EXPERT COMMITTEE ON
BIOLOGICAL STANDARDIZATION

Geneva, 27 September - 3 October 1983

A COLLABORATIVE INVESTIGATION OF:
- A PROPOSED STANDARD FOR ANTI-C SALINE AGGLUTINATING BLOOD-TYPING SERUM
- A PROPOSED STANDARD FOR ANTI-D SALINE AGGLUTINATING BLOOD-TYPING SERUM
- A PROPOSED STANDARD FOR ANTI-E SALINE AGGLUTINATING BLOOD-TYPING SERUM

by

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INTRODUCTION

In the meeting in 1976 the Committee on Biological Standardization noted that the WHO Working Group on the Standardization of the Human Blood Products and Related Substances had expressed the need of reference materials for anti-A, B, anti-C and anti-E blood-typing sera. At the same time it was informed that the stock of the International Standards for anti-A and anti-B blood-typing sera were depleted.

The Committee requested the Central Laboratory to obtain materials suitable to establish or to replace these preparations and to arrange for collaborative assays (Technical Report Series, No. 610, 1977).


This report gives the results of a collaborative assay to test a proposed anti-C, a proposed anti-D and a proposed anti-E blood-typing serum standard, saline agglutinating.

ANTI-C

Human plasma of different ABO groups was collected by plasmapheresis and blood donation from donors found positive for anti-C saline agglutinating antibodies. ACD was used as anticoagulant.

The plasma was recalcified with CaCl₂ (final concentration 0.013 M), incubated at 37°C for one hour and then stored overnight at +4°C. The formed clot was removed by filtration through filter paper. To this "serum" was added Na₂EDTA (final concentration 0.015 M) and NaN₃ (final concentration 0.1%). Hetero-agglutinins were removed by adsorption at room temperature with erythrocytes of different ABO groups, washed three times with phosphate buffered saline. In order to enhance agglutination, up to 3% potentiating medium (Ortho, Raritan, United States of America) was added to the serum. All the serum was pooled and stored at +4°C. The fat layer that appeared on top of the serum upon storage at +4°C for two weeks, was removed and the serum was then filtered through asbestos filters (Seitz K7 and Seitz EKS) and through a presterilized 0.22 µm membrane filter (Schleicher & Schull, 1121). The pool
was distributed in 1 ml quantities into colourless Fiolax ampoules (capacity 2 ml), frozen, freeze-dried, filled with nitrogen and sealed. The dried content of the ampoules was determined by weighing 11 ampoules with and without the dried serum. The water content of the ampoules was determined by the Karl Fischer method (USP). Accelerated degradation tests were done at different temperatures, the ampoules being stored at those temperatures for various periods of time.

ANTI-D

Human plasma of different ABO groups was collected by plasmapheresis from hyperimmune donors, stimulated with group O, RzR erythrocytes. ACD was used as anticoagulant. The anti-D plasma was treated in the same way as described for anti-C.

ANTI-E

Human plasma of different ABO groups was collected by plasmapheresis and blood donation from donors found positive for anti-E saline agglutinating antibodies. ACD was used as anticoagulant. The anti-E plasma was treated in the same way as described for anti-C, however in this case no potentiating medium was added.

RESULTS

Stability

Anti-C - Samples were stored for 4, 8 and 12 weeks at 4°C and 37°C. Although the strength of the agglutinates (score) was somewhat less when stored at 37°C (10 versus 12), there was no difference in titre between the two temperatures. The titre fell from 1:32 for the reference and the 4-weeks samples to 1:16 for the 8- and 12-weeks of storage samples.

Anti-D - Samples were stored for 4, 8 and 12 weeks at 4°C and 37°C. The score of the samples stored at 4°C was higher than when stored at 37°C (15 versus 13). All samples, whether stored at 4°C or at 37°C, for 4 or for 12 weeks, all had a titre of 1:16, the same as the reference.

Anti-E - Samples were stored for 4, 8 and 12 weeks at 4°C and 25°C. The samples stored at 37°C did not dissolve completely and therefore were not used. The titre of the 4°C samples was 1:16, score 14; when stored at 25°C the titre dropped to 1:8. The score and titre of the 4°C samples was not influenced by the time of storage. When stored at 25°C, the titre was not influenced, the score dropped from 10 to 9 to 8 for 4, 8 and 12 weeks, respectively. There was no difference between the reference and the 4°C samples.

Water content

The water content of the ampoules after freeze-drying was found to be 2.5% for anti-C, 1.8% for anti-D and 2.7% for anti-E.

Distribution

Anti-C - The average content of 11 ampoules was 66.3 mg per ampoule, with a coefficient of variation of 1.8%. The 95% confidence limits are 63.6 ≤ X ≤ 69.0.

Anti-D - The average content of 11 ampoules was 112.4 mg per ampoule, with a coefficient of variation of 0.8%. The 95% confidence limits are 110.4 ≤ X ≤ 114.4.

Anti-E - The average content of 11 ampoules was 66.9 mg per ampoule, with a coefficient of variation of 1.5%. The 95% confidence limits are 64.7 ≤ X ≤ 69.1.
Specificity

The specificity of the proposed standards was tested at three different temperatures (4°C, 20-25°C and 37°C) according to a given protocol and to the laboratories' own methods. The proposed standards were tested with R0R (ccDee), r'r (Ccdee) and r"r (ccdEe) cells of mainly group 0 and with rr (ccdee) cells of group A, B and C. Most cells came from blood collected with CPD as anticoagulant, only a few were from ACD or clotted blood.

Anti-C - With one exception, the laboratories found only positive reaction with the r'r cells. The one exception found dubious-positive results with the r"r cells, both in the laboratory and the prescribed method.

Anti-D and anti-E - The proposed standards only reacted with the R0R and r"r cells, respectively.

Two laboratories tested the proposed standards not only with saline techniques but also in an indirect antiglobulin technique and for enzyme technique. They found the anti-C and anti-E to contain incomplete "(anti-D)" antibodies, and also anti-E to contain incomplete anti-C antibodies.

Potency

The potency of the proposed standards has been determined by two methods, the prescribed and the laboratory method. Differences between the prescribed and the laboratory method included time of incubation, time and relative centrifugal force of centrifugation, and ratio cells-antibodies.

Anti-C - All laboratories found positive reactions, in general relatively strong ones with the r'r cells and relatively weak ones with the R0R (CCDEE) and the R2R2 (CcDEE) cells. One of the laboratories commented on this and mentioned the anti-C serum contains much anti-Ce. Geometrical mean titre: 1:9. With Ccdee cells, only 1:20.

Anti-D - One laboratory found extremely weak reactions with the proposed standard, while they reacted strongly with other complete anti-D sera. Three out of the five ampoules were tested. The other laboratories found good positive reactions. Geometrical mean titre: 1:5

Anti-E - In all cases the anti-E serum gave good strong reactions. Geometrical mean titre: 1:23

DISCUSSION

The weight of the content of the ampoules shows only a small variation themselves are normal, except for anti-D which is relatively high.

The water content of the ampoules is normal. The accelerated degradation studies did not give results from which the theoretical half-time at different storage temperatures could be calculated. Although all three proposed standards showed differences between the 44°C and the 37°C or 25°C samples, the time of storage at that temperature had no or very little influence on the titre and the score. This suggests that the deterioration is hardly time-dependent, but occurs relatively quick to a certain extent at elevated temperature. Stored at low temperature (-20°C) the proposed standards will be stable.

Anti-C - Although one laboratory found unwanted dubious-positive reactions with one cell, the overall results indicate that the specificity of the anti-C serum, saline agglutinating, is good when the antiserum is used in the techniques it is meant for. The presence of anti-Ce may be less desirable because it causes reactions with Ce-positive cells to be more strongly, the overall titre with C-positive/Ce-negative cells is satisfactory.

The presence of incomplete anti-D antibodies does not interfere with the results in saline agglutinating techniques.
Anti-D - With the exception of the one laboratory that found extremely weak reactions and in one case even no reaction, the strength of the reactions found by the other laboratories is just acceptable. The reason for these very weak reactions is not clear, perhaps it is caused by incomplete dissolving of the dried content. However, the investigator did not mention this and tried several ampoules. The failure to give a positive reaction with one of the test cells makes this proposed standard less suitable.

Anti-E - The specificity of this proposed standard and its strength make it suitable as standard serum. The presence of antibodies against the C and D antigen, which are only demonstrable in techniques for incomplete antibodies, does not interfere with the characteristics of this antiserum in its proposed technique.

Tested in techniques for incomplete blood typing reagents, the two laboratories found high specific reactions. According to their comments, reagents used for saline agglutination should also be specific in all other tests. They therefore found the anti-C and anti-E less suitable for standards.

CONCLUSIONS

From the data presented, the three proposed standards have been shown to be stable reagents, to be specific in the saline agglutination technique and to have, with the exception of anti-D, acceptable potency.

We therefore propose

Anti-C - To accept the proposed standard as standard serum for anti-C blood-typing serum, saline agglutinating, and to assign to it 100 I.U. per ampoule.

Anti-D - Not to accept the proposed standard.

Anti-E - To accept the proposed standard as standard serum for anti-E blood-typing serum, saline agglutinating, and to assign to it 100 I.U. per ampoule.
SPECIFICITY TESTS

ANTI-C

The specificity of the proposed standard for anti-C shall be tested according to the following protocol and according to the laboratory method. This test shall be done with cells of the following ABO and rhesus groups:

A, B or O Ror (ccDee)
A, B or O r'r (Cccddee)
A, B or O r'r (ccddEe)
A1 rr (ccddee)
B rr (ccdee)
O rr (ccdee)

1. Wash the cells at least twice with an excess of a physiological phosphate buffered saline solution (PBS, pH 7.1-7.4).
2. Prepare a 3-5% suspension of these washed cells in PBS to which 2% of AB serum has been added (PBS-AB).
3. Put 0.2 ml of the undiluted anti-C serum in a clean, all glass test-tube (10 x 75 mm), add 0.1 ml of the 3-5% cell suspension, thoroughly mix the contents of the tube and incubate for one hour at 37°C (use a water-bath of 37°C for this incubation). A second tube shall be incubated for one hour at 20-25°C and a third tube shall be incubated for one hour at 2-8°C.
4. Centrifuge the tubes for 45-60 seconds at a relative centrifugal force of 120 g.
5. Examine microscopically for agglutination.

ANTI-D

The specificity of the proposed standard for anti-D shall be tested according to the following protocol and according to the laboratory method. This test shall be done with cells of the following ABO and rhesus groups:

A, B or O Ror (ccDee)
A, B or O r'r (Cccddee)
A, B or O r'r (ccddEe)
A1 rr (ccddee)
B rr (ccdee)
O rr (ccdee)

1. Wash the cells at least twice with an excess of a physiological phosphate buffered saline solution (PBS, pH 7.1-7.4).
2. Prepare a 3-5% suspension of these washed cells in PBS to which 2% of AB serum has been added (PBS-AB).
3. Put 0.2 ml of the undiluted anti-D serum in a clean, all glass test-tube (10 x 75 mm), add 0.1 ml of the 3-5% cell suspension, thoroughly mix the contents of the tube and incubate for one hour at 37°C (use a water-bath of 37°C for this incubation). A second tube shall be incubated for one hour at 20-25°C and a third tube shall be incubated for one hour at 2-8°C.
Appendix 1

4. Centrifuge the tubes for 45-60 seconds at a relative centrifugal force of 120 g.

Examine microscopically for agglutination

ANTI-E

The specificity of the proposed standard for anti-E shall be tested according to the following protocol and according to the laboratory method. This test shall be done with cells of the following ABO and rhesus groups:

- A, B or O R₀ r (ccDee)
- A, B or O r'r (Ccddee)
- A, B or O r"r (ccddEe)
- A₁ rr (ccddee)
- B rr (ccddee)
- O rr (ccddee)

1. Wash the cells at least twice with an excess of a physiological phosphate buffered saline solution (PBS, pH 7.1-7.4).

2. Prepare a 3-5% suspension of these washed cells in PBS to which 2% of AB serum has been added (PBS-AB).

3. Put 0.2 ml of the undiluted anti-E serum in a clean, all glass test-tube (10 x 75 mm), add 0.1 ml of the 3-5% cell suspension, thoroughly mix the contents of the tube and incubate for one hour at 37°C (use a water-bath of 37°C for this incubation). A second tube shall be incubated for one hour at 20-25°C and a third tube shall be incubated for one hour at 2-8°C.

4. Centrifuge the tubes for 45-60 seconds at a relative centrifugal force of 120 g.

5. Examine microscopically for agglutination.
PROTOCOLS OF THE PRESCRIBED METHODS OF THE POTENCY TESTS

Antiserum: anti-C saline agglutinating blood-typing serum

1. Wash fresh r'r cells (Ccdeen) at least twice with an excess of a physiological phosphate buffered saline solution (PBS, pH 7.1-7.4).

2. Prepare a 2% suspension of these washed cells in PBS to which 2% of AB serum has been added (PBS-AB).

3. Prepare a twofold serial dilution series of the anti-C serum in PBS-AB (1:1 to 1:128). To avoid carry-over, a clean pipette shall be used for each transfer.

4. Put 0.1 ml of each serum dilution in a separate clean, all glass test-tube (10 x 75 mm), add 0.1 ml of the 2% cell suspension, thoroughly mix the content of each tube and incubate for 15 minutes at 37°C (use a water-bath of 37°C for this incubation).

5. Centrifuge the tubes for 45-60 seconds at a relative centrifugal force of 120 g.

6. Examine macroscopically for agglutination by gently dislodging the cell-buttons of each tube. The reactions shall be graded according to the given notation.

Antiserum: anti-D saline agglutinating blood-typing serum

1. Wash fresh Rr cells (ccDee) at least twice with an excess of a physiological phosphate buffered saline solution (PBS, pH 7.1-7.4).

2. Prepare a 2% suspension of these washed cells in PBS to which 2% of AB serum has been added (PBS-AB).

3. Prepare a twofold serial dilution series of the anti-D serum in PBS-AB (1:1 to 1:128). To avoid carry-over, a clean pipette shall be used for each transfer.

4. Put 0.1 ml of each serum dilution in a separate clean, all glass test-tube (10 x 75 mm), add 0.1 ml of the 2% cell suspension, thoroughly mix the content of each tube and incubate for 15 minutes at 37°C (use a water-bath of 37°C for this incubation).

5. Centrifuge the tubes for 45-60 seconds at a relative centrifugal force of 120 g.

6. Examine macroscopically for agglutination by gently dislodging the cell-buttons of each tube. The reactions shall be graded according to the given notation.

Antiserum: anti-E saline agglutinating blood-typing serum

1. Wash fresh r'r cells (ccdddEe) at least twice with an excess of a physiological phosphate buffered saline solution (PBS, pH 7.1-7.4).

2. Prepare a 2% suspension of these washed cells in PBS to which 2% of AB serum has been added (PBS-AB).

3. Prepare a twofold serial dilution series of the anti-E serum in PBS-AB (1:1 to 1:128). To avoid carry-over, a clean pipette shall be used for each transfer.

4. Put 0.1 ml of each serum dilution in a separate clean, all glass test-tube (10 x 75 mm), add 0.1 ml of the 2% cell suspension, thoroughly mix the content of each tube and incubate for 15 minutes at 37°C (use a water-bath of 37°C for this incubation).
Appendix 2

5. Centrifuge the tubes for 45-60 seconds at a relative centrifugal force of 120 g.

6. Examine macroscopically for agglutination by gently dislodging the cell-buttons of each tube. The reactions shall be graded according to the given notation.
POTENCY TESTS

ANTI-C

Determine the titre of the proposed standard for anti-C saline agglutinating blood-typing serum according to the prescribed method and according to your own laboratory method.

The determination shall be done with three different cells. At least one of these cells has to be of the rhesus-type r'r (Ccdee). The other cells may be of the types R_2R_2 (CcDEE), R_2R_2 (CcDEE) or rYrY (CcDEE).

Use the following notation for grading your reactions:

4+ : cell-button remains one clump;
3+ : cell-button dislodges into several clumps;
2+ : cell-button dislodges into many small clumps of about equal size;
1+ : cell-button dislodges into finely granular, but definitely small clumps.

ANTI-D

Determine the titre of the proposed standard for anti-D saline agglutinating blood-typing serum according to the prescribed method and according to your own laboratory method.

The determination shall be done with three different cells. One of these cells has to be a group 0 cell. All three cells have to be of the rhesus-type R_0r (CcDee).

Use the following notation for grading your reactions:

4+ : cell-button remains one clump;
3+ : cell-button dislodges into several clumps;
2+ : cell-button dislodges into many small clumps of about equal size;
1+ : cell-button dislodges into finely granular, but definitely small clumps.

ANTI-E

Determine the titre of the proposed standard for anti-E saline agglutinating blood-typing serum according to the prescribed method and according to your own laboratory method.

The determination shall be done with three different cells. One of these cells has to be a group 0 cell. All three cells have to be of the rhesus-type r'r (CcddEe).

Use the following notation for grading your reactions:

4+ : cell-button remains one clump;
3+ : cell-button dislodges into several clumps;
2+ : cell-button dislodges into many small clumps of about equal size;
1+ : cell-button dislodges into finely granular, but definitely small clumps.
### SPECIFICITY OF THE PROPOSED STANDARDS IN SALINE TECHNIQUES ANTI-C, ANTI-D AND ANTI-E (SALINE AGGLUTINATING) AS SHOWN BY THE USE OF CELLS FROM DIFFERENT RHESUS PHENOTYPES

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<tr>
<td>C</td>
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<td>D³</td>
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<td>F³</td>
<td>good</td>
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<td>G</td>
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1. Rhesus phenotypes of used cells: ccDee, Ccdee, ccdEe, 3x ccdee.
2. Laboratory B found a + reaction with the ccdEecells when tested with the anti-C serum at all three temperatures (4, 20 and 37°C) in both the prescribed and the laboratory method.
3. When the reagents were tested in an antiglobulin technique, laboratories D & F found that anti-C and anti-E were shown to contain incomplete anti-D antibodies, and that anti-E also contained incomplete anti-C antibodies.

### POTENCY OF THE PROPOSED STANDARD FOR ANTI-C (SALINE AGGLUTINATING) EXPRESSED AS LOG₂ TITRE

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### POTENCY OF THE PROPOSED STANDARD FOR ANTI-E (SALINE AGGLUTINATING) EXPRESSED AS LOG₂ TITRE

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APPENDIX 5

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