetected when unbuffered saline was used. Six of 19 antibodies reacted more strongly (difference > 10 score units) in pH 7.0 and pH 7.5 buffered saline than in unbuffered saline (two examples of anti-Jk^b and -D, one of anti-c, and one of anti-Fy^a). Another five antibodies reacted better (score > 10) either at pH 7.0 or pH 7.5 (one example of anti-e, -Fy^a, and -S, and two examples of anti-Fy^b). Table 2 shows the p values calculated for salines at pH 6.5, 7.0, and 7.5. The values for pH 7.0 and 7.5 (.0007251 and .0002795, respectively) are highly significant.

Table 3 shows data obtained in HA antiglobulin tests washed with unbuffered saline and phosphate-buffered saline at a pH of 7.0 to 7.5. Of the 20 antibodies tested, three (two examples of anti-Fy^a and one example of anti-K) produced titration score values in phosphate-buffered saline that were significantly different from those obtained when unbuffered saline was used.

Discussion

Parallel tests show that the pH of saline can affect the results of SP and HA antibody detection tests. The titration scores of antibodies were higher in saline with a pH of 7.0 to 7.5 than in unbuffered saline with a pH of 6.4 to 6.6. In SP tests, titration score values obtained with saline at pH 5.5, 6.0, and 8.0 were higher than those obtained with saline at a pH of 6.5. However, these data are considered misleading since negative endpoints were not obtained in many tests. Further, the data do not show that inert group AB sera were more likely to produce falsely positive results in

tests washed with buffered saline at pH 5.5, 6.0, and 8.0. Thus, the scores obtained with these solutions are artificially high because of falsely positive reactions at high antibody dilutions.

Falsely positive results can occur in Capture-R SP tests when reagent screening RBCs dissociate from immobilized monolayers during the test process. Loss of red cells exposes the chemical coupling agent used to anchor them to the bottoms of the test wells. The exposed coupling agent can then bind IgG non-specifically, leading to the unwanted attachment of IgG-coated indicator red cells via the antiglobulin component. Alternatively, the exposed coupling agent can bind indicator red cells directly by attaching to their membranes during centrifugation.

SP results obtained with buffered saline at a pH of 6.5 did not differ significantly from results obtained with unbuffered saline. However, results obtained with saline at a pH of 7.0 and 7.5 were significantly different. Since saline at a pH of 7.0 to 7.5 was found to improve positive SP test results, its effect on HA tests was also evaluated. Our findings at this pH support those of Bruce et al.[4] We also support the suggestion of these authors that saline solutions used in antibody detection tests be considered as important as the potentiating media, reagent red cell phenotype, or antiglobulin reagent employed. Thus, the pH of saline should be strictly controlled at 7.0 to 7.5 when either SP testing or HA testing is performed.

Table 2. Totals of score values for each buffered saline tested and of p values calculated for salines at pH 6.5, 7.0, and 7.5

Saline pH	Saline score	t value	df*	p value
5.5	1113			
6.0	1111			
6.5	1092	2.528016	18	.0189414
7.0	1137	4.065991	18	.0007251
7.5	1182	4.495873	18	.0002795
8.0	1127			
Unbuffered (pH 6.4 to 6.6)	972			

*Degrees of freedom

Table 3. Results obtained with unbuffered saline and buffered saline (pH 7.0 to 7.5) in HA tests

Anti-	Code	pH 7.0 score	Unbuffered saline score
K	10004	37	27
K	85-132	30	28
S	9E4709	19	14
S	12171	19	16
Jk^a	61	23	20
Jk^a	5901A	19	16
c	588F	24	24
c	5109	24	24
e	LD1115	28	27
D	123B	28	27
D	1549M	27	21
Fy^a	067-81P	35	24
Fy^a	3151	39	26
Fy^b	LD150	38	35
Fy^b	LD123	21	15
Jk^b	568F	23	19
Jk^b	5984A	15	15
s	200-67	30	29
s	6175	18	16
Score totals		513	439
t value		–4.33992	
degree of freedom		19	
p value		0.003528	

Table 1. Titration results obtained in SP tests using buffered and unbuffered saline

		Buffered saline pH						
Anti-	Code	5.5	6.0	6.5	7.0	7.5	8.0	Unbuffered saline pH 6.5–6.6
K	85-132	50	31	28	31	44	38	35
K	10004	42	28	29	40	39	34	40
Jk^a	61	42	56	48	54	60	58	57
Jk^a	5901A	36	45	40	25	49	48	32
Jk^b	5984A	37	26	22	23	32	23	03
Jk^b	568F	37	37	26	25	42	41	00
c	588F	56	58	52	64	61	51	58
c	5109	61	64	64	74	70	71	59
e	LD115	68	70	77	82	75	69	68
e	2154D	59	80	81	75	71	64	74
D	123B	75	69	75	72	67	70	52
D	1549M	57	56	50	59	62	61	48
Fy^a	067-81P	72	79	71	71	68	73	48
Fy^a	3151	75	71	66	72	68	70	60
Fy^b	LD150	74	67	71	79	75	76	69
Fy^b	LD123	49	69	70	70	75	71	65
s	200-67	79	83	87	79	84	92	77
s	6175A	80	52	63	69	62	49	61
S	12171	64	70	72	73	78	68	66

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