ONDERZOEKINGEN EN MEDEDELINGEN
UIT HET NEDERLANDS INSTITUUT
VOOR PRAEVENTIEVE GENEESKUNDE

No. 15

STUDIES ON THE ANTIGENIC COMPOSITION OF THE INFLUENZA VIRUS-A STRAINS

ISOLATED IN THE
NETHERLANDS IN THE PERIOD 1947–1953

by

J. MULDER M. D., L. M. BRANS M. D.

and

N. MASUREL

H. E. STENFERT KROESE N.V. - LEIDEN

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Up to now, the cross haemagglutination inhibition test has proven to be a satisfactory procedure for a rapid broad grouping of influenza virus strains. Disregarding minor discrepancies, the results of this method have been about the same in different countries. However, the results must be critically considered if incorrect conclusions are to be avoided.

Influence of the laboratory animal used to produce the antiserum. In contrast to the use of roosters in American laboratories, many centers in European countries use ferret antisera. Generally speaking, the titres of the ferret sera are higher than those of rooster sera (SAMPAIO (1952)) so that overlapping with heterologous strains will be easier to detect with ferret sera. Also the avidity for antisera of egg-lines with homologous and heterologous ferret antisera can be elicited more clearly than with rooster sera (ISAACS, GLEDHILL and ANDREWES (1952)), which fact might be important in the epidemiological study of influenza. Exclusively ferret sera were used in this study.

Elimination of non-specific inhibitors in antisera. This elimination is absolutely necessary to avoid incorrect conclusions. It is imperative that all inhibition be abolished prior to the use of the antisera. This is especially necessary for freshly isolated egg-lines. We have used enzymes from Vibrio cholera for this elimination, up to the present time (survey by BRANS, HERTZBERGER and BINKHORST (1953)).

Egg-lines and mouse-adapted egg-lines of influenza virus strains. The use of the term "mouse-line" in this study denotes an egg-line which has been adapted to the mouse lung and subsequently cultured further on egg. Those influenza virus strains from the same subgroup which have been adapted to the mouse are antigenically more "homogenic" than pure egg-lines. This is partially due to a better "avidity" for homologous and heterologous antisera (see below).

It is important to take into account the fact that antisera from mouse-lines often give a lower (sometimes much lower) titre with the homologous and heterologous egg-lines from strains of the same subgroup (MULDER and BRANS (1952); (see below)). However, the time and work involved in mouse adaptation presents a practical difficulty which makes it impossible for all laboratories to pass every egg-strain on mice. In the event of doubt in the grouping of certain strains important for epidemiological study, it is necesary, in our opinion, to include mouse-line strains and the antisera produced from them in the tests. If a certain strain, isolated during an epidemic, is chosen as a representative, one must be certain that this strain has a high avidity for the homologous antiserum and heterologous antisera of strains of the same subgroup (see below).

The P-Q variation of influenza viruses. Certain egg-lines of influenza viruses show a low titre with homologous and heterologous ferret antisera from strains of the same subgroup. Others show a high titre only with the homologous serum and a lower one with heterologous sera from the same subgroup. Still other strains demonstrate high titres against homologous as well as against heterologous sera from the same subgroup. We have provisionally denoted this phenomenon as "P-Q-R variation" (Diagram 1).

DIAGRAM 1

Avidity variations in influenza-virus strains, belonging to the same subgroup, using ferret-antisera (according to MULDER *and* VAN DER VEEN (1949)*).*

ANTISERUM	STRAINS		
	P	Q	R
P	++++	+	++++
Q	+	+	++++
R	+	+	++++

However, since the difference between a P- and an R-variant can be regarded as a (small) difference in antigenic composition, we believe that, in extended study of the cross haemagglutination inhibition tests, the diagram of Table 1 cannot be composed exclusively from avidity factors alone. In as much as the term "P-Q variation" is already established in literature, the substitution of the term "R-S relation" for "P-R" and "Q-R" variation merits perhaps recommendation (Diagram 2).

DIAGRAM 2

Diagram representing a frequently occurring antigenic cross pattern from two influenza virus strains R and S, being closely related or derived from each other by mouse adaptation (strain S).

ANTISERUM	STRAINS	
	R	S
R	++++	++++
S	+	++++

The R-S relation between two influenza virus strains. A typical R-S relation within a strain arises very often after adaptation of an egg-line strain to the mouse (MULDER and BRANS (1952)). We also found that reciprocal crossing of two different lines of the influenza-B LEE strain (one strain from the laboratory of Dr. P. VON MAGNUS in Copenhagen and one from our own lab-

oratory) showed an R-S relation (The anti-Copenhagen serum gave a much lower titre with the LEYDEN strain (Table 1)). R-S relations were found between ferret-mouse-egg-lines from the A PR_8 group. For example, the strain Talmey (1937 England) showed this relation with the strains PR_8 (1934 U.S.A.), Christie (1937 England), Burr (1937 England) and A (1941 Netherlands). The antisera from the afore mentioned strains all showed a low titre with the Talmey strain (Table 2). The same was observed between certain of the egg-lines of 6 A strains, isolated during the influenza epidemic of 1949 in the Netherlands (see Table 3). It is probable that the R-S relation is based on a "reduction" of an antigen present in the R strain. The antigenic composition of an R strain could, for example, be denoted as "rs" and that of the S strain chiefly as "s". Likewise, then, we must accept the supposition that the "s"-antigen in the R strain is "blocked" for antibodies; which could be indicated with "r(s)". R-S relations often appear in strains which are closely related antigenically and which have been isolated in the same epidemic. On the other hand, it is certain that the antigens of Q strains are difficultly acceptable to antiserum (ISAACS, GLEDHILL and ANDREWES (1952)); FISET and DEPOUX (1954)). Q strains can be changed into P strains by passage on the ferret, and, in turn, P strains can acquire Q phase characteristics by passage in eggs in the presence of homologous antiserum (ISAACS, GLEDHILL and ANDREWES (1952)). Egg-lines from freshly isolated P phase strains can show Q characteristics in continued egg passages. Cross absorption tests are necessary for full appreciation of the above-mentioned serological patterns of influenza virus strains (HIRST (1952); JENSEN and FRANCIS (1953); ISAACS, DEPOUX and FISET (1954); FISET and DEPOUX (1954)). Nevertheless, when we are concerned with P-Q variations and R-S relations, the results of these absorption tests can be predicted from the common cross haemagglutination-inhibition tests. The cross absorption test is however indispensable for the demonstration of "new" antigens.

The combination of Q phase characteristics and R-S relations. Whenever egg-lines of Q strains are crossed with P strains of the same subgroup and an R-S relation exists at the same time, mistakes can be made in grouping. Diagram 3 illustrates this case of 2 egg-lines with an R-S relation of which the strain S is in the Q phase.

DIAGRAM 3

Crossing of egg-lines of Q and P phase strains (S and R) with R-S relation.

SERUM	STRAIN	
	R (P) (egg)	(S (Q) (egg)
R (P) (egg)	++++	+
S (Q) (egg)	+	+

Only after mouse-adaptation can the serological relationship be demonstrated (diagram 4).

DIAGRAM 4

The same crossing from diagram 3 after mouse-adaptation of the strains R and S.

SERUM	STRAIN	
	R (P) (mouse)	S (P) (mouse)
R (P) (mouse)	++++	++++
S (P) (mouse)	+	++++

The phenomenon of egg-line antisera showing low titres with egg- and high titres with mouse-lines. We have found repeatedly that egg-line antisera have lower titres with respect to the homologous and heterologous egg-lines of the same subgroup than with mouse-lines of those strains. In two series of experiments, we found that the ratio between titres of egg-line antisera against the homologous egg- and mouse-lines amounted to 1 : 1.5 and 1 : 1.4 respectively. However, we also saw cases in which this ratio was 1 : 4. Using heterologous egg-line antisera from strains of the same subgroup, these ratios were 1 : 2 and 1 : 1.3 respectively. As far as Q strains are concerned, it is self-evident that the titres of egg-line antisera against egg-lines are much lower than those against mouse-lines. Using Q strains one also sees, however, that the egg-line antiserum can have a very low titre against an egg-line of a heterologous P phase strain and a much higher titre against the mouse-line of this strain. This phenomenon therefore is identical to the one described in the R-S relation, such as diagram 5 shows.

DIAGRAM 5

Crossing of Q- and P-phase strains using the egg-line of Q and the egg- and mouse-line of P.

SERUM	STRAINS		
	Q (egg)	P (egg)	P (mouse)
Q (egg)	+	+	++++
P (egg)	+	++++	++++
P (mouse)	+	+	++++

In such a case, the Q egg-line antiserum reacts against the P egg-strain as the P mouse antiserum does. This relationship exists between the Scandinavian and the Liverpool type of the 1951 strains (see below).

Materials and Methods. Isolation and passage of strains. Primary isolation of strains was carried out by inoculation of sputum and garglings from patients in the acute phase of the illness into the amniotic cavity of 13 day old chick embroys. To prevent bacterial growth, penicillin (500 U/ml), streptomycin (500 U/ml) and sulfamethylpyrimidine (200 mg%) were added. After one or more amniotic passages, the strains were then passed by inoculation in the allantoic cavity of 10 day old chick embryos. Tests pools of virus infected allantoic fluid, diluted 1 : 4 in merthiolate (0.01 pct) were used for the haemagglutination inhibition tests.

Preparation of ferret antisera. Anesthetized ferrets were infected intranasally with 1 ml. virus infected allantoic fluid (0.5 ml per nostril) and blood was taken by heart puncture a few days before, and twelve days after the inoculation. The pre-infection sera were tested for antibodies against the strain A WS (1933 England), A PR_8 (1934 U.S.A.), A FM_1 (1947 U.S.A.), A_5 (1953 Netherlands), B Lee (1940 U.S.A.) and B Bon (1943 Australia) for the purpose of excluding a previous spontaneous influenza infection in the ferret. Part of each post infection serum was freeze dried.

Mouse adaptation. For the adaptation of a strain to mice, anesthetized animals were infected intranasally, and serial passage with lung tissue was done until all the mice infected died spontaneously of typical influenza pneumonia within ten days.

Treatment of sera with enzymes of V. Cholera. All patients' and ferrets' sera were treated with crude cholera filtrate (1 part serum on 5 parts cholera filtrate), so that all non-specific inhibitors were completely neutralized.

Haemagglutination inhibition technique. The haemagglutination inhibition technique was performed according to the micro-method as described by VAN DER VEEN and MULDER (1950). Each experiment was checked to assure that all non-specific inhibitors were eliminated by the cholera filtrate used in the test. Normal ferret serum with a high non-specific inhibition was employed for this purpose. Titres of the haemagglutination inhibition tests are expressed as the reciprocals of those serum dilutions actually reacting with the virus. The figures were theoretically corrected to 50 pCt agglutination and the use of 3 A.U. of virus.

Chicken red cells. Blood was aspirated from anesthetized chickens into Na-citrate by heart puncture. The erythrocytes were then washed three times with saline and stored under refrigeration as a 10 pCt suspension. The erythrocytes were used within three days after being collected.

The 1947-A strains. In 1947 only one A strain was isolated. It was obtained from the trachea of a 26 year old patient who died within three days from a superimposed Staphylococcus aureus infection in the air passages and lungs. The strain clearly belonged to the FM_1 subgroup (VAN DER VEEN and MULDER (1950), table 30) and has not been considered further in this study. The egg-line was in the Q-phase.

The 1949-A strains. Thirty-eight A strains were isolated in January and February of 1949. All clearly belonged to the FM_1 subgroup. Egg-lines of 6 strains were cross tested with the FM_1 strain (table 3). This table shows the following particulars. The FM_1 strain shows an R-S relation with all six

strains, possibly as a consequence of the fact that our FM_1 is a mouse-line strain. R-S relations are found between A_1 (1949 Netherlands) and the strains Hof, Wagter, Hes and Vr. The strain Hof also shows R-S relations with the strains Hes and Vr. The strain Heer shows an R-S relation with the strains Hof, Wagt, Hes and Vr. The strain Heer is strongly neutralized by all antisera, and is the "strongest" antigen of all the strains tested. This strain is therefore suited for serving as a representative of the afore mentioned 6 1949 strains which were studied. The strains A_1 and Heer are practically identical. None of the 6 egg-line strains are in the Q-phase.

The 1951-A strains. During testing of the 1951 strains, we met with difficulties which remained unresolvable until the appearance of the 1953 strains. For this reason, the 1953 strains are discussed first.

The 1953-A strains. Twenty-five strains were isolated during the epidemic of the winter of 1953. The strain A_5 (1953 Netherlands) (egg- and mouse-line) was crossed with representatives of the subgroups from preceeding years. Table 4 gives the results, from which can be concluded that the strain A_5 (1953 Netherlands) is a "separate" one. The same crossings were performed with the strain Vro (1953 Netherlands) with the same results. Cross-tests of the strain A_5 with the strains Heer (1949 Netherlands) and FM_1 (1947 U.S.A.) are found in tables 9, 11, 12, 13, 15 and 16. From the titres obtained it can be concluded that the strain A_5 (1953 Netherlands) for the most part has lost the FM_1 antigen and has acquired a "new" antigen. A_1 (1953 England) is closely related to A_5 (1953 Netherlands) and the remaining 1953 strains (table 5).

Thirteen strains from 1953 were cross tested mutually and with strain A_1 (1953 Denmark) and A_1 (1953 England) (table 5). It appears from this table that all these strains can be regarded as belonging to the same subgroup. R-S relations are present but, since the tests were made on different days, one must be cautious about drawing too many conclusions from the table. Probably 4 of the 25 strains are Q-phase strains. The strain A_5 (1953 Netherlands) was chosen as representative of the group and for the production of vaccine (N.V. Philips Roxane, Weesp), since this strain showed a good virus production in the chick embryo.

The 1951-A strains. In the winter 1950-1951, ISAACS, GLEDHILL, and ANDREWES (1952) found 2 different antigenic subgroups amongst the influenza A strains which they investigated: namely a Liverpool-type and a Scandinavian-type. The Scandinavian-type was identical to strains which were isolated in the summer of 1950 during an epidemic in Stockholm. There were sufficient grounds to assume that the Liverpool type had made its way from the southern hemisphere (Melbourne, Johannesburg) and the Scandinavian type from Scandinavia. The Liverpool type was responsible for severe influenza epidemics in Liverpool and Belfast. It was isolated together with strains of the Scandinavian type in England, Ireland, the Netherlands and Italy. Only the Liverpool type was identified in most of the countries of southern Europe as well as in the U.S.A., Canada and Japan. Both the

Liverpool and Scandinavian types were related to the FM_1 subgroup. A single strain in Denmark and England was an intermediary between the Liverpool and Scandinavian types (epidemiologic survey by ISAACS and ARCHETTI (1954)).

The 1951-A strains isolated in the Netherlands. In 1951 17 A strains were isolated which were provisionally classified in the FM_1 subgroup. There was, however, the difficulty that 14 of the 17 strains were Q-phase viruses with low titres against homologous and heterologous antisera.

Table 6 shows the crossing of 8 strains of which 6 are Q-phase strains. The egg-line of the Liverpool strain was also included. From table 6 it is apparent that the P-phase strains Stre and Ba are very closely related to the Liverpool strain. The titres of the 6 remaining strains are low.

The antisera of 17 1951 strains were further tested against a series of other strains isolated in 1947, 1949, 1951 and 1953. The antiserum A_3 (1950 Sweden) was also included in this study. The egg- and mouse-line of Barratt (1947 England), Heer (1949 Netherlands), Liverpool (1951 England) and A_5 (1953 Netherlands) were included as well as the mouse-line antisera of the strain Pru (1951 Netherlands). Table 7 gives the results. It follows, from this table, that the 14 Q-phase 1951 strains can be placed in the FM_1 subgroup. The antisera Ro, Stre, and Ba behave practically identically in their relationship with respect to all strains which were investigated, and their antisera are the only ones which show a high titre with the egg-line of the Liverpool strain. The titres of the remaining 1951 antisera are low with the Liverpool egg-line, but high with the mouse-line of this strain. The antisera of Pru (egg- and mouse-line) and Mo have a rather high titre against A_5 (1953 Netherlands). A_3 (1950 Sweden) behaves like most Netherlands' 1951 strains. However, the antiserum of this strain does not show a high titre against A_5 (1953 Netherlands).

The grouping of the Liverpool strain and 2 strains identical to it (Stre (1951 Netherlands) and Ba (1951 Netherlands)). The antisera of two mouse-lines of the Liverpool strain were crossed with the FM_1 strain (egg-mouse-egg-line; 8th mouse passage). Table 8 shows the results. Some relationship exists between both strains in as much as the antiserum FM_1 ($E_xM_8E_{35}$) shows a moderate titre against the Liverpool strain.

From tables 9 and 16 it appears that the antiserum of the Liverpool strain shows a rather high titre with the strain Heer (1949 Netherlands). Compared to FM_1 and A Heer (1949 Netherlands) the strains Stre (1951 Netherlands) and Ba (1951 Netherlands) (which are practically identical to the Liverpool strain) have approximately the same ratios as the Liverpoolstrain (tables 9 and 13). In an experiment previously performed, a stronger relationship was found between the strain Barratt (1947 England), resp. Heer (1949 Netherlands) and Ba (1951 Netherlands) (table 10).

Overlapping of the 1951-strains with those isolated in 1953. In table 7 it is already strikingly apparent that the antisera of 2 1951 Q-phase strains show a rather high titre against the strain A_5 (1953 Netherlands). The highest titre

is shown by the strain Pru (1951 Netherlands). Several other crossings were done with the Pru strain, the results of which were not always identical. In table 11, a ferret-line antiserum from the Pru strain shows a titre approximately as high as with the egg-line of A_5 (1953 Netherlands) as with FM_1 (1949 U.S.A.) and in table 12 one sees that the strain has a ratio about the same with Heer (1949 Netherlands) and A_5 (1953 Netherlands). The titres with the mouse-line of this last strain are lower than those with the egg-line. In the experiment of table 13, in which only crossing with mouse-lines had been done, the titre of the 1951-antisera against the mouse-line of A_5 (1953 Netherlands) also remains lower than against Heer (1949 Netherlands). In contrast to this, the mouse-line of A_1 (1953 England) shows higher titres (table 14). Table 15 shows the titres of the strain Bi (1951 Netherlands) against A_5 (1953 Netherlands). Again these are lower than those against the strain Heer (1949 Netherlands). Crossings with the strain A_3 (Sweden 1950) are found in tables 7, 14 and 16. Overlapping titres with A_5 (1953 Netherlands) and with A_1 (1953 England) is present, but these are lower than against Barratt (1947 England) and Heer (1949 Netherlands). Also, the mouse-line antisera from Liverpool (1951 England), Stre (1951 Netherlands) and Ba (1951 Netherlands) show a rather high titre against A_5 (1953 Netherlands) and A_1 (1953 England). The antisera of A_5 (1953 Netherlands) and A_1 (1953 England) do not however, have a high titre against these strains (tables 9, 13, 14 and 16).

Antibodies in patients' sera from the years 1949, 1951 and 1953. Table 17 shows the results of the haemagglutination inhibition test with pairs of sera, obtained from influenza patients during the illness and during the convalescent period of the disease, against FM_1 (1947 U.S.A.) and A_5 (1953 Netherlands). Here too it appears that these strains possess a different antigenic composition, since the titre rises against both differ sharply, and was negative in 1953 with 2 of the 10 pairs with respect to the FM_1 strain. It is noteworthy that in 1949 antibody rises were also seen with respect to A_5 (1953 Netherlands). It remains uncertain as to whether this means that the antigen concerned was already developed in 1949 in some strains, or whether it is the result of the circumstantiality in which some individuals show a "wide" antibody re ponse.

Prae- and post-vaccination sera. Table 18 shows the results of the haemagglutination inhibition test of 19 pairs of sera obtained from patients who were vaccinated with a vaccine containing the strains FM_1 and Liverpool (Philips Roxane, Weesp). Pre-vaccination titres against the strain A_5 (1953 Netherlands) were present in 5 of the 10 individuals. In 4 patients, the antibody rise was negative or very small against the strain A_5 (1953 Netherlands). Two patients showed a higher post vaccination titre for A_5 (1953 Netherlands) than for FM_1. In judging the results of the vaccination experiments in 1953, one must bear in mind that the vaccine employed was not optimal.