THE DETERMINATION OF ANTISTREPTOLYSIN

BY

GERALD D. JOHNSON

From the Special Unit for Juvenile Rheumatism, Canadian Red Cross Memorial Hospital, Taplow, Bucks

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Hodge and Swift (1933) found that the haemolytic activity of a given batch of streptolysin O did not give a satisfactory index of its ability to combine with antibody. For the purpose of standardizing the antistreptolysin titration (A.S.T.), therefore, it is necessary to determine the combining power of the lysin by reference to antistreptolysin of known concentration. The use of standardized whole serum from patients with raised antistreptolysin titres has not proved satisfactory in this laboratory for this purpose, owing to the fluctuations in titre which such serum has been found to show over a period of time. The following is an account of a method designed for routine use which employs standard antistreptolysin globulin derived from pooled serum.

Materials

Preparation of Streptococcal Haemolysin.—The medium is a modification of that originally described by Todd and Hewitt (1932) and further modified by Hodge and Swift (1933).

Lean beef, freed from fat, is minced and added to tap water in the proportion of 500 g. to 1 litre of water. After infusion overnight at 4° C. the temperature is carefully raised to 85° C. and maintained at this level for one hour. Filtration through muslin and finally glass-wool yields a clear golden-yellow broth. To this is added proteose peptone (Difco) in the proportion of 20 g. per litre, and normal sodium hydroxide solution, 30 ml. per litre. The broth is reheated to 85° C. and held at this temperature for a further half-hour, after which time the precipitated phosphates are removed by filtration through Chardin paper. Sterilization is effected by autoclaving at 15 lb. pressure for 20 minutes. The final pH should be between 7.2 and 7.6.

The broth is enriched and buffered with the following solution :

Dextrose	...	...	...	3 g.
NaHCO ₃	...	...	...	2 g.
NaCl	...	...	...	2 g.
Na ₂ HPO ₄ .12H ₂ O	...	...	...	2 g.
Aqua dest.	...	...	...	50 ml.

This solution must not be heated, and is sterilised by filtration through a bacteriological grade sintered

glass filter. For use the buffer solution is added in the proportion of 1 part to 19 parts of broth.

The organism employed for the production of lysin is the Richards strain of *Str. pyogenes* Group A Type 3, and is obtainable from the National Collection of Type Cultures, Colindale (N.C.T.C. 8191).

In order to obtain maximum growth attention is drawn to the following practical points concerning the actual cultivation of the streptococcus. It is essential to confirm the purity of the culture used for inoculating the broth by preliminary subculture, as contaminating organisms frequently elaborate haemolysins, the presence of which would interfere with the specificity of the reaction. For the same reason it is advisable to check the sterility of the broth by incubating it at 37° C. for 24 hours before inoculation. Approximately 10 ml. of an actively growing (eight hour) culture is added to 1½ litres of broth whose temperature has been previously raised to 37° C. The optimum incubation time is 18 hours, and if this is exceeded the lysin titre tends to fall. It has been found convenient to carry out the incubation in a water-bath. After the specified time has elapsed the broth is centrifuged at about 2,000 r.p.m. to remove the bulk of the organisms, and the supernatant filtered through a bacteriological grade sintered glass filter. Sterility is maintained by the addition of sodium ethyl mercurithiosalicylate ("merthiolate," B.D.H.), 1:10,000.

In view of the variation in the haemolytic activity of streptolysin during storage, fresh lysin is not used until at least one week after its preparation.

Stored at 4° C. streptolysin O has retained its activity for over 12 months, and it has not been found necessary to reduce it in bulk as has been advocated by other workers.

Buffered Saline Solution.—The activity of streptolysin O is maximum at pH 6.5, and the isotonic saline used in all titrations is accordingly buffered at this pH using the following phosphate solution as recommended by Herbert and Todd (1941).*

Na ₂ HPO ₄ .12H ₂ O	...	...	18.11 g.
NaH ₂ PO ₄ .2H ₂ O	...	...	16.76 g.

These ingredients are dissolved in distilled water with the aid of gentle heat and made up to 1 litre.

* This paper contains a misprint, as a result of which the ratio of the phosphate salts has been reversed. The formula here described has been corrected accordingly.

For use 1 volume of the buffer solution is added to 1 volume of 0.158 M sodium chloride solution in distilled water. All subsequent references to "saline" in this paper are to this mixture.

Red Cell Suspension.—Fresh rabbit blood, collected in 3.8% sodium citrate solution, is "washed" three times by centrifuging with saline and finally made up to a 5% suspension.

Standard Antistreptolysin Globulin.—In this laboratory the test was standardized against a sample of antistreptolysin globulin from Dr. B. F. Massell.

Subsequently the preparation of standard globulin derived from patients' serum has been carried out according to the following method, which was described by Kendall (1937).

One hundred millilitres of undiluted pooled serum from patients found to have a raised A.S.T. is added to 50 ml. of saturated ammonium sulphate solution, and the precipitated euglobulin recovered by centrifugation. It is dissolved in 50 ml. of distilled water and reprecipitated with 25 ml. of saturated ammonium sulphate solution, after which the precipitate is finally dissolved in the minimum volume of distilled water necessary to effect complete solution. For the purpose of the work described here it is not necessary to repeat the precipitation five times as recommended by Kendall. The dissolved euglobulin fraction, after the addition of a drop of chloroform, is dialysed against normal saline until sulphate is undetectable (with barium chloride solution) in the saline. Continued dialysis using distilled water results in precipitation of the water-insoluble euglobulin which is removed from the solution by centrifugation and discarded. After the addition of "merthiolate" (1:10,000) the solution is filtered through a bacteriological grade sintered glass filter.

It is essential that all stages in the preparation of globulin solutions involving the use of high salt concentrations should be carried out in a cold-room (4° C.), but from the dialysis stage room temperature is permissible.

Prepared in this way batches of antistreptolysin globulin have maintained a constant titre, when stored at ordinary refrigerator temperature (4° C.), for at least 18 months.

Method

Principle.—The haemolytic activity of the reduced lysin against rabbit red cells is determined in the presence of 1 unit of antistreptolysin globulin. The patient's serum is then titrated for its haemolysis-inhibiting power against the amount of lysin which just fails to produce haemolysis in the presence of 1 unit of standard globulin, the reciprocal of this dilution of the serum giving the number of units of antistreptolysin per millilitre.

It will be appreciated that, as the test is carried out using a standardized amount of lysin which gives no haemolysis in the presence of 1 unit of antistreptolysin, the production of any degree of lysis in a mixture which includes (diluted) serum indicates that

the serum contains less than 1 unit. Accordingly, actual measurement of the degree of haemolysis is unnecessary, and, furthermore, the number of red cells added need not be accurately determined. It has been found that this system is capable of giving reproducible results over long periods without showing any deviation from the original standard, and, furthermore, in view of the stability of the lysin it is not necessary to repeat the titration before each batch of tests but only at intervals of at least a month.

Reduction of Lysin.—Thioglycollic acid (thiol-acetic acid, B.D.H., $\text{CH}_2\text{SH.COOH}$) has been found to be a convenient reducing agent, and is used in the following way:

In a small conical flask 0.3 ml. thioglycollic acid is placed and 5.7 ml. distilled water. A few drops of B.D.H. "universal indicator" are added, and the solution neutralized with 60% sodium hydroxide solution to a pale straw colour (pH 6.0), and made up to a total volume of 15 ml. with (buffered) saline. This constitutes the reducing agent. Four parts of lysin are treated with one part of this solution at least 15 minutes before the lysin is required. Prepared in this way the reduced lysin is stable for two or three days, although it is preferable to prepare a fresh batch each day. Other workers use various reducing agents, e.g., sodium hydrosulphite, cysteine, sodium nitrite. The above is recommended for its consistent effect and ease of use.

Standardization of Lysin.—Having obtained the standard globulin of known combining activity in terms of units of antistreptolysin the first step is to determine the degree to which the lysin has to be diluted in order that 2 volumes of globulin solution diluted to contain 1 unit of antistreptolysin globulin per ml. just inhibits the haemolytic effect of 1 volume of the diluted lysin.

An example of a lysin titration will make this clear:

Reduced lysin (ml.)	..	1.0	1.0	1.0	1.0	1.0	1.0
Saline	..	..	1.0	1.2	1.4	1.6	2.0

Take 0.25 ml. of each dilution

Diluted lysin	..	0.25	0.25	0.25	0.25	0.25	0.25
Standard globulin containing 1 unit/ml.	..	0.5	0.5	0.5	0.5	0.5	0.5

Water-bath at 37° C. for 15 min.

5% cell suspension	..	0.25	0.25	0.25	0.25	0.25	0.25
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Water-bath at 37° C. for 60 min.

Degree of haemolysis	..	+	+	±	—	—	—
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Thus, at a dilution of lysin 1.0:1.6, haemolysis is inhibited by 1 unit of antistreptolysin. It will be noted that the time allowed for the combination of lysin with its antibody is 15 minutes at 37° C. and 60 minutes for the haemolytic reaction.

For each serum to be tested 1 ml. of diluted lysin is required in the first instance; therefore, in the example above, for 50 tests, 20 ml. of reduced lysin is added to 32 ml. of saline.

While variations in the potency of different batches of lysin may be encountered, by adherence to the method of preparation described above, titres ranging from 1 in 1.6 to 1 in 3.6 can be consistently obtained.

Dilution of Patient's Serum.—Each serum, after inactivation at 56° C. for half an hour, is tested in varying dilutions for its inhibition of streptolysin diluted according to the titration value. This is carried out in two stages, the first of which is designed to show the range within which the titre lies, the second to determine more accurately the end-point of those sera found to have an A.S.T. of 200 units/ml. or more.

The scheme of serum dilution to be described limits the possible number of readings per test to seven, thus enabling as many as 70 or 80 sera to be conveniently examined in one batch.

Stage I.—The following dilutions of serum are prepared in four tubes :

	Dilution
0.25 ml. serum + 4.75 ml. saline	1 : 20
1 ml. of 1 : 20 dilution + 1.5 ml. saline	1 : 50
0.5 ml. of 1 : 20 dilution + 4.5 ml. saline	1 : 200
1 ml. of 1 : 200 dilution + 1.5 ml. saline	1 : 500

It has been found expedient, when large numbers of tests are being carried out, to use an automatic pipetting device adjusted to deliver a constant volume of 1.5 ml. for the saline. Thus, the first and third tubes in the above dilution series receive 3 such volumes and the second and fourth 1 volume. The additional 0.25 ml. is then added to the first tube with an ordinary pipette.

Having prepared these dilutions of the sera, the following volumes are taken into 3 in. $\times$ $\frac{3}{8}$ in. tubes :

Tube		Final Dilution
1	0.5 ml. of 1 : 50 dilution	1 : 50
2	0.2 " " 1 : 50 " + 0.3 ml. saline	1 : 125
3	0.5 " " 1 : 200 "	1 : 200
4	0.5 " " 1 : 500 "	1 : 500

To each tube is added 0.25 ml. of the lysin diluted according to its titration result, and after being shaken the tubes are left in a water-bath at 37° C. for 15 minutes.

After this time 0.25 ml. of 5% rabbit cell suspension is added to all tubes, which are shaken and returned to the water-bath for a further hour. It is desirable to shake the tubes again 15 minutes after the beginning of this stage.

When the tubes are finally removed from the water-bath they are left to stand at room temperature for about an hour in order to complete the sedimentation of unlysed cells, thus facilitating the examination of

the supernatant. If this precaution is not observed inaccurate readings may result. Each tube is then inspected for haemolysis, and, as discussed above, the slightest degree detectable is regarded as significant.

Result	A.S.T.
(a) Haemolysis in all tubes	Less than 50 units
(b) " " tubes 2, 3, and 4	50 units
(c) " " " 3, 4	125 units
(d) Haemolysis in tube 4	200-500 units
(e) No haemolysis	500 units or more

Of these five categories, sera which fall into (d) and (e) are further titrated to determine their end-point more accurately.

Stage II : Category (d) Sera.—Of 1:200 dilution, 0.3 ml. + 0.2 ml. of saline is titrated, giving a final dilution of 1:333.

Category (e) Sera	Final Dilution
0.3 ml. of 1 : 500 dilution + 0.2 ml. of saline	1 : 833
0.2 " " 1 : 500 " + 0.3 " " "	1 : 1,250
0.1 " " 1 : 500 " + 0.4 " " "	1 : 2,500

Having prepared these extended dilutions the test is carried out as before, adding first lysin, incubating, and then cell suspension, finally reading in the same way. The A.S.T. is taken as the reciprocal of the dilution in the last tube showing no haemolysis.

Titres below 200 units per ml. are regarded as being within normal limits, and those of 200 units and above as being raised.

It should be noted that the titres resulting from this scheme of dilution are equally spaced logarithmically, with the exception of the first and last values, viz. :

Titre	Log.
50	1.7
125	2.1
200	2.3
333	2.5
500	2.7
833	2.9
1,250	3.1
2,500	3.4

Results are therefore suitable for expression as a geometric mean according to the usual formula :

$$\text{Geometric mean} = \text{Antilog } \frac{\sum (f \cdot \log x)}{n}$$

The application of this statistical method as a means of controlling the standardization of the test has proved useful.

Comment

This method has been designed to enable batches of 70 or 80 specimens to be titrated completely in one day, and it is felt that the range of values

chosen in the dilution series meets the clinical requirements in this Unit. The scheme may be modified to meet any required range.

Summary

A method suitable for the routine determination of the serum antistreptolysin O level is described.

Based on inhibition by the antibody of haemolysis in a system comprising streptococcal lysin and red blood cells, the test is standardized by reference to a globulin solution of known antistreptolysin content.

I am indebted to Dr. E. J. Holborow not only for his constant encouragement in the preparation of this paper but also for his generosity in allowing me to describe the serum dilution system which he devised. My thanks are also due to Dr. L. E. Glynn for his helpful criticism.

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