ONDERZOEKINGEN EN MEDEDELINGEN
UIT HET INSTITUUT
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LEIDEN - HOLLAND

No. 6

STUDIES ON THE ANTIGENIC COMPOSITION OF HUMAN INFLUENZA VIRUS A STRAINS

with the aid of the haemagglutination inhibition technique

by

J. van der Veen, M.D. and J. Mulder, M.D.

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ERRATA

P. 18, Line 5 from below. The following solutions were used . . . .
Read: the following physiological solutions were used . . . .

P. 23, fig. 5. NON SPECIFIC TITRES: 0 . . . 9 . . . 10
Read: 0 . . . 5 . . . 10.

P. 25, line 3 from below. Read: In the treated post-infection sera the titre is about as high as or lower than that of the non-specific inhibition of the corresponding untreated pre-infection sera.

P. 37, line 5. Read: table 20 shows the results of these tests.

P. 51, line 6 from below. Read: this will probably ensure that the standard strains will give a high titre with heterologous antisera from the same subgroup.

P. 54, table 29, Right half. Rhodes 1947 E. Read: Rhodes 1947 U.S.A.

Table 30, virus strain Gat 1937 E. Read: Gat (1937 E) FME_7

Graph. 1, Strain Gg (1941 Sw). Read: Gg (1947 Sw).

Graph. 1, Note [1]) 2 = 18—96. Read: 2 = 18—36.

Graph. 2, Virus strain Gat 1937 E. Read: Gat (1937 E) FME_7

CONTENTS

I.

FORMER STUDIES ON THE ANTIGENIC COMPOSITION OF HUMAN INFLUENZA VIRUS A STRAINS

Introduction. In the year 1933 a virus causative of human influenza was demonstrated for the first time by inoculating ferrets intranasally with garglings from patients suffering from the clinical disease (SMITH, ANDREWES and LAIDLAW(1933)). Later a similar virus was recovered in many different countries during influenza epidemics (survey by VAN BRUGGEN *et al.*(1947)).

Since 1940 two groups of influenza viruses — influenza virus A and B — have been recognized. As groups they are quite distinct from each other in their antigenic composition (FRANCIS(1940), MAGILL(1940)) but in both groups there exist strains with individual antigenic properties. The first communications on this subject, as regards the A group, were made by MAGILL and FRANCIS(1936, 1938) and WILSON SMITH and ANDREWES(1938).

In the present communication the A strains are indicated by the name originally given by the author, the year, and the country where the strain was isolated (table 1 shows the data on strains and sera).

Differences of antigenic composition in human influenza A strains, employing the cross-neutralization test in mice. By means of cross-neutralization tests in mice with ferret antisera 28 A strains (17 from England, 3 from the continent of Europe, and 8 from America), were classified by WILSON SMITH and ANDREWES(1938). These authors considered that there are 4 principal antigenic components present in the A group, and that these occur in most strains but in unequal proportions.

They therefore classified the strains into 3 groups. The first or "specific" group comprised 4 strains, WS(1933 E), TALMEY(1937 E), CHRISTIE (1937 E) and GATENBY(1937 E). In addition, they indicated a second group of "intermediate" strains, which showed a close relationship to more than one specific strain. Finally they described a third group, comprising strains which were related to all four specific strains and were relatively non-specific antigenically.

MAGILL and FRANCIS(1938) examined 23 human strains (17 from USA, 1 from Australia, 1 from Hungary, and 4 from England: WS(1933 E), TALMEY(1937 E), GATENBY(1937 E) and BH(1935 E)), with the aid of rabbit sera. They arrived at a classification in 5 groups, merging however more or less into each other. In their study WS(1933 E) also represented a distinct group, to which, however, none of the other strains belonged. TALMEY(1937 E) and GATENBY(1937 E) also fell into two different groups. One of the American strains, PR8(i.e. Puerto Rico 8,

TABLE 1

Data on the influenza virus strains used in this investigation

Strain designation	Location of donor	Year of isolation	Passage formula of the strain used for preparation of ferret antisera [1])
Lee(1940 USA)	United States of America	1940	$F_8 M_{137}$ and $F_8 M_{137} E_{146}$
Swine-15(1930 USA)	United States of America	1930	F M E_7
WS(1933 E)	England	1933	F M E_{19} and F M E_{26}
PR8(1934 USA)	United States of America	1934	$F_{198} M_{593} E_{59}$ and $F_{198} M_{593} E_{66}$
Phila(1935 USA)	United States of America	1935	$F_{246} M_{96} E_5$
Melbourne(1935 Au)	Australia	1935	F M E (E_1) [2])
Christie(1937 E)	England	1937	F M and F M E_6 and F M E_{33}
Talmey(1937 E)	England	1937	F M and F M E_1 and F M E_{21}
Gatenby(1937 E)	England	1937	F M E_7 and F M E_{30}
Burr(1937 E)	England	1937	F M E_3
Bos(1939 Ned)	Netherlands	1939	F_7 M
A(1941 Ned)	Netherlands	1941	$F_3 M_{38} E_{21}$
Sl(1941 Ned)	Netherlands	1941	$F_4 M_{36} E_{17}$ and $F_4 M_{36} E_{25}$
Schn(1941 Ned)	Netherlands	1941	$F_3 E_8$
Schn(1941 Ned), mouse-adapted	Netherlands	1941	$F_3 E_6 M_{12} E_5$
Weiss(1943 USA)	United States of America	1943	$F_3 M_{32} E_{64}$
965(1943 USA)	United States of America	1943	E_8
Hemsbury(1943 E)	England	1943	F M E_1
A 128(1943 Sw)	Sweden	1943	F E_9
Cam(1946 Au)	Australia	1946	E_{69}
Gg(1947 Sw)	Sweden	1947	$F_5 E_{15}$

[1]) The strain used as antigen in serum inhibition tests has the same passage formula, only having some more final egg-passages.

[2]) F M E (E_1): Number of previous egg-passages unknown and one egg-passage done in our laboratory.

TABLE 1 (continued)

Strain designation	Location of donor	Year of isolation	Passage formula of the strain used for preparation of ferret antisera
Barratt(1947 E)	England	1947	E (E_2)
Barratt(1947 E), mouse-adapted	England	1947	E M_{20}
Kunz(1947 E)	England	1947	E_7
Woiteki(1947 E)	England	1947	E_{13}
K(1947 Ned)	Netherlands	1947	F_3 E_{39}
K(1947 Ned), mouse-adapted	Netherlands	1947	F_3 E_{24} M_{13} E_9
FM1(1947 USA)	United States of America	1947	E M_8 E_{14}
Rhodes(1947 USA)	United States of America	1947	F_4 M_{14} E_{22}
1236(1947 USA)	United States of America	1947	E_{15}
1265(1948 USA)	United States of America	1948	E_{14}
FJS(1948 Au)	Australia	1948	E_{14}
AI(1949 Ned)	Netherlands	1949	E_8 and E_{16}
Hof(1949 Ned)	Netherlands	1949	E_8
Vr(1949 Ned)	Netherlands	1949	E_8 and E_{10}
Hes(1949 Ned)	Netherlands	1949	E_8 and E_{10}
Wagt(1949 Ned)	Netherlands	1949	E_8 and E_{14}
Heer(1949 Ned)	Netherlands	1949	E_8 and E_{15} and E_{20}
Heer(1949 Ned), mouse-adapted	Netherlands	1949	E_8 M_{20}

1934), was also by itself a representative of one group, but with the peculiarity of polyvalence, both in regard to the strain and to the antiserum against it [1]). There were considerable differences between the classifications of SMITH and ANDREWES and of MAGILL and FRANCIS, although with regard to some strains they resembled each other. SMITH and ANDREWES tried to account for the differences by means of the circumstance that different species of animals had been used for the production of the antisera, and also that the American investigators

[1]) Swine influenza (Swine-15 (SHOPE 1930)) was included in their experiment and though belonging to the A group stood out clearly from the above mentioned groups of human influenza A strains.

relied on differences which they were inclined to ignore. This investigation on a large scale with the mouse protection test has not been repeated since 1938. The British workers and the other European Centres provisionally held to the English classification, and compared newly isolated strains with the four "specific" strains.

BURNET and CLARK(1942) developed a third theory of the antigenic composition of human A-strains. They employed ferret sera, and defined the strains WS(1933 E), a strain isolated by them in Melbourne in 1935 (MELBOURNE(1935 Au)), and GATENBY(1937 E) as prototype of three groups, within which more specific but unstable differences may be observed. While ANDREWES had found (quoted from BURNET and CLARK(1942)) that Melbourne was an intermediate strain with TALMEY and CHRISTIE components, these investigators found no difference in antigenic composition in the three strains mentioned. To explain this, they assumed that after repeated mouse passages the strains gradually lost their more specific antigenic structure and passed into an intermediate form, represented by Melbourne. It remains an open question, however, whether this view has been adequately proved. Still, BURNET and CLARK's interpretation remains important, and it was put forward again by HIRST in 1947.

The PR8(1934 USA) strain. (FRANCIS(1934)). Smith and Andrewes' analysis placed this strain in the intermediate group, with a close relationship to the strains TALMEY(1937 E) and CHRISTIE(1937 E). They found more strains of this kind. From Magill and Francis' analysis there appears to be some relationship to TALMEY, and generally a closer relationship to most of the other strains that were examined. In consequence of this polyvalence and the fact that the strain is higher virulent for mice, it has been widely used in America to investigate the antigenic structure of newly isolated A strains.

New A strains isolated in Europe after 1937. After the great 1936/1937 epidemic in Europe numerous fresh influenza epidemics were recorded, and new strains were isolated. (PATOCKA(1939), MULDER(1940), STUART HARRIS *et al.*(1940), ANDREWES *et al.*(1941), DAVOLI and PARODI(1941), MULDER *et al.*(1941), TAYLOR and DREGUSS(1941), DREGUSS(1942), STUART HARRIS *et al.*(1943)). In so far as they were analysed, all showed the antigenic composition of the intermediate strains, which puts them in close proximity to the strains TALMEY(1937 E) and CHRISTIE(1937 E). In America new strains were found related to PR8. It can hardly be doubted that on both continents more or less identical A strains were recovered between 1937 and 1943, as PR8 is closely related to TALMEY and CHRISTIE.

The employment of polyvalent strains in the analyses of the antigenic structure. For a provisional analysis of the antigenic structure of newly isolated strains the employment of polyvalent strains had advantages. The English authors mentioned this already in 1938, but their most polyvalent strains (BURR(1937 E) and KOPP(1936 Germany)) showed a low virulence for mice. Afterwards the PR8-strain was also employed in the National Institute for Medical Research in London (STUART HARRIS *et al.*(1940)). It is also the strain now generally incorporated in influenza vaccines.

The epidemiological significance of the variations in the A group encountered in the year 1937. The epidemiological significance of the variations in the A group has never been clearly understood. During the big influenza epidemic of 1936/1937 in England the three specific strains, TALMEY, CHRISTIE and GATENBY were recovered in towns near each other (Chatham, Shorncliffe and Windsor). In addition a strain of the WS type (EO) was recovered in London, a polyvalent strain (BURR) in Uxbridge and also many intermediate strains. Nor has it been possible since 1937 to draw up a classification of epidemics caused by certain "specific" A strains.

Other methods of analysis of antigenic composition. The use of other procedures to study the antigenic properties of influenza strains within the A or the B group was not generally applied until the in vitro haemagglutination inhibition test became known. The complement fixation test with antigen from infected mouse lung did not prove sufficiently specific to differentiate the strains within the A group owing to "soluble antigen" (LENNETTE and HORSFALL(1941), EATON(1941), HOYLE(1945)). FULTON and DUMBELL(1949) showed however, that when using elementary bodies from allantoic fluid (by adsorption and elution from red cells or high-speed centrifugation) complement-fixation though elaborate does permit recognition of antigenic differences within the principal groups of A viruses.

The cross-immunity test in mice was performed on a large scale by SMITH and ANDREWES(1938) en MAGILL and FRANCIS(1938) and showed strain differences which in principle agreed with those obtained with the cross neutralization test in mice.

Analysis of the antigenic composition of strains of influenza virus with the haemagglutination inhibition test of Hirst. The newer methods of study of influenza virus firstly by means of propagation on the chick embryo (BURNET(1936)) and secondly by in vitro haemagglutination (HIRST(1941), MCCLELLAND and HARE(1941)) have provided totally different techniques for studying the antigenic composition of strains. This is due to the fact that the inhibition of haemagglutination by antisera proved to be very specific (HIRST(1943)).

Influence of the passage formula of strains and antisera on the antigenic structure. Owing to the propagation of old stock strains on embryonated eggs, and the fact that after 1941 numerous new strains were isolated directly in eggs, the adaptation to mice fell into disuse, and a series of strains has been developed with different "passage formulae". The "old" stock strains isolated before 1941 have all undergone ferret- and mouse-passages. By propagation on eggs their passage formula becomes F_p, M_q, E_r, with different values for p, q and r. Many strains isolated after 1941 have E_r as formula (including passages via amnion and allantois). Further there may exist F_p, E_r and E_rM_q strains, and possibly also M_q and M_qE_r strains. The antisera, too, have different formulae, according to their preparation from ferret-, ferret-mouse, ferret-mouse-egg, ferret-egg, or egg-passages of virus.

HIRST(1943) has pointed out that all A strains isolated by him in 1941 in the amnion of the chick embryo had the same antigenic com-

position (ferret sera being employed), and he supposed that the differences in antigenic structure of strains isolated in a single epidemic (such as in 1936/1937) may have been caused by repeated ferret- and mouse-passages. Moreover he has found (HIRST(1947 a)) that two egg strains had actually changed their antigenic properties after passage in mice. From this it would follow that the passage formula of the strain may influence the antigenic structure.

Analysis of the antigenic composition of human A strains investigated with the haemagglutination inhibition test. The second world war was responsible for the fact that the analysis of the antigenic structure of a large number of A strains by means of the agglutination inhibition test has as yet been little studied. The first investigation on this subject was made by HIRST(1943). He used for this purpose three American A strains of 1941 isolated in the chick embryo, the strain PR8(1934 USA) and the strains WS(1933 E), TALMEY(1937 E), GATENBY(1937 E), CHRISTIE (1937 E) and the swine influenza strain SHOPE(1930 USA). The old laboratory strains all had the formula FME, and the ferret antisera had been prepared with infected allantoic fluids. In his hands the 1941 strains differed sometimes considerably in antigenic structure from the other strains and had the closest relationship to PR8 and CHRISTIE.The antigenic relationship of the older laboratory strains that were examined agreed in the main with the results of SMITH and ANDREWES. The four "specific" strains of these workers were antigenically remote from each other. PR8 was most closely related to CHRISTIE and TALMEY. The swine virus of SHOPE differed greatly from the other strains. HIRST pointed out that, although clear differences between strains will always be found, the degree in which they appear to deviate from each other may differ considerably according to the particular antisera which are employed.

A new influenza A subgroup. In the years 1945, 1946 and 1947 influenza strains were recovered in Australia, Sweden, England, USA and Holland, belonging to the A group and showing considerable antigenic differences from the established laboratory strains as demonstrated by the cross-haemagglutination inhibition test (ANDERSON and BURNET (1947), ANDERSON(1947 a), FRANCIS *et al.*(1947), STUART HARRIS and MILLER(1947), DUDGEON *et al.*(1948), KALTER *et al.*(1948), LÖFSTRÖM (1949), MULDER and van der VEEN(1948), RASMUSSEN *et al.*(1948), SIGEL *et at.*(1948), TAYLOR(1949)) or by the cross-complement fixation test (FULTON and DUMBELL(1949)).

TAYLOR(1949) performed cross-agglutination inhibition tests with different strains from the American epidemic in the spring of 1947 and compared them with an A strain isolated in 1943, a swine strain and a strain of influenza B (LEE). Hamster and rabbit antisera were employed. With the exception of one strain, the 1947 strains comprised a separate coherent group, being not only quite different from the classic strains PR8 and LEE and the swine virus, but also from a 1943 strain of type A. The new type is denoted by American investigators as Influenza A-prime.

It was the aim of our study to make a more detailed investigation of the antigenic composition of human A strains by means of the new method of haemagglutination inhibition.

II.

MICRO HIRST TECHNIQUE FOR THE HAEMAGGLUTINATION INHIBITION TESTS WITH INFLUENZA VIRUS STRAINS

In 1941 HIRST(1941) and MCCLELLAND and HARE(1941) found that chicken red cells were agglutinated by influenza virus, and this discovery was immediately applied to measure virus and serum antibodies quantitatively in vitro (HIRST (1942 a)). The technique of the virus and serum titration with Hirst's phenomenon varies slightly in the various laboratories (HIRST and PICKELS(1942), BURNET and CLARK(1942), SALK(1944)). In the virus laboratory of the Clinic for Internal Medicine, Leyden University, a modified micro method is practised (MULDER and GOSLINGS (1948)). The technique is as follows:

Preparation of dilutions. As diluting fluid for virus, serum and red cells we employ a 0.85 per cent NaCl solution, buffered with a 20 per cent sodium carbonate solution at pH 7.0. Comparative virus titrations in phosphate buffer (pH 7.0) and buffered saline yield the same results.

Preparation of red cell suspension. Blood of fullgrown hens is collected in a solution of 3.8 per cent sodium citrate, washed three times with saline, and next centrifuged for 10 minutes (at the rate of 2000 r.p.m.) in a graduated centrifuge tube. The red cell volume is made up with saline to make a 10 per cent suspension for storage. The required dilution is made immediately before the test. The red cells are not kept longer than five days at 2° C.

Micro technique for the titration of virus. The virus dilutions are made with bevelled Pasteur pipettes with nearly constant diameter in the concavities of a porcelain tile. The diameter of the holes is 11 mm and their depth is 9 mm. The final dilution of the red cells yields excellent sedimentation and agglutination patterns. The tiles are placed in a Petri dish with moistened filtering paper at the bottom to prevent desiccation. A series of twofold virus dilutions in saline is mixed with an equal volume (four drops) of one per cent red cell suspension (final dilution 0.5 per cent). The patterns, which are distinctly visible against the white background, are read after standing for half an hour at 2° C (plate 1, figure 1 and 2, opposite p. 8). Partial agglutination of the red cells, which can be clearly judged, is expressed as 1, 2 and 3 plus agglutination, depending on the size of the patterns. At 2° C the patterns remain unaltered for many hours. The titre of a virus suspension is recorded as the reciprocal value of the highest final dilution that gives complete agglutination,

and this is termed one agglutination unit (AU). As in the case of the HIRST-SALK method, the titres of the virus dilutions in the micro technique are inversely proportional to the concentration of the final red cell dilution (table 2).

TABLE 2

Titrations of the strain PR8 with a micro method using different final concentrations of red cells

Concentration of red cells (per cent)	Dilutions of virus							
	40	80	160	320	640	1280	2560	5120
1.5	++++	++++	++++	++++	+	0	0	0
1.0	++++	++++	++++	++++	+++	0	0	0
0.75	++++	++++	++++	++++	++++	++	0	0
0.50	++++	++++	++++	++++	++++	+++	0	0
0.375	++++	++++	++++	++++	++++	++++	++	0

Micro technique for the titration of serum. With a 1 ml pipette the serum is first diluted in saline, to make a 1 : 6 dilution. From this solution, sufficient for titrations with several strains, a series of twofold serum dilutions is made in saline in a porcelain tile, and next mixed with half a volume (two drops) of virus suspension. After interaction for half an hour at 2° C two drops of a 2 per cent red cell suspension are added. Readings are made after the tiles have remained undisturbed for half an hour at 2° C. The titre of the serum is expressed as the reciprocal value of the final serum dilution that gives partial (2 plus) agglutination. If this pattern does not occur, the dilution with 2 plus agglutination is calculated by means of interpolation from the dilutions showing larger or smaller agglutination patterns.

Concentration of the virus in the haemagglutination inhibition test. At least two AU of the virus are always employed. In order to determine the exact quantity of virus used in the serum tests, we perform, simultaneously with the latter, a virus titration with the same virus dilution and red cell dilution as that added to the serum. The number of AU units employed in the test is calculated as follows: if the virus dilution t gives 4 plus agglutination, and in the dilution $\frac{1}{2}$ t the red cells settle out completely (0), the dilution t contains 1 AU virus. If the dilution $\frac{1}{2}$ t shows partial agglutination (1, 2 or 3 plus), the virus dilution t contains respectively $1\frac{1}{4}$, $1\frac{1}{2}$ or $1\frac{3}{4}$ AU virus. From the number of AU virus present in the dilution t it may then be calculated how many AU have been employed in the serum dilutions as final concentration. The final concentration of the virus that is employed generally varies from $2\frac{1}{2}$ to $3\frac{1}{2}$ AU.

Ultimately the serum titre found by test is adjusted to a value equivalent to 3 AU virus, it being assumed that an inverse proportion exists between the serum titre and the number of AU of the virus that is added (table 3).

Accuracy of the micro titrations. Apart from the virus, the serum and the red cells, the following variables are of importance:

(1) *Pasteur pipette.* In order to prevent where possible errors arising from the employment of this pipette, each series of experiments is performed with the same pipette, which between the various actions is cleaned with saline, alcohol and ether, afterwards being dried in a flame. We also endeavour to keep constant other factors which may influence the size of the drops, such as the space of the opening, the position of the pipette, the rate of dropping, the volume that is present in the pipette, the absence of fluid on the outside of the pipette, and the temperature of the pipette.

TABLE 3

Ferret antiserum titration against the homologous strain PR8 with a micro method using increasing numbers of agglutinating units

Number of AU	Final dilutions of antiserum (ferret)							
	190	380	770	1540	3070	6140	12290	24580
3	0	0	0	0	0	++++	++++	++++
6	0	0	0	0	+++	++++	++++	++++
12	0	0	0	+++	++++	++++	++++	++++
24	0	0	+++	++++	++++	++++	++++	++++

(2) *The porcelain tiles.* After each experiment the tiles are cleaned with water, alcohol and ether, and then dried. All the tiles that are used, have been examined beforehand to see whether they yield good agglutination patterns of the red cells.

(3) *The size of the drops.* The drops are not always quite identical in volume but vary according to whether saline, serum or red cell suspension is used. The differences however proved to be slight [1]).

(4) *The temperature.* Unless stated otherwise, all tests took place at 2° C.

Determination of the standard deviation of the dilution errors resulting from the micro method. The standard deviation of the method was determined in a series of twofold dilutions of red cell suspension, mixed with

[1]) With the graduated pipette described by M. VAN RIEMSDIJK (1917) it is possible to drop more accurately than with a Pasteur pipette. As, however, the use of the van Riemsdijk pipette took longer than the Pasteur pipette, we have provisionally preferred the latter.

an equal volume (four drops) of saline. The number of red cells per cubic millimetre in certain red cell dilutions was counted by using a B-T-Haemocyter. This procedure was repeated ten times with 4 to 5 countings per dilution. The mean dilution of the red cells was then expressed as the ratio of this dilution to the original undiluted suspension (table 4). The experimental averages deviated from the dilutions that were theoretically expected. We have to consider the fact, however, that the red cell suspension has a different drop size and a different viscosity from the saline. In view of the purpose of these tests it is especially the standard deviation that is important, giving a measure of the accuracy of the drop technique. The test here described was performed in the same way as the antibody titration of serum. As it may be assumed that in both cases the errors of the drop technique are equal, we may conclude from the results that if a serum titration is performed 100 times, the deviation of the mean dilution will in 95 cases not exceed 30 per cent. As in titrations of different sera each dilution in itself always shows

TABLE 4

Standard deviation of a red cell dilution using a micro method

Theoretical dilution	Mean experimental dilution of red cells	Standard deviation	Standard deviation expressed in per cent
2	2.0	± 0.09	± 4.5
8	9.1	± 1.0	± 11
32	35	± 4.1	± 12
128	117	±14	± 15

practically the same deviations, and also because the absolute figures are of secondary importance for the serology of influenza, we have assumed that in practice the serum dilutions range in a purely geometrical progression.

In order to obtain an idea of the magnitude of the standard deviation in virus titrations and serum titrations, ten virus titrations were performed twice, side by side, and in exactly the same way, with one strain (PR8). The standard deviations of the ten titres were respectively ± 7 and ± 10 per cent. The standard deviations of two series of ten serum titrations performed side by side were respectively ± 14 and ± 14 per cent.

From these tests it is clear that the micro technique yields a reasonable degree of accuracy.

Comparison of the micro technique and Hirst method. In order to compare the two methods, virus suspensions of different strains were titrated simultaneously by the HIRST method and by the micro method.

Titration of virus by Hirst-Salk technique. We followed the technique of HIRST-SALK(SALK 1944), except that a 0.20 per cent final dilution of red cell suspension was employed. In the comparative agglutination tests the HIRST-SALK method yielded a titre which was two to six times