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(54) DILUENTS FOR RUBELLA VIRUS HAEMAGGLUTINATION-INHIBITION TEST

(71) We, TAKEDA YAKUHIN KOGYO KABUSHIKI KAISHA, also known as TAKEDA CHEMICAL INDUSTRIES, LTD., a body corporate organised according to the laws of Japan, of 27, Doshomachi 2-chome, Higashi-ku, Osaka, Japan, do hereby declare the invention, for which we pray that a patent may be granted to us, and the method by which it is to be performed, to be particularly described in and by the following statement:—

The present invention relates to diluents for rubella virus haemagglutination-inhibition test.

The haemagglutination-inhibition (hereinafter briefly referred to as HI) test for rubella virus is an important tool in serological diagnosis because of its comparatively high sensitivity, expedience and convenience in use compared with other diagnostic procedures for rubella virus, for example, neutralization, complement fixation, and fluorescent antibody tests.

Heretofore, in rubella virus HI tests, the three reactants, that is to say, the rubella virus haemagglutinating antigen (hereinafter briefly referred to as rubella virus HA antigen), the test serum and the erythrocytes, have each been diluted with the same type of diluent, and various diluents for this purpose have been proposed. Each of the proposed diluents, however, has its own disadvantages. Currently veronal buffer solution is most commonly employed, but the sensitivity of the rubella virus HI test is about 10 times higher in the pH range of about 6.0 to 6.5 than at pH 7.2, the pH level established by veronal buffer [Journal of Immunology *104*, 818 (1970)]. Thus, use has often been made of diluents buffered by N-2-hydroxyethylpiperazine-N′-2′-ethanesulphonic acid (hereinafter referred to briefly as HEPES), which has high buffering capacity in the pH range of 6.0 to 6.5 and which helps to produce a stable, well-defined erythrocyte agglutination pattern. This diluent contains not only HEPES in a molar concentration of 0.025 but also NaCl in a molar concentration of 0.14, 1% (weight/volume; also hereinafter) of bovine serum albumin, 0.00025% of gelatin and $CaCl_2$ in a molar concentration of 0.001. Each of these ingredients has been considered to be essential because of its invariable major influence upon the sensitivity of the HI reaction, the stability of the resultant erythrocyte agglutination pattern and the distinctness of the end-point of the reaction. Generally the rubella virus HA antigen is rapidly inactivated on dilution with a diluent, and when using the above HEPES buffer, as is the case with other buffers, it is not practicable to use a stored, previously diluted, rubella virus HA antigen fluid, instead the antigen must be diluted to the prescribed concentration of 4 haemagglutinating (HA) units just before conducting the reaction with a test serum. Moreover, the inactivation of the HA antigen takes place even on such an occasion, thus interfering with an accurate assessment of the end-point of the HI reaction of rubella HI antibody in the test serum, with the result that the HI antibody titre thus determined is neither accurate nor reproducible.

The present invention is based on the surprising observation that the inactivation of rubella virus HA antigen in a system of rubella virus HA antigen diluted with HEPES buffer is dependent upon, and results from, the concentration of HEPES and the presence of $CaCl_2$.

The present invention accordingly provides a diluent suitable for rubella virus HA antigen and for rubella virus test sera, which comprises HEPES in a molar concentration from 0.001 to 0.01 and NaCl, the pH of which is in the range of 6.0 to

7.5, and which is free from calcium ions. This diluent is essentially characterised by containing HEPES in a low concentration, i.e. the molar concentration is from 0.001 to 0.01, and by being free from calcium ions. By means of this composition it is possible to preclude inactivation of rubella virus HA antigen almost completely and thereby to ensure an effective use of stored, previously diluted HA antigen. It is particularly advantageous that this diluent contains HEPES in a molar concentration of from 0.004 to 0.006 and has a pH in the range of 6.0 to 6.5, especially near 6.2. NaCl is preferably present in a molar concentration of from 0.05 to 0.3, and when a rubella virus HI test is to be carried out using fresh erythrocytes HaCl is advantageously present in a molar concentration of 0.14 to 0.15.

It is preferable that the diluent of the invention also comprises albumin, which preferably is serum albumin, especially bovine serum albumin. The concentration of albumin is preferably in the range of from 0.05 percent to 1 percent.

The diluent for the erythrocytes in the rubella virus HI test may comprise calcium ions in a sufficient concentration to let the rubella virus HI reaction take place with high sensitivity and HEPES in a sufficient concentration to let the diluent display a high buffering capacity, thus helping produce a stable, well-defined erythrocyte agglutination pattern. The molar concentration of HEPES in this diluent may be in the range of from 0.01 to 0.05, and is preferably 0.025. The molar concentration of $CaCl_2$ is generally in the range of from 0.0005 to 0.01, preferably 0.002. The pH of the diluent is generally in the range of from 6.0 to 6.5, preferably 6.2. The molar concentration of NaCl in this diluent is preferably in the range of from 0.05 to 0.3 and, where fresh erythrocytes are employed, a range of from 0.14 to 0.15 molar is particularly desirable. This diluent preferably contains gelatin, the concentration of which may be in the range of from 0.001 percent to 0.01 percent, preferably 0.005 percent.

The test serum may be any type of serum provided that it lends itself to the determination of rubella virus HI antibodies, for example, the sera of warm-blooded animals, for example, human beings, cattle, rabbits, and rats. Particularly, the test serum is the human serum taken for the purpose of determining the titre of rubella virus HI antibodies.

It is preferable that, prior to dilution with the diluent according to this invention, the test serum is subjected to a pretreatment adapted to remove or inactivate the non-specific inhibitors of agglutination occurring in the serum. Examples of such pretreatment are adsorption on kaolin, extraction with acetone, and precipitation with heparin-$MnCl_2$. Particularly advantageous is the method wherein phospholipase C is permitted to act upon the test serum to inactivate its non-specific inhibitors of agglutination.

The erythrocytes may be any erythrocytes which lead themselves to agglutination with rubella virus HA antigen, for example, the erythrocytes of fowls, for example, one-day-old chicks, geese, pigeons, and quails. Such erythrocytes may be fresh cells or fixed erythrocytes. The fixed erythrocytes are preferably formalinized erythrocytes. The formalinized erythrocytes of one-day-old chicks described in e.g. Japanese Patent Publication No. 37724/1975 may be used with advantage.

This invention also provides a method for carrying out a rubella virus HI test, which comprises admixing a rubella virus HA antigen and a test serum, both of which have been previously diluted with the diluent of the invention for rubella virus HA antigen and for test serum and, then, admixing the resultant mixture with a suspension of erythrocytes diluted with the above-mentioned diluent for erythrocytes to assess the agglutination or non-agglutination of the erythrocytes. By this method it is possible to determine the titre of rubella virus HI antibody in the test serum with accuracy and high sensitivity.

To perform the HI reaction according to this invention, use may be made of any suitable procedure, a typical procedure being by the microtitre method which, for example, comprises taking 0.025 ml. of a test serum or inactivated, diluting it serially in two-fold steps on a micro-plate with the diluent of the invention for rubella HA antigen and for test serum, and adding 0.025 ml. of a rubella HA antigen fluid previous diluted to 4 HA units with the same diluent. Then, after 60 minutes' standing at room temperature, the mixture is further admixed with 0.050 ml. of a 0.25% suspension of erythrocytes previously diluted with the aforementioned diluent for erythrocytes. The entire mixture is allowed to stand at 40°C for a further 60 minutes and, then examined for the agglutination or non-agglutination of the erythrocytes. The rubella virus HI antibody titre is expressed in

terms of the maximum dilution factor of the test serum at which the agglutination of erythrocytes has been completely inhibited.

This invention further provides a kit for the above-mentioned rubella virus HI test, which comprises (1) the diluent of the invention for rubella virus HA antigen and for rubella virus HI test sera, (2) the aforementioned diluent for erythrocytes and (3) rubella virus HA antigen, together with instructions for carrying out a haemagglutination inhibition test. This kit may further comprise the erythrocytes, particularly, the fixed erythrocytes, and may also comprise a vessel in which to carry out the test, for example, a microtitre plate. The antigen is preferably in unit dosage form, a unit dose advantageously comprising 4 units of the antigen.

The following Examples and Experimentals illustrate the invention.

Throughout the present specification as well as in claims, the "mg.", "g.", "ml.", "°C", "M" and "N" respectively refer to "milligram(s)", "gram(s)", "millilitre(s)", "degrees centigrade", "molar concentration" and "normality", and percentages are weight/volume unless otherwise specified.

Example 1.

HEPES	594 mg.
NaCl	4.09 g.
Bovine serum albumin	1.0 g.

The above components are dissolved in 450 ml. of distilled water, adjusted to pH 6.2 with 0.1N—NaOH and made up to 500 ml. with a further amount of distilled water. By the above procedure is obtained a diluent for rubella virus HA antigen and for test sera for use in rubella virus HI test, which contains 0.005 M—HEPES, 0.14M—NaCl and 0.2% of bovine serum albumin and is of pH 6.2.

Example 2.

HEPES	2.97 g.
$CaCl_2$ (anhydrous)	111 mg.
NaCl	4.09 g.
Gelatin	25 mg.

The above components are dissolved in 450 ml. of distilled water, adjusted to pH 6.2 with 1N—NaOH and made up to 500 ml. with a further amount of distilled water to prepare a diluent for erythrocytes, which contains 0.025M—HEPES, 0.002M—$CaCl_2$, 0.14M—NaCl and 0.005% of gelatin and is of pH 6.2.

Experimental 1.

Portions of a rubella virus HA antigen were dissolved in the diluent of Example 1 for rubella virus HA antigen and test sera and in a conventional diluent, HSAGC*[1], respectively. Aliquots of each antigen fluids were stored at 5°C and room temperature (25°C), respectively, and samples taken at timed intervals were assayed for HA titres to compare the relative stability of the HA antigen dilutions.

The HA titres were determined in the following manner. Thus, the formalinized erythrocytes of one-day-old chicks (a suspension in distilled water of the lyophilized erythrocytes obtained by the procedure of Example 1 of Japanese Patent Publication No. 37724/1975; the same hereinafter) were diluted with with diluent of Example 2 for erythrocytes and the rubella virus HA titre was determined by Sever's microtitre method on a permanent V-plate (Journal of Immunology *88*, 320—329 (1962)). The dilution of the HA antigen was performed using the diluent of Example 1.

TABLE 1

HA antigen fluid stored	Storage temperature	Rubella virus HA titre			
		Storage time			
		0 hr.	1 hr.	2 hrs.	4 hrs.
Diluent of Example 1	5°C	64	64	64	32
	Room temperature	64	64	128	64
HSAGC *1)	5°C	64	8	4	<4
	Room temperature	64	16	8	4

*1) HSAGC stands for HEPES-Saline-Albumin-Gelatin-$CaCl_2$, the composition of which is particularized below.

HEPES	2.97 g.
NaCl	4.09 g.
Bovine serum albumin	1.0 g.
Gelatin	1.25 mg.
$CaCl_2$ (anhydrous)	55.5 mg.

The above components were dissolved in distilled water, adjusted to pH 6.2 with 1N—NaOH and made up to 500 ml. with distilled water.

Experimental 2.

Aliquots of a test human serum were each subjected to one of the following pretreatments (a) to (e) to prepare seral samples for rubella virus HI tests.

(a) Untreated control serum
To 0.1 ml. of human serum were added 0.3 ml. of HS*2).

(b) Kaolin-treated serum
To 0.1 ml. of human serum was added 0.3 ml. of HS, followed by addition of 0.4 ml. of 25% kaolin-HS suspension to the resultant serum dilution. The mixture was incubated at 30°C for 30 minutes, at the end of which time it was centrifuged. The supernatant fluid was taken and heated at 56°C for 30 minutes to inactivate the serum.

(c) Acetone-treated serum
To 0.1 ml. of human serum was added about 4 ml. of acetone and the mixture was centrifuged at 1500 r.p.m. for 5 minutes. The supernatant fluid was discarded and, following addition of about 4 ml. of acetone, the mixture was similarly centrifuged again. The supernatant was discarded. The sediment was dried and 0.4 ml. of HS was added. The mixture was allowed to stand at 4°C overnight and, then, heated at 56°C for 30 minutes to inactivate the serum.

(d) Heparin-$MnCl_2$-treated serum
To 0.1 ml. of human serum was added 0.2 ml. of HS, followed by addition of 0.05 ml. each of heparin (1,000 units/ml.) and 0.5M—$MnOl_2$. The mixture was allowed to stand at 4°C for 60 minutes and centrifuged. The supernatant fluid was taken and heated at 56°C for 30 minutes to inactivate the serum.

(e) Phospholipase C-treated serum

To 0.1 ml. of human serum was added 0.2 ml. of HS, followed by addition of 0.1 ml. of 0.06 units/ml. of phospholipase C*3). The mixture was incubated at 37°C overnight and, then, heated at 56°C for 30 minutes to inactivate the serum.

*2) HS stands for HEPES-Saline, the composition of which is as follows.

HEPES	238 mg.
NaCl	4.09 g.

The above components were dissolved in 450 ml. of distilled water, adjusted to pH 7.2 with 0.1N—NaOH and made up to 500 ml. with distilled water.

*3) The phospholipase C used is a commercial product prepared from *Clostridium perfringens* ("Phospholipase C" prepared and sold by P—L Biochemicals, Inc., Milwaukee, U.S.A.), one unit of this enzyme being capable of liberating 1 micromole of the acid-soluble phosphorus (phosphorus of phosphorylcholine) per minute when allowed to act upon lecithin at pH 7.3 and 37°C.

To each of the various HI test serum samples obtained as above was added 0.1 ml. of a 10% suspension of the formalinized erythrocytes of one-day-old chicks, whereby the naturally-occurring agglutinins were absorbed before conducting an HI test.

In this HI test, using the diluent of Example 1 for the dilution of serum and antigen and the diluent of Example 2 for the dilution of formalinized erythrocytes, the rubella virus HI antibody titre was determined by the above-mentioned Sever's microtitre method on a permanent V-plate. The results are set forth in Table 2.

TABLE 2

Test serum No.	Rubella virus HI antibody titre				
	Untreated	Kaolin-treated	Acetone-treated	Heparin-$MnCl_2$-treated	Phospholipase C-treated
1	640	16	20	40	40
2	640	32	40	40	80
3	640	16	40	20	40
4	640	16	20	20	20
5	320	8	5	10	5
6	320	16	20	20	20
7	640	16	20	40	40
8	640	16	20	40	40
9	1280	16	40	80	80
10	640	<8	5	<5	<5
11	640	64	40	40	80
12	1280	64	80	160	160
13	1280	64	40	80	80
14	640	32	40	40	40
15	640	<8	<5	<5	<5

Experimental 3.

Aliquots of a rubella virus HA antigen were diluted with the diluent of Example 1 and HSAGC, respectively, to an HA titre of 4 units and stored at 5°C for 2 hours. Using each of thus-diluted and stored HA antigen fluids, the rubella virus HI antibody titre was determined for the phospholipase C-treated sera of Experimental 2 in the same manner as Experimental 2. Where the HA antigen fluids diluted with the diluent of Example 1 were employed, the test sera were diluted with the diluent of Example 1 while the formalinized erythrocytes were diluted with the diluent of Example 2. Where the HA antigen diluted with HSAGC was employed, both the serum and formalinized erythrocytes were diluted with HSAGC. The results obtained are set forth in Table 3.

TABLE 3

Test serum No.	Rubella virus HI antibody titre	
	HA antigen diluted with the diluent of Example 1	HA antigen diluted with HSAGC
1	40	80*
2	80	80*
3	40	80*
4	20	40*
5	5	10*
6	20	20*
7	40	40*
8	40	80*
9	80	320*
10	<5	<5
11	80	160*
12	160	160*
13	80	80*
14	40	160*
15	<5	<5

*Because of the inactivation of HA antigen during storage (a reduction in HA titre from 4 units to 1 unit), no adequate erythrocyte agglutination pattern was obtained. This meant an ill-defined end-point of the HI reaction, which interferred with an accurate assessment of the HI titre.

WHAT WE CLAIM IS:—

1. A diluent suitable for rubella virus haemagglutinating antigen and for rubella virus haemagglutination-inhibition test sera, which comprises N-2-hydroxyethylpiperazine-N'-2'-ethanesulphonic acid in a molar concentration of from 0.001 to 0.01 and NaCl, the pH of which is in the range of from 6.0 to 7.5, and which is free from calcium ions.

2. A diluent as claimed in claim 1, wherein the molar concentration of N-2-

Experimental 3.

Aliquots of a rubella virus HA antigen were diluted with the diluent of Example 1 and HSAGC, respectively, to an HA titre of 4 units and stored at 5°C for 2 hours. Using each of thus-diluted and stored HA antigen fluids, the rubella virus HI antibody titre was determined for the phospholipase C-treated sera of Experimental 2 in the same manner as Experimental 2. Where the HA antigen fluids diluted with the diluent of Example 1 were employed, the test sera were diluted with the diluent of Example 1 while the formalinized erythrocytes were diluted with the diluent of Example 2. Where the HA antigen diluted with HSAGC was employed, both the serum and formalinized erythrocytes were diluted with HSAGC. The results obtained are set forth in Table 3.

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Test serum No.	Rubella virus HI antibody titre	
	HA antigen diluted with the diluent of Example 1	HA antigen diluted with HSAGC
1	40	80*
2	80	80*
3	40	80*
4	20	40*
5	5	10*
6	20	20*
7	40	40*
8	40	80*
9	80	320*
10	<5	<5
11	80	160*
12	160	160*
13	80	80*
14	40	160*
15	<5	<5

*Because of the inactivation of HA antigen during storage (a reduction in HA titre from 4 units to 1 unit), no adequate erythrocyte agglutination pattern was obtained. This meant an ill-defined end-point of the HI reaction, which interferred with an accurate assessment of the HI titre.

WHAT WE CLAIM IS:—

1. A diluent suitable for rubella virus haemagglutinating antigen and for rubella virus haemagglutination-inhibition test sera, which comprises N-2-hydroxyethylpiperazine-N'-2'-ethanesulphonic acid in a molar concentration of from 0.001 to 0.01 and NaCl, the pH of which is in the range of from 6.0 to 7.5, and which is free from calcium ions.

2. A diluent as claimed in claim 1, wherein the molar concentration of N-2-

hydroxyethylpiperazine-N'-2'-ethanesulphonic acid is in the range of from 0.004 to 0.006.

3. A diluent as claimed in claim 1, or claim 2, wherein the pH range is from 6.0 to 6.5.

4. A diluent as claimed in any one of claims 1 to 3, which also comprises albumin.

5. A diluent as claimed in claim 4, wherein the albumin is serum albumin.

6. A diluent as claimed in claim 4 or claim 5, wherein the albumin is present in a concentration of from 0.05 to 1 percent.

7. A diluent as claimed in any one of claims 1 to 6, wherein the molar concentration of NaCl is in the range of from 0.05 to 0.3.

8. A diluent as claimed in claim 7, wherein the molar concentration of NaCl is in the range of from 0.14 to 0.15.

9. A diluent as claimed in claim 1, substantially as described in Example 1 herein.

10. A reagent which comprises rubella haemagglutinating antigen diluted with a diluent as claimed in any one of claims 1 to 9.

11. A reagent as claimed in claim 10, which comprises 4 haemagglutinating units of the antigen.

12. A method for carrying out a rubella virus haemagglutination-inhibition test, which comprises admixing a test serum previously diluted with a diluent as claimed in any one of claims 1 to 9, with rubella virus haemagglutinating antigen previously diluted with a diluent as claimed in any one of claims 1 to 9, and, then, admixing the resultant mixture with a suspension of erythrocytes previously diluted with a diluent which comprises N-2-hydroxyethylpiperazine-N'-2'-ethanesulphonic acid in a molar comcentration of from 0.01 to 0.05, $CaCl_2$ in a molar concentration of from 0.0005 to 0.01 and NaCl, the pH of which is in the range of from 6.0 to 6.5, to assess agglutination or non-agglutination of the erythrocytes.

13. A method as claimed in claim 12, wherein in the diluent for the erythrocytes the molar concentration of the N-2-hydroxyethylpiperazine-N'-2'-ethanesulphonic acid is 0.025 and that of the $CaCl_2$ is 0.002.

14. A method as claimed in claim 12 or claim 12, wherein the molar concentration of NaCl in the diluent for the erythrocytes is from 0.05 to 0.3.

15. A method as claimed in claim 14, wherein the molar concentration of NaCl is from 0.14 to 0.15.

16. A method as claimed in any one of claims 12 to 15, wherein the diluent for erythrocytes also comprises gelatin.

17. A method as claimed in claim 16, wherein the diluent for the erythrocytes comprises form 0.001% to 0.01% gelatin.

18. A method as claimed in any one of claims 12 to 17, wherein the diluent for erythrocytes is substantially as described in Example 2 herein.

19. A method as claimed in any one of claims 12 to 18, wherein the test serum is human serum.

20. A method as claimed in any one of claims 12 to 19, wherein the erythrocytes are fowl erythrocytes.

21. A method as claimed in claim 20, wherein the erythrocytes are chick erythrocytes.

22. A method as claimed in any one of claims 12 to 21, wherein the erythrocytes are fixed erythrocytes.

23. A method as claimed in any one of claims 12 to 21, wherein the test serum has been subjected to a pretreatment adapted to remove or inactivate any non-specific inhibitors of agglutination occurring in the serum.

24. A method as claimed in any one of claims 12 to 23, wherein microtitre procedures are used.

25. A method as claimed in claim 12, carried out substantially as described in Experimental 2 or Experimental 3 herein.

26. A kit for rubella virus haemagglutination-inhibition tests, which comprises (1) a diluent for rubella virus haemagglutinating antigen and for rubella virus haemagglutination-inhibition test sera as claimed in any one of claims 1 to 9, (2) a diluent for erythrocytes as defined in any one of claims 12 to 18, and (3) rubella virus haemagglutinating antigen, together with instructions for carrying out a haemagglutination inhibition test.

27. A kit as claimed in claim 26, which further comprises fixed erythrocytes.

28. A kit as claimed in claim 26 or claim 27, wherein the antigen is in unit dosage form.

29. A kit as claimed in claim 28, wherein a unit dose comprises 4 units of the antigen.

30. A kit as claimed in any one of claims 26 to 29, which also comprise a vessel in which to carry out the test.

31. A kit as claimed in claim 30, wherein the vessel is a microtitre plate.

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