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IMMUNOLOGICAL TYPES OF HORSESICKNESS VIRUS AND THEIR SIGNIFICANCE IN IMMUNIZATION.

B. M. Mc INTOSH, Onderstepoort Laboratory.*

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CONTENTS

* Present address: Poliomyelitis Foundation Laboratories, South African Institute for Medical Research, Johannesburg.

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CONTENTS (*continued*).

I. Introduction

Horsesickness is still one of the major infectious diseases with which the stockowner has to contend in Africa, despite the decrease in economic importance of horses and mules in recent years. Moreover, with the future development of this continent it must continue to occupy, possibly to an increasing degree, the attention of those concerned with the welfare of our livestock.

In the past, severe epizootics of the disease have occurred in parts of Africa and Asia outside the recognised areas of distribution and these occurrences should serve as a warning that, in the future, outbreaks may well occur in continents hitherto free from the disease. In this connection the diagnosis of bluetongue in sheep in the United States of America (Alexander, Haig, Kaschula and McIntosh, 1952; McKercher, McGowan, Howart and Saito, 1953) is an example of how infectious diseases may make their appearance in continents considered to be free from infection.

Horsesickness is a seasonal, insect-borne virus disease of solipeds and is noted chiefly for the high mortality it causes in horses. In this species the course is usually acute and in fully susceptible animals more than 90 per cent of those affected may succumb. Clinically the disease is characterized by hyperthermia, oedema of the lungs, pleura and subcutaneous tissues and haemorrhages in the internal organs. During the febrile stage the blood contains virus in high concentration and the transmission of the disease to other susceptible horses by injection of infected blood is accomplished with ease.

Mules are somewhat less susceptible than horses. In Southern Africa clinical signs of infection are extremely rare in the donkey but during the epizootic in 1944 in the Middle East about 4 per cent of the donkeys died (Alexander, 1948). According to Alexander the greater susceptibility of the Egyptian donkey (*Equus asinus africanum domesticus*) is explained by the fact that this donkey is of a different variety from the donkey (*Equus asinus asinus*) found in Southern Africa.

Outbreaks of horsesickness have been known to occur in dogs fed on the flesh or organs of diseased horses (Bevan, 1911; Piercy, 1951). Experimentally, the dog has been shown to be susceptible and of 54 dogs infected parenterally (Theiler, 1906, 1910) 30 developed fever reactions, and nine died showing pulmonary oedema.

Injection of virus into goats produces a febrile reaction but apparently the virus cannot be passaged serially in this species (Theiler, 1930).

Horsesickness is commonly confined to the continent of Africa south of latitude 10° N. except in the East where it extends into Abyssinia, Eritrea and the Anglo-Egyptian Sudan. It occurs rarely in northern West Africa, the Cameroons and Nigeria (Knuth and du Toit, 1921; von Ostertag and Kulenkampf, 1941; Curasson, 1942; Alexander, 1948; Henning, 1949). Within the Union of South Africa it is widespread although it occurs more frequently in the eastern portion of this country. It is rare in the western and southern parts of the Cape Province (Theiler, 1921). At infrequent intervals severe epizootics have occurred in Egypt, (Carpano, 1933) the Middle East (Alexander, 1948) and Yemen along the southern Red Sea coast of Arabia (Carpano, 1930). In these latter countries the disease has failed to establish itself and the epizootics, which have occurred at intervals of about fifteen years are the result of infection having spread from the adjoining enzootic areas (Alexander, 1948). In the same way, outbreaks of the disease in the south-western areas of the Cape are the result of infection introduced from other parts of the Union (Theiler, 1921).

Within the recognised enzootic areas outbreaks may be expected annually in the late summer; the severity of these outbreaks apparently is dependent upon climatic and probably other unknown factors related to the insect vector or hypothetical virus reservoir. Approximately ten days after the first frost the disease disappears with dramatic suddenness. No further cases normally occur until the following summer, although isolated instances of infection during winter after the occurrence of frosts have been reported (du Toit, 1940). As a general rule the relatively lower-lying parts of a given area are most severely affected. Late and heavy rains after periods of prolonged drought favour the occurrence of epizootics.

In the eighteenth and nineteenth centuries it was observed that the severest epizootics occurred at irregular intervals varying from ten to twenty years. Seventeen hundred horses died of horsesickness in 1769 in the Cape of Good Hope and heavy losses in Southern Africa were reported in 1780, 1801, 1839, 1854, 1862 and 1891. The worst outbreak on record was that in 1854 when some 70,000 horses out of a poulation of 160,000 in the Cape were reported as having died from the disease (Theiler, 1921). More recently, severe outbreaks occurred in 1914, 1918, 1923 (Henning, 1948), in 1940 and 1946 (Alexander, 1954*a*) and also in 1953.

It would appear that the virus is maintained during the winter in some reservoir host which is confined to the recognised areas of distribution of the disease. Past experience indicates that neither domestic solipeds, dogs, goats nor *Culicoides* spp., which are now commonly accepted as the vectors (du Toit, 1944) can be considered as reservoir hosts. If this were the case it would follow that areas such as Egypt and the south-western Cape Province would be enzootic areas. Once the disease had been introduced into these areas it would become established. Horses from South Africa have been introduced into Madagascar and India without the appearance of the disease in those countries which indicates that, since *Culicoides* have a world-wide distribution, the reappearance of the disease in enzootic areas during summer cannot be attributed to the recovered horse acting as a reservoir of the virus. That horsesickness has been known to occur in horses introduced into parts from which all horses, mules and donkeys had been excluded for many years would also point to the virus being maintained in some non-equine host (Theiler, 1921). In a search for this host Theiler carried out extensive transmission experiments from a variety of wild mammals, birds and amphibians that were caught or shot at random in enzootic horsesickness area, all with negative results. It appears that equidae are not essential for the maintenance of the virus in nature and that infection of this species is a purely fortuitous event. Once the first case occurs amongst these animals it may be assumed, at least in a fully susceptible population, that a horse-to-horse transmission by the vector will readily occur.

The identity of the virus reservoir is still unknown and while its discovery may not simplify the control of the disease in domestic animals in the enzootic areas this knowledge might be of extreme value in preventing the introduction of the disease into countries at present free.

By intracerebral injection mice are susceptible and with serial passage in this species neurotropic adaptation of the virus occurs (Nieschulz, 1932; Alexander, 1933). Usually from the second passage the virus is 100 per cent fatal for mice and, once adapted, infective mouse brain gives titres of $10^{-5.5}$ to $10^{-6.5}$ when titrated intracerebrally in mice. With early mouse passage virus guinea-pigs have been shown to be susceptible by the intracerebral route and in this species also the virus becomes neurotropic. However, the concentration of virus in brain tissue of this host is considerably less than in mice (Alexander, 1933, 1935). With neurotropic adaptation in mice and guinea-pigs horsesickness virus becomes modified for the horse and by the 100th passage in mice and apparently much earlier in guinea-pigs it is attenuated, and may be injected into susceptible horses with complete safety (Alexander, 1935).

The embryonated hen's egg has been shown to be susceptible to both neurotropic and viscerotropic virus (Alexander, 1938; McIntosh, 1954*a*) although a detailed investigation of the behaviour of the virus in this host remains to be completed.

As might be expected no form of specific therapy has been successful in the treatment of horsesickness. Some success in its prevention may be achieved by methods based upon the elimination of insect attack (Theiler, 1921; Alexander, 1948).

II. Antigenic plurality of horsesickness virus strains

It has long been known that antigenic heterogeneity exists amongst different strains of horsesickness virus. Theiler (1908, 1915, 1921) working with viscerotropic strains in horses and mules showed by means of cross-immunity tests that while the immunity resulting from infection was durable and solid to the same virus strain a variable percentage of horses contracted the disease in a mild, severe or even fatal form when challenged with a different strain or subsequently were exposed to natural infection. In the early days farmers had noticed that horses may suffer several attacks of the disease and the subsequent infections were spoken of as " aanmanings " or relapses. From cross-immunity tests Theiler (1930) concluded that from 26 per cent to 81 per cent of horses immune to one strain are likely to react later to other strains. The wide variation apparently was dependent upon both the virulence of the challenging strain and the antigenic relationship between the strains. It was evident also from Theiler's studies that there exists a high degree of antigenic relationship between all strains of horsesickness virus. A group of horses immune to any one strain also possessed some degree of immunity to all other available strains. It was this factor which enabled Theiler, using only two immunizing strains in the horse and one in the mule, to produce a fairly successful vaccine with his serum-virus method of immunization.

However, despite the success achieved by Theiler in selecting suitable and effective strains for immunization based upon his conception of antigenic heterogeneity it was evident, that due to the expense and the difficulty of obtaining susceptible horses, no systematic immunological study of all strains would be possible by cross-immunity tests in horses. The variation in susceptibility of different horses to the same virus also served to confuse the position.

Not until it had been shown that the mouse is susceptible to the virus of horsesickness was an antigenic study of all strains possible. Numerous strains have been isolated and adapted to mice and it is possible that by suitable technique all strains might be so adapted. With these mouse-adapted strains Alexander (1933, 1935, 1936) and Alexander and du Toit (1934) were able to confirm the existence of antigenic multiplicity amongst horsesickness viruses. Horses immune to attenuated mouse-adapted viruses were shown to be solidly immune to the homologous viscerotropic virus but on challenge with heterologous strains sometimes developed a severe and fatal reaction. At the same time the development of the intracerebral neutralization test in mice (Alexander, 1935) indicated that an economical and reliable method was available for the immunological differentiation of strains. An intensive study by Alexander of numerous strains isolated since 1933 made possible the selection of antigenically different strains for incorporation in a vaccine.

The selection of these vaccine strains has been based on the result of intracerebral neutralization tests in mice with antisera prepared in known susceptible horses. For the production of these antisera the procedure is to inject a 10 per cent suspension of infective mouse brain into a horse which is then bled for serum three months later, by which time the neutralizing antibodies against the injected virus usually have reached the maximum titre. Cross-neutralization tests with these horse sera in some instances showed unilateral neutralization which added to the difficulty of accurate antigenic grouping of strains. The antibody titre against heterologous strains may be enhanced by repeated injections of the same virus and this is illustrated by the results of neutralization tests with three strains of virus reported by Alexander (1935).

Although the irregular results obtained in neutralization tests with horse antisera made the classification of strains into immunological groups difficult it was still possible, with some accuracy, to select suitable strains for incorporation in a polyvalent vaccine.

Polson and Dent (1950) showed that some strains of neurotropic horsesickness virus are characterized by different rates of inactivation when exposed to ultra-violet irradiation. Since strains of similar antigenic structure show closely related rates of inactivation it appeared that strains with divergent antigenic strucure could be differentiated by the significantly different rates of inactivation. However, no extensive use has been made of this phenomenon to classify virus strains antigenically.

From the results of filtration and centrifugation experiments Polson (1941) showed that the size of the horsesickness virus particle did not vary with the antigenic structure. From these experiments a particle size of 40--60mμ was obtained.

III. The history of immunization

The absence of any knowledge as to the identity of the permanent or primary virus reservoir, the lack of any detailed information on the breeding habits and bionomics of the insect vector which nullifies all efforts aimed at its elimination or control, and the continued failure of specific chemotherapy have meant that the control of horsesickness has been based upon the application of a suitable method of immunization Early recognition of the durable immunity following recovery from a natural attack encouraged research in this direction.

Several early methods of immunization now are of historical interest only, as little of the methods used or the results obtained have been published (Edington, 1900; Koch, 1904; Bevan, 1918, cited by Theiler, 1930).

The first effective method of immunization was the serum-virus method introduced in 1905 by Theiler (1908, 1909) devised in the first instance for the immunization only of mules. It consisted of the simultaneous injection of virulent virus (Strain O) and 300 ml. of immune serum (Serum O) obtained from horses by a process of hyperimmunization. Due to the relative resistance of the mule the losses as a result of this method of immunization based upon the use of a virulent virus were not prohibitive, averaging 1 per cent. The immunity produced was fairly good. Failures in immunity as a result of exposure to natural infection were approximately 2 per cent and the mortality was as low as 0·4 per cent (Theiler, 1930). However, instances are known where severe mortality was experienced. For instance a mortality of approximately 15 per cent was reported in several hundred exposed, immunized mules at Losperfontein in 1932 (Neitz, 1954).

In horses also the serum-virus method was used but was more involved. It was first introduced in 1910 (Theiler, 1921, 1930) and was used until 1933. The method consisted of the simultaneous injection of a virus, T, (replaced in 1923 by Strain N), and 400 ml. of O serum followed three days later by a second virus O, and a further 400 ml. of O serum. The selection of these viruses as the most suitable to use was the outcome of a considerable number of cross-immunity tests in horses by Theiler. Both the T and N strains of virus were relatively avirulent and the O virus, while extremely virulent, gave the best immunity. The method was far from safe; the mortality from the immunization varied from 2 to 10 per cent and in many horses that recovered convalescence was protracted. Rare cases of shock occurred following the intravenous injection of large quantities of serum and a small percentage of horses died from " staggers " some time after immunization (Theiler, 1921). The transmission of equine infectious anaemia also was traced to the serum.

On exposure to natural infection a percentage of immunized horses developed horsesickness. Figures compiled from reports by Theiler (1921) and du Toit (1924), shown in Table 1, give an indication of the extent to which horsesickness occurred in immunized horses.

The severe Union-wide losses experienced during the 1922-23 epizootic resulted in an enquiry into the efficacy of the method of immunization. This enquiry revealed that the higher losses experienced in non-immunized horses, estimated at 70 per cent, justified continuation of the use of this vaccine (du Toit, 1924). In these reports on failures or breakdowns in immunity the time which the animals was exposed to natural infection is not specified. If this period only concerned the first horsesickness season following immunization the final percentages of failures in immunity would, very likely, be higher than the figures given in Table 1, for some horses which survive one season may succumb during subsequent horsesickness seasons.

TABLE 1

Failures in immunity of horses immunized by the serum-virus method

Year	Locality	No. of Horses	Failures in Immunity	Deaths
1914–15	Onderstepoort	101	16*	10*
1916–17	Onderstepoort	224	6	3
1916–17	Zululand	50	†	4
1917–18	Zululand	208	30	9
1922–23	Union	†	29	17

* Figures indicate percentages.
† Figures not available.

Despite the success achieved, the serum-virus method was expensive, cumbersome, and precluded the use of several strains to confer a wide polyvalent immunity.

In efforts to find a safer vaccine the possibility of using formalized virus was investigated in Kenya (Whitworth, 1929; Walker, 1930, 1931) and at Onderstepoort (du Toit and Alexander, 1930; du Toit and Neitz, 1932; du Toit, Alexander and Neitz, 1933*a*, *b*). These studies showed that although formalized infective horse spleen was antigenic and gave a solid immunity to challenge with virulent homologous virus the immunity was transient and the margin of safety was small. For the production of a durable immunity an injection of virulent blood had to be given after the vaccine. The keeping qualities of the vaccine were poor. The safest method involved multiple injections of vaccine, inactivated by progressively lower concentrations of formaldehyde, at intervals of 14 to 21 days. These injections were followed by an injection of virulent blood. The immunity produced was fairly good in mules but poor in horses. No attempt was made to replace the serum-virus method with formalized spleen virus.

Kind (1934) also carried out immunization experiments with formalized virus but with inconclusive results.

Only after the adaptation and attenuation of horsesickness virus to mice was it possible to produce a vaccine containing several antigenically different strains which could be used for immunizing large numbers of animals. (Alexander and van der Vyver, 1935; Alexander, Neitz and du Toit, 1936). Immunization with neurotropic virus was introduced in 1934 and is being used extensively to-day. Some 130,000 doses are issued annually from Onderstepoort and a few other laboratories in Africa produce small quantities of vaccine. Initially four strains were incorporated in the vaccine issued from Onderstepoort but at present as a result of additions which have been made from time to time there are eight strains in the vaccine. No change in the strains used has been made since 1949. The vaccine is entirely safe and a wide polyvalent immunity is obtained from a single injection.

It has been shown (Alexander, 1936*a*) that after simultaneous injection of several antigenically different strains of neurotropic virus a horse responds with the production of antibody against each strain, although the degree of response of individual horses varies and, in some cases antibody against one or more of the injected strains may not be detectable by neutralization tests (Alexander and Mason, 1941).

After injection of neurotropic virus the immunity produced is solid. This is evidenced by the fact that in no single instance has the immunity been broken in the laboratory when challenged with the homologous virulent virus (Alexander, 1936*b*, 1954*b*). The immunity is known to last for at least six years and is very probably life-long (Alexander, 1949). However, annual immunization is recommended so as to ensure the production of the widest possible polyvalent immunity.

The results obtained from the immunization of horses and mules with neurotropic virus strains have been satisfactory and the use of this vaccine on a large scale has revolutionized the control of horsesickness. It is extremely difficult to obtain reliable figures but from various reports it is doubtful whether the average annual mortality from natural infection in horses immunized with the present vaccine exceeds one per cent although it is possible that in years when severe epizootics occur this figure may be slightly higher. For instance, in the annual returns of the Director of Veterinary Services for the Union of South Africa it is reported that 741 immunized horses died from horsesickness in Natal during the severe epizootic of 1953. This figure represents approximately 3 per cent of the total number of horses which were reported as having been immunized.

While these figures are unreliable in that they are compiled mainly from hearsay evidence it must be accepted that several hundreds of immunized horses died during this epizootic, although it is not possible to ascertain the percentage mortality with any degree of certainty.

A point which should be emphasized is that the majority of these horses possessed some immunity from either earlier injections of vaccine or natural attacks of the disease from which they had recovered. Hence these figures of mortality do not represent the mortality in a group of susceptible horses immunized with a single injection of vaccine.

Many horses in the Union are carriers of *Babesia equi* (Laveran, 1901) and *B. caballi* (Nuttall, 1910) and suffer relapses of biliary fever during attacks of horsesickness. Undoubtedly some immunized horses which would recover from the horsesickness infection succumb as a result of the dual infection (Theiler, 1906*b*, *c*).

During the epizootic of horsesickness in the Middle East in 1944 the routine polyvalent neurotropic vaccine produced at Onderstepoort was used with considerable success (Alexander, 1948). It was used to immunize horses, mules and donkeys in Egypt and Palestine and resulted in complete control of the epizootic. It was noticed that deaths ceased approximately 21 days after immunization. If allowance is made for an incubation period of 7 days and an illness of 4 days it follows that immunity developed from about the 10th day after immunization. This is in agreement with results obtained in the laboratory which showed that horses immunized with neurotropic strains were solidly immune to challenge with homologous virulent strains three weeks later (Alexander, 1949).

The vaccine used to immunize the animals in the Middle East contained seven strains, viz., 449, 1180, VRY, OD, KA, O, VH, (see Table 3). Neutralization tests with three virus strains isolated from animals in Egypt and one strain from Palestine indicated that the Middle East strains were very closely related and that there was a strong relationship between these strains and the vaccine strains VH and OD (Alexander, 1948).

Further data on the protection provided by the neurotropic vaccine is available from the Onderstepoort Laboratory farm, Kaalplaas, where a fairly large number of horses are kept under conditions which expose them to horsesickness infection. During the months February, March and April, 1953, eleven horses died from horsesickness on this farm, and an unknown number suffered from an attack of horsesickness and recovered. Of the eleven horses that died, seven had been introduced recently from Wakkerstroom in the Transvaal Highveld, an area which is considered to be relatively free from horsesickness. In view of this it is possible that on arrival on Kaalplaas these horses were all fully susceptible animals, having never been immunized or exposed to natural infection. They were immunized on 17/3/53 shortly after their arrival and seven of them died between 2/4/53 and 21/4/53.

Examination of the Onderstepoort records showed that six of these horses died after 7/4/53 i.e. 21 days or more after infection. While this period is long enough for an immunity to develop against homologous virus strains, neutralization tests indicate that a period of five months is necessary for the maximum development of antibody (Alexander, 1935). It is believed that not until this period has elapsed is the maximum immunity developed against heterologous strains. Hence, the deaths of these horses should not be regarded as true failures in immunity although the heavy mortality amongst these horses is an indication of the severe infection present on this farm during these three months.

The remaining four horses which died on Kaalplaas had each received two or more injections of vaccine. The dates of these injections are given in Table 2. From the number of inoculations these horses received it is evident that the mortality in these four horses must be considered to be the result of true breakdowns in immunity. Sera were collected from horses 1582 and 1096 just before they died. Neutralization tests on these sera (*vide infra*) showed that these two horses possessed high titre antibody against each vaccine strain at the time of their illness. While the total number of immunized horses present on Kaalplaas during these three months varied from time to time, the average number was 110. Hence the loss of these four horses represents a mortality of 3·6 per cent.

TABLE 2

Immunization history of some horses that died on Kaalplaas

Horse No.	Date of Death	Dates of Immunization
1803	19/2/53	19/6/51, 29/10/52.
1590	9/3/53	5/3/52, 18/4/52, 1/7/52, 27/7/52.
1582	27/3/53	5/3/52, 18/11/52.
1096	28/3/53	16/7/48, 21/9/48, 6/12/48, 17/12/48, 8/11/49, 8/11/50, 12/9/51, 29/10/52.

Although these breakdowns in immunity are extremely disappointing to individual owners, viewed from the broad aspect of the control of the disease, active immunization with neurotropic attenuated vaccine must be considered as a highly effective method of control. At present it is the only method of control which can be considered as practical.

IV. THE SCOPE OF THE PRESENT WORK

During the epizootic of horsesickness experienced throughout the Union in 1953, the reported mortality in immunized horses was the cause of some concern. During this outbreak numerous blood samples from breakdown cases of horsesickness in immunized horses were received at Onderstepoort. From these samples ten viruses were isolated and adapted to mice. There was some urgency that these viruses be classified antigenically as the possibility could not be overlooked that the presence of a large vaccine immune equine population may have resulted in the selection and propagation of variant antigenic strains which were the cause of the mortality in immunized horses during this epizootic. Apart from this possibility it is important in horsesickness, where the virus exists as multiple

antigenic types, that from time to time the antigenic composition of the prevalent viruses in nature be submitted to critical laboratory examination. The large number of viruses isolated in 1953 from widely separated parts of the country provided an opportunity for such a study.

Due to the unsatisfactory tendency of horse antisera to develop antibodies to heterologous strains it was considered advisable to investigate the suitability of rabbit antisera. Also, with the large number of virus strains now isolated it was becoming increasingly difficult to obtain enough susceptible horses to produce antisera against all these strains.

Although the rabbit is insusceptible to either viscerotropic or neurotropic horsesickness virus it was known that an antiserum could be prepared by hyperimmunization with mouse-adapted virus (Alexander, Polson and van Rooy, 1950). However, no extensive neutralization tests with rabbit antisera had been carried out and so the degree of their specificity was unknown. It was hoped that, possibly, the rabbit being insusceptible would develop antibodies only against the major antigenic components of each horsesickness strain and thus would facilitate the grouping of strains into the main immunological groups. In the work reported here on the typing of strains, rabbit antisera have been used exclusively. With these sera it has been possible to group the forty-two virus strains examined into seven immunological types.

It has often been noticed that viruses isolated from cases of horsesickness in immunized horses, on antigenic examination in the laboratory, proved to be closely related to strains included in the vaccine used to immunize the virus donor (Alexander, 1954*c*; McIntosh, 1953). Unless the immune status of the animal at the time of infection could be determined, the significance of this finding is obscure since the possibility of unsuccessful immunization could not be excluded with certainty. In order to investigate this aspect further sera were obtained from several breakdown cases as early as possible during the illness—before neutralizing antibodies to the infecting virus would be detectable in a neutralization test. The results of the investigation on these sera are included in this work.

In addition some experimental work on the use of the ferret and dog for the isolation of virus from breakdown cases in immunized horses is described.

V. The virus strains

It may be stated with confidence that all the viruses examined in the present study are in fact horsesickness viruses. All, except two which originated from trapped *Culicoides* spp., were isolated from horses showing clinical symptoms of horsesickness. The symptoms of this disease are usually characteristic and the diagnosis is not often in doubt. Furthermore, it is likely that specimens submitted to the laboratory for virus isolation would be collected only from cases in which the diagnosis was obvious. However, with our present knowledge of horsesickness viruses certainty in regard to mouse-adapted strains can exist only with those viruses in which the parent strains have been known to produce horsesickness on inoculation into horses or which can be shown to be related antigenically to viruses falling within this category. It must be accepted that the eight viruses at present included in the horsesickness vaccine issued from Onderstepoort fulfil one or the other of these requirements. From the results submitted in this report the antigenic relationship of all the remaining viruses examined, except four, to these vaccine strains should justify their identification as horsesickness viruses. The

four strains which showed no antigenic relationship to the vaccine strains, are recent isolates which constitute a single antigenic type. So far none of these four strains has been inoculated into horses but from the history of their origin as well as their behaviour in mice and ferrets it is felt that their identification as horsesickness viruses should be accepted without doubt.

Preliminary work with the complement fixation test supports this opinion. With this test, using acetone-ether extracted baby mouse brain antigens (Casals, 1949) and antisera prepared in guinea-pigs, apparently all strains of horsesickness virus are cross-reactive and no grouping of strains as is obtained with the neutralization test with rabbit antisera is evident. With the complement fixation test strains PMB and Karen were cross-reactive with all the vaccine strains against which they were tested. These two strains are two of the four strains which showed no antigenic relationship to vaccine strains with the neutralization test.

The two strains, isolated from *Culicoides* spp., should be considered as horsesickness viruses in view of their demonstrated antigenic relationship to two of the vaccine strains.

All the viruses examined were mouse-adapted and at various passage levels. The earlier isolated viruses had had between 100 and 150 mouse passages but most of the more recently isolated viruses were at a much lower level. Many of the viruses had been passaged once through the ferret followed by several serial passages in baby mice, as well as adult mice as shown in Table 3. Some strains were passed from thirty to forty times in baby mice.

It is customary to refer to the different horsesickness viruses as strains but it should be emphasized that the word as used refers merely to different isolates and does not take into account any properties of the various viruses such as virulence for the horse, growth characteristics in the mouse or egg embryo, or antigenic composition. For lack of any rational system of classification the symbols used to denote the various viruses have their origin from the laboratory number of the horse infected with the particular virus or from the locality where the strains made their first appearance.

The 42 viruses investigated may be considered for convenience in three groups.

(1) The eight vaccine strains. These strains are incorporated in the present vaccine issued from Onderstepoort. They had been selected for inclusion in the vaccine partly on grounds of their divergent antigenicity, a fact which made them suitable for inclusion as a comparative group in a plan of cross-neutralization experiments.

(2) Sixteen recently isolated strains. Ten of these strains were isolated during the epizootic in 1953.

(3) A group of 18 strains isolated at various times over the past 20 years. Most of these strains were isolated from cases of horsesickness in immunized horses. They had been investigated and for a variety of reasons had been found unsuitable for inclusion in the vaccine. They had been freeze dried as infected brain material and kept at 4° C. Some had been stored in this way for 16 years.

To facilitate reference, the laboratory history and other relevant data, of every strain examined, are listed in Table 3 in the three groups as outlined above.

Virus for the neutralization tests was prepared from the highest available mouse passage level of virus of each strain.

The vaccine strains and most of the earlier strains were isolated and adapted to mice by Dr. R. A. Alexander. From 1949 to 1950 the isolation and adaptation of strains in mice were done by Dr. K. E. Weiss. All these strains were available at Onderstepoort as dried mouse brain material. Strain Karen was isolated from an immunized foal in Kenya and was sent to Onderstepoort by the Director of Veterinary Services, Kabete, Kenya, when in its 21st mouse passage.

TABLE 3

Particulars of the virus strains

Strain	Isolated	Lab. Passages	Locality First Appeared	History of Virus Donor
1. *The Vaccine Strains—*				
1180.....	1933	100 to 120AM.....	South Africa.....	?
KA.....	1937	100 to 120AM.....	Onderstepoort.....	?
OD.....	1937	100 to 120AM.....	Pretoria District.....	?
Vry.....	1938	100 to 120AM.....	Vryheid, Natal.....	?
VH.....	1940	100 to 120AM.....	Vaalhartz, Cape.....	?
L.....	1940	100 to 120AM.....	Ladysmith, Natal.....	?
A501.....	1948	100 to 120AM.....	Andalusia, Cape.....	?
114.....	1940	100 to 120AM.....	South Africa.....	?
2. *Recently Isolated Strains—*				
Rhod.....	1949	68AM.....	Rhodesia.....	Imm.
454.....	1949	68AM.....	Onderstepoort.....	Imm. 6X.
1513.....	1950	1H, 37AM.....	Onderstepoort.....	Imm. 3X.
1408.....	1950	1H, 27AM.....	Onderstepoort.....	Imm. 3X.
MFK.....	1952	1F, 3BM, 27AM....	Mafeking, Cape.....	Imm. ?X.
FR.....	1952	1F, 3BM, 38AM....	Vryheid, Natal.....	Imm. 3X.
ES.....	1953	1F, 42BM.....	Eshowe, Natal.....	Imm. 3X. Natural attack in 1952.
1100.....	1953	1F, 38BM.....	Onderstepoort.....	Imm. 5X.
1582.....	1953	37BM.....	Onderstepoort.....	Imm. 2X.
PMB.....	1953	30BM.....	Maritzburg, Natal....	?
VR.....	1953	1F, 39BM.....	Vryheid, Natal.....	Imm. ?X.
P2.....	1953	1F, 25BM.....	Pretoria District.....	Imm. 3X.
P1.....	1953	1F, 20BM, 7AM....	Same farm as P2.....	Imm. 3X.
1096.....	1953	25BM, 10AM.....	Onderstepoort.....	Imm. 8X.
2627.....	1953	30BM, 10AM.....	Onderstepoort.....	Imm. 3 Weeks before infection
ERM.....	1953	1F, 20BM, 10AM...	Ermelo, Transvaal....	Imm. ?X.

TABLE III. (*continued*).

Strain	Isolated	Lab. Passages	Locality First Appeared	History of Virus Donor
3. *Various Strains Isolated since* 1933—				
449.....	1932	3H, 150AM.....	Onderstepoort.....	Hyp. horse with O virus.
'O'.....	1933	193H, 150AM.....	Pretoria District.....	Theiler's O strain.
1157.....	1933	50AM.....	Onderstepoort.....	?
Pirie.....	1936	130AM.....	South Africa.....	?
Westerman.	1936	102AM.....	South Africa.....	?
Theiler.....	1937	100AM.....	South Africa.....	?
Keppel.....	1937	?	South Africa.....	?
Galpin.....	1938	100AM.....	Northern Transvaal...	Imm. ?X.
Cedara.....	1940	54AM.....	Cedara, Natal.....	?
H409.....	1947	?	South Africa.....	?
Mataffin...	1948	40AM.....	E. Transvaal.....	?
1397.....	1949	35AM.....	Onderstepoort.....	Imm. ?X.
1145.....	1949	35AM.....	Onderstepoort.....	Imm. 2X.
30B.....	1949	3BM, 70AM.....	Onderstepoort.....	*Culicoides* spp.
CA.....	1949	3MB, 70AM.....	Onderstepoort.....	*Culicoides* spp.
Potch.....	1950	30AM.....	Potchefstroom.....	Imm. ?X.
1144.....	1950	1H, 3BM, 30AM...	Onderstepoort.....	Imm. ?X.
Karen.....	1952	MA23.....	Kabete, Kenya.....	Imm. foal.

'O' Strain isolated in 1905 and maintained in horse passage until 1933 when adapted to mice.
1H=1 horse passage.
1F=one ferret passage.
30AM=Thirty adult mice passages.
10BM=Ten baby mice passages.
Imm.=Immunized.
Imm. 3X=Horse immunized with 3 inoculations of polyvalent neurotropic mouse vaccine.
Hyp.=Hyperimmunized

VI. THE EXPERIMENTAL ANIMALS

(*a*) *Rabbits*

For the preparation of antisera adult rabbits obtained from the Institute were used. These rabbits have been inbred for the past thirteen years and have reached a high degree of uniformity.

(*b*) *Mice*

All the mice were supplied by the Institute and were the Swiss strain of white mouse. Adults were from five to six weeks old and baby mice were from three to six days old.

(*c*) *Ferrets*

The ferrets (*Mustela eversmanni furo* Linn.) were from the stock kept at the Institute and prior to their use in experiments were housed under conditions which unavoidably exposed them to possible attacks by *Culicoides* spp. This apparently did not influence the experimental results. After introduction into experiments they were housed in buildings which excluded attack from these insects.

(*d*) *Horses*

The donors of the serum used in the preparation of the normal serum-saline diluent were susceptible horses maintained at this Institute under horsesickness-free conditions.

(*e*) *Dogs*

These were mongrel animals purchased locally.

VII. Laboratory methods

(*a*) *Preparation of the viral antigens*

To obtain a supply of virus of each strain for the neutralization tests five or six mice were sacrificed *in extremis*. To the freshly harvested brains chilled diluent was added in sufficient quantity to make a 5 per cent suspension. After blending, the suspension was centrifuged at 3,000 r.p.m. for 15 minutes and the supernatant was distributed in 1·2 ml. amounts into tubes, which were then sealed and stored in a solid carbon dioxide cabinet. Lots of each virus were prepared in this manner in sufficient quantity for all the intended experiments and with one or two exceptions the same lot of each virus was used throughout the series of experiments. After preparation, each lot of virus was tested for potency, after being held under the identical conditions of the neutralization test.

The diluent for the virus was a mixture containing 5 per cent lactose, 1 per cent peptone in M/50 phosphate buffer in distilled water. Ten per cent horse serum in normal saline was found to be unsatisfactory as a diluent for horse-sickness virus preserved in the frozen state. Freeze-dried virus using the lactose-peptone mixture as diluent also proved to be unsatisfactory. On titration of the dried virus immediately after reconstituting, the titre was satisfactory but on being exposed to the conditions of the neutralization test there was a marked decrease in titre. With a serum-saline diluent the rate of freezing appeared to be important as quick-freezing in a solid carbon dioxide alcohol mixture appeared to be more detrimental to the virus than a slow freeze in a mechanical deep-freeze.

(*b*) *Sera*

Rabbit

Antisera against each of the eight vaccine strains as well as the 16 recently isolated viruses, enumerated in Group 2 above, were prepared by the hyperimmunization of rabbits. Pooled antisera from two rabbits were used for each strain. The rabbits were given 10 weekly, intravenous injections of 1 ml. each of a freshly prepared half per cent infected brain suspension in normal saline. These suspensions were prepared just prior to injection from lots of infected brain material stored at —15° C. After blending, the suspensions were centrifuged at 3,000 r.p.m. for half an hour and the supernatant was injected into the rabbits. The rabbits were bled 10 days after the last injection. The sera were stored at −15° C without preservative in tubes with airtight rubber stoppers. To avoid repeated freezing and thawing, lots of each antiserum were stored at the same temperature in 0·4 ml. amounts. These volumes were just sufficient for one test. All rabbit sera were used unheated.

Horse

Sera for neutralization tests were collected from eight immunized horses reacting to natural infection. Six of these horses were maintained at the laboratory or on the adjoining farm Kaalplaas. The numbers of these horses are 1513, 1408, 1100, 1582, 1096 and 2627. The other two horses were Percherons from a privately owned farm near Pretoria. Merthiolate at a concentration of 1/10,000 was added to these sera and they were either stored in a refrigerator at 5° C or in a deep-freeze at −15° C until tested. The sera were inactivated at 56° C for half an hour before testing.

Ferret

In the case of the three ferrets injected with viscerotropic virus pre-inoculation sera were collected. The antisera were collected five weeks after the injection of viscerotropic virus. These sera were stored at −15° C and were heated at 56° C for half an hour before testing.

(*c*) *Technique of the neutralization test*

The method employed was, with minor modifications, that described by Alexander (1935) in which a constant amount of virus was added to serial dilutions of serum.

With the rabbit sera serial five-fold dilutions of serum were used over the range, 1/5, 1/25, 1/125, 1/625. The dilutions of serum were prepared at double strength in volumes of 0·8 ml. so that the addition of 0·8 ml. of virus suspension to each serum dilution would result in the required serum dilution.

In the case of the horse sera four-fold dilutions over the range 1/2, 1/8, 1/32, 1/128, 1/512 were used. These dilutions were also prepared initially at double strength and the volume of each serum dilution with these sera was 0·75 ml. and to each dilution 0·75 ml. of virus suspension was added.

With the ferret sera both the five-fold and the four-fold system of serum dilutions were used.

The virus antigens were diluted so as to provide between 50 to 100 LD50 of virus in each intracerebral dose of ·05 ml. of serum-virus mixture. Although in the majority of neutralization tests the LD50 of virus was between 40 and 160, the extreme limits were 20 and 320 LD50. Where it was considered that abnormal amounts of virus had resulted in a misleading result the test was repeated.

With each neutralization run (i.e. the series of tests done on the same day with the same virus) the exact titre of the virus test dose was determined by titration in mice. For this titration a series of dilutions of the virus was prepared as follows: 1/2, 1/5, 1/10, 1/20, 1/40, 1/80, 1/160, 1/320. After completion of the virus dilutions an equivalent volume of serum-saline was added to each dilution except the first. This serum-saline was added to take the place of the antiserum dilution. The virus dilutions were injected into mice after injection of the serum virus mixtures.

The serum-virus mixtures together with the virus titration were incubated at 37° C for 2 hours, held in the refrigerator at 5° C overnight, and injected into mice the following day.

In the case of the serum-virus mixtures a group of six mice was used for each mixture, and with the virus titrations four mice were used for each dilution. The dose for all mice was 0·05 ml., given intracerebrally. The mice were observed for a period of ten days and deaths from the third day were included in the death score. In the case of strains which had been passaged in baby mice an observation period of 14 days was necessary.

(d) Collection of material from horses and ferrets for virus isolation

For isolation of virus from horses blood, collected in an anti-coagulant preservative (O.C.G.), (water 8240 ml., glycerine, 5,000 ml., potassium oxalate 50 grm., Carbolic acid 50 grm.), was used. This blood was collected usually at the height of the febrile reaction.

In the case of the six immunized horses maintained at the laboratory or Kaalplaas (Nos. 1513, 1408, 1100, 1582, 1096, 2627) and the two immunized Percherons from which serum was obtained for neutralization tests blood was also collected in O.C.G. for virus isolation. The two bleedings, for serum and for virus isolation, were made on the same day. From all these horses viruses were eventually isolated and adapted to mice. Following the usual custom the strains from the laboratory horses were designated 1513, 1408, 1100, 1582, 1096 and 2627 and those from the Percherons P1 and P2.

Several virus strains were isolated from O.C.G.—blood samples submitted by veterinarians in various parts of South Africa and Rhodesia. These samples were sent through the post and no refrigeration during transit was attempted. On arrival at the laboratory they were stored in a refrigerator at 5° C until injected into mice, ferrets or horses for the purpose of virus isolation.

For isolation of virus from ferrets, the ferret was anaesthetized with ether and blood was withdrawn by cardiac puncture into O.C.G. When spleen was used as a source of virus pieces of this organ were macerated in a Waring blendor with an equal amount of serum-saline and then lightly centrifuged. The undiluted supernatant was used to infect mice.

(e) Injection of mice and method of passage

As the O.C.G. blood mixture is toxic when injected intracerebrally into mice, it was diluted to 1/5 with serumsaline prior to injection. For serial passage a 10 per cent suspension of infective brains was used. All infective brain material was stored at −15° C until used. The brains were harvested from mice when *in extremis*. The suspension was clarified by centrifugation at 3,000 r.p.m. for 15 minutes before injection into mice. With infant mice it was possible to make a passage every second day and with adults every third or fourth day depending upon the particular strain. The strains isolated in 1953 were passaged mainly in infant mice.

In the passage of strains in baby instead of in adult mice it was felt that there was a greater danger of accidental mixing of the strains due to the higher susceptibility of baby mice and certain routine precautions were adopted to reduce this hazard. With a view to ascertaining the chances of accidental infection during passage the infectivity of horsesickness virus for baby mice by various routes was determined. These experiments showed that the percentage mortality from the injection of 10^5 intracerebral doses by the intraperitoneal route was 21 per cent and by the intranasal 100 per cent. Comparative titrations by the intracerebral and intranasal routes showed that the infectivity by the latter route was consider-

ably less since titres of only $10^{-1.5}$ were obtained by intranasal infection. Amongst several families in which only half the litter was infected by the intracerebral route none of the non-infected litter mates became sick. No virus could be demonstrated in the muscle and blood of baby mice when *in extremis*.

(*f*) *Serum-saline diluent*

All dilutions were made in 10 per cent horse serum in normal physiological saline. The serum was inactivated at 56° C for 30 minutes and merthiolate added at a concentration of 1/10,000.

(*g*) *Calculation of titres*

Serum and virus titres were calculated according to the method of Reed and Muench (1937) and are expressed as the reciprocal of the dilution end-point. Due to the fact that the heterologous titres were often higher than the homologous it was considered that no advantage could be gained by determining antigenic ratios or R values of related strains (Hillman and Horsfall, 1952).

VIII. Experimental procedures and results

(*a*) *Isolation of virus in mice from immunized horses*

It will have been noticed that among the viruses isolated since 1952 several were passed once through the ferret. It is felt that some explanation should be given for this procedure and accordingly, the work done on the isolation of virus from immunized horses is given in some detail.

From infective blood drawn at the height of the disease in non-immune horses virus isolation in mice is comparatively simple. In adult mice injected with such blood the incubation period will vary from ten days to three weeks and usually only about half the mice will become sick (Alexander, 1935). Usually within the second or third passage, the mortality is 100 per cent and the strain can be maintained without difficulty in serial passage. Baby mice are more susceptible (Weiss, 1949) and even from the first passage the mortality in mice of this age is usually 100 per cent.

However, when blood is drawn from a reacting immunized horse it is often impossible to infect either adult or baby mice. Mulligan (1938) has reported the same difficulty in Kenya. Such immunized horses usually contain serum antibodies to high titre against the eight virus strains incorporated in the vaccine and apparently these antibodies interfere with multiplication of virus in the mouse.

Injection of blood from a reacting immunized horse into a susceptible horse results in infection, and so this difficulty of isolation in mice may be overcome by sub-inoculation of the blood sample into a horse and from virus circulating in the blood of this horse during the febrile reaction the mouse can readily be infected. Even though susceptible horses may be available the method is expensive and unless some alternative can be found it will not be possible to isolate all strains received at the laboratory. For obvious reasons it is of considerable importance that as many strains as possible be isolated from breakdown cases in immunized horses and the submission of blood samples from such cases is encouraged. The result of the injection of blood from reacting immune horses into susceptible horses shows that failure in these cases to isolate the virus in mice is not due to absence of viable virus but is probably due to the presence of antibodies which develop in response to immunization. These blood samples are apparently a neutral serum-virus mixture for the mouse but an infective mixture for the horse.

If such were the case it was thought that is might be possible with certain procedures to reactivate sufficient virus in the blood samples to enable infection in the mouse to occur. Numerous workers have demonstrated that reactivation of neutralized virus may be accomplished in several ways. Taylor (1941) and Goyal (1935) showed that by simple dilution, virus in neutral serum-virus mixtures could be reactivated. Morgan and Olitsky (1940) showed that high speed centrifugation was another method which may be employed while blind passage in mice was used by McKee and Hale (1946) to reactivate neutral mixtures in influenza. Accordingly, these three methods were attempted on O.C.G.-blood mixtures obtained from reacting immunized horses, and which were known to be infective for susceptible horses but which had not proved infective for either adult or baby mice.

The donors of these blood samples were two laboratory horses Nos. 1513 and 1408 and a horse from the Vryheid district (donor for strain FR). The veterinary histories of the two laboratory horses showed that these animals had each received three annual injections of vaccine prior to their breakdown infection. The precise history of the Vryheid horse is not known although it had been reported by the State Veterinarian, Vryheid, who submitted the sample that the horse had been immunized several times. The blood samples from horses Nos. 1513 and 1408 had been injected into susceptible horses which had reacted and been bled during this reaction and so both these strains were available in two blood samples; the one sample from an immunized horse (non-infectious or "neutral" for the mouse) and the other from a susceptible horse (infectious or "active" for the mouse). The FR virus used in these experiments was that contained in the blood sample obtained directly from the breakdown case.

Centrifugation

The O.C.G.-blood mixture obtained from horse No. 1408 was centrifuged in an anglehead centrifuge for half an hour at 3,000 r.p.m. to remove gross particulate matter and the supernatant centrifuged for half an hour at 15,000 r.p.m., thus applying a centrifugal force sufficient to sediment the particles of horsesickness virus (Polson and Alexander, 1945). The sediment obtained was resuspended in 10 per cent serum-saline and again centrifuged for half an hour at 15,000 r.p.m. This sediment was resuspended in serum-saline and was used to inject baby mice. In this manner it was hoped that sufficient antibody would be removed to allow infection of mice to take place. The attempt was unsuccessful. This procedure was repeated with the O.C.G.-blood mixture obtained from the horse in the Vryheid district with a similar negative result.

Dilution

Infective O.C.G.-blood obtained from horse No. 1513 was injected into families of baby mice in the following dilutions: 1/100, 1/500, 1/1000, 1/10,000. No deaths which could be attributed to horsesickness occurred in any of the mice. Infective blood (Strain 1513) obtained from a reacting susceptible horse was subsequently titrated in baby mice and the infective titre proved to be only 100 LD50. In view of the slight dilutions such low titres would allow further attempts at activating virus by this method were abandoned.

Blind passage

A 1/10 dilution of O.C.G.-blood obtained from horse No. 1513 was injected intracerebrally into two families of baby mice. Brains from groups of apparently healthy mice were harvested on the fifth and seventh day for further passage into baby mice. The second passage mice and the remaining mice of the first passage were observed for several weeks but no mortality occurred.

Result

From these three experiments it did not appear possible to adapt the viruses contained in the above blood mixtures to mice even if techniques involving centrifugation, dilution or blind passage are used.

Following the above failures attempts were made to propagate these " neutral " viruses in an animal, other than the horse, which is susceptible to viscerotropic virus and thence to proceed with neurotropic adaptation in the mouse.

The dog

Two dogs were given intravenously 1 ml. of O.C.G.-blood mixture from horse No. 1513 and another two dogs were given 1 ml. of O.C.G.-blood obtained from a susceptible horse infected with strain 1513. The results of these injections are given in Table 4. One of the dogs, No. 2, injected with " neutral " virus showed only a doubtful febrile reaction and was discarded from the experiment. The other three all reacted and were bled into O.C.G. at what was thought to be the height of the reaction and these blood mixtures were injected into families of baby mice.

TABLE 4

Injection of dogs with blood obtained from immunized and susceptible horses

Dog	State of Virus	Reaction in Dog	Injection of Mice
1	" Neutral "	Sick 4th day. Temp. for 4 days	Unsuccessful.
2	" Neutral "	Doubtful reaction	Not attempted.
3	" Active "	Sick 3rd day. Temp. for 4 days	Successful.
4	" Active "	Slight temp. 3rd and 6th day	Unsuccessful.

Result and discussion

It will be seen that infection of mice occurred with blood from only one dog. No. 3, which received blood from the susceptible horse. The reason for the failure to isolate virus from dog No. 1 which was clinically sick when bled on the fourth day and from dog No. 4 bled at the same time cannot be explained. No pre-inoculation sera were available from these dogs for testing for antibody to determine whether they had experienced earlier infections with horsesickness virus but tests on sera from other dogs in enzootic areas indicate that natural infection in dogs is not common. Rather than antibody having interfered with isolation of virus from these dogs it is likely that the cause was the low titre of virus in the peripheral blood. Due to a shortage of dogs no further attempts were made in this animal but it would appear that the dog may be used to isolate these " neutral " viruses. Probably spleen material harvested during the febrile reaction would be more suitable than blood to infect mice.

The Ferret

To determine whether ferrets are susceptible to viscerotropic virus each of two ferrets was given 1 ml. of O.C.G.-blood obtained from a susceptible horse reacting to infection with strain 1408. One ferret reacted with a temperature of 107·8° F on the morning of the fourth day after injection. That afternoon the temperature had dropped to 103·6° F and from blood collected at this time virus was isolated in baby mice. The other ferret showed no definite febrile reaction and no blood was collected from this one.

As it was apparent that ferrets are susceptible to viscerotropic virus attempts were made to infect these animals with blood samples containing " neutral " virus. Each of a group of four ferrets was given 1 ml. of blood containing " neutral " 1513 virus and another group of four received blood containing " neutral " FR virus. Injections were either by the intracardial (6 ferrets) or intraperitoneal (2 ferrets) route. Blood or spleen were harvested from the ferrets at various times after infection and injected into baby mice. The reactions of the ferrets, and the infectivity for mice of materials collected are shown in Table 5.

Results and discussion

Neither ferret injected intraperitoneally showed any apparent reaction nor could virus be isolated from them. The other six ferrets, injected intracardially showed a definite temperature reaction and virus was isolated from each one. The duration of the febrile reaction varied from two to four days. No other clinical symptoms were evident although one ferret was off its feed for one day. Blood collected during the febrile reaction and spleen collected either during the febrile reaction or on the following day, proved infective for mice. The incubation period in baby mice injected with ferret material varied from four to eleven days and usually all the injected mice died.

TABLE 5

The isolation of virus in mice from ferrets injected with " neutral " virus

Ferret	Virus	Route of Injection	Reaction in Ferret	Material Collected	Infection in Mice
1	1513	IP.....	None.....	Spleen 10*.	—
2	1513	IP.....	None.....	Blood 5....	—
				Spleen 10...	—
3	1513	I. Card....	Temp. 4th to 7th day†.....	Blood 4....	+
				Spleen 10..	—
4	1513	I. Card....	Temp. 5th to 9th day. Off feed 8th day..	Blood 5....	+
				Spleen 9...	+
5	FR..	I. Card....	Temp. 4th day.....	Spleen 6...	+
6	FR..	I. Card....	Temp. 4th and 5th day.....	Blood 4....	+
				Blood 5....	+
				Spleen 6...	+
7	FR..	I. Card....	Temp. 4th and 5th day.....	Spleen 6...	+
8	FR..	I. Card....	Temp. 4th day.....	Spleen 5...	+

* = Number refers to days after injection that material was collected.
† = Refers to days after injection.
IP = Intraperitoneal.
I. Card = Intracardial.
— = Infection of mice unsuccessful.
+ = Infection of mice successful.

From this experiment it was evident that the ferret could be used to isolate virus from blood samples which were not infective for mice. The former animal has subsequently been used on several occasions to isolate virus. Subsequent work showed that the infection in the ferret was often inapparent with no febrile reaction. From these ferrets which failed to show any reaction virus usually could be isolated in mice from a blind passage of spleen harvested on the sixth day after infection.

Certain experiments conducted in an attempt to show that it was the antibodies in blood from immunized horses which prevented isolation of the virus in mice were inconclusive. However, it is felt that this is the most likely explanation. Neutralization tests on sera obtained from horses Nos. 1513 and 1408 showed that they possessed high titre antibody at the time of their illness.

Isaacs (1948) has reported that an influenza virus-serum mixture can be prepared which will not show multiplication of virus when injected intranasally into mice, but will produce infection in the more sensitive chick embryo. It is probably a question of the degree of neutralization together with the susceptibility of the particular host-cell system. Results obtained by Tyrrell and Horsfall (1953) during quantitative studies of serum-virus mixtures with Newcastle disease, influenza A and bacterial virus, T. 3, showed that the route of inoculation and the host-cell system used to test for the presence of unneutralized virus had a striking effect on the result obtained. With influenza A virus there appeared to be an inverse relationship between the degree of susceptibility of a host to infection with the virus and the neutralizing titre of a serum as measured in that host. These results suggest that the blood samples from reacting immunized horses contain a certain amount of unneutralized virus which is sufficient to infect the horse and ferret but not the mouse.

(*b*) *Effect on the virus of passage in baby mice*

As a result of the passage of the newly isolated strains in infant instead of in adult mice, it became apparent that the modification of the virus during serial passage was not the same in mice of both age groups. It was noticed that after 30 or more passages in infant mice these strains on subsequent propagation in adult mice gave titres much lower than what was usually expected from adult mouse virus. More striking was the fact that the average survival time (from time of infection to death) in adult mice from infection with baby mouse-adapted virus was a day or two longer. For this reason it was necessary to allow an observation period of 14 instead of 10 days in the neutralization tests when baby mouse-adapted strains were used. Unfortunately, no one strain with baby and adult mouse lines at similar passage levels was available for comparative experiments but the results of titrations of strain PMB in adult and baby mice at various stages of passage are illustrative of the titre changes noted. These results are shown in Table 6. Also a detailed protocol of a titration in adult mice of this strain at the 69th baby mouse passage is given in Table 7 to show the abnormally long incubation period of the disease in adult mice.

Result and discussion

The titres in Table 6 illustrate clearly the marked drop in titre obtained when baby mouse virus is propagated in adult mice. As no adult mouse passaged line of this strain was available it is not known what titres would have been obtained

TABLE 6

Titrations of strain PMB at various passage levels

Passage History	Titres	
	In Adult Mice	In Baby Mice
39BM	7·0*	7·0
39BM+22AM	4·3	5·4
39MB+25AM+1BM	6·5	8·0
69BM	6·7	7·8
69BM+1AM	4·5	5·2

* Titres expressed as log. of dilution end-point.
BM = Baby mice.
AM = Adult mice.

TABLE 7

Protocol of titration of strain PMB (69th baby mouse passage) in adult mice

Virus Dilut.	Days after Injection								
	6	7	8	9	10	11	12	13	14
10^{-2}	0/4*	3/1	1/0	—	—	—	—	—	—
$^{-3}$	0/4	2/2	2/0	—	—	—	—	—	—
$^{-4}$	0/4	2/2	2/0	—	—	—	—	—	—
$^{-5}$	0/4	0/4	0/4	1/3	2/1	0/1	1/0	—	—
$^{-6}$	0/3	0/3	0/3	0/3	0/3	0/3	1/2	0/2	0/2
$^{-7}$	0/4	0/4	0/4	0/4	0/4	0/4	2/2	0/2	0/2
$^{-8}$	0/4	0/4	0/4	0/4	0/4	1/3	0/3	0/3	0/3
$^{-9}$	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4

* Mice dead/mice surviving.

with this line when titrated in adults. From experience with many adult mouse passage strains it can be stated that the titres of $10^{-4.3}$ and $10^{-4.5}$ obtained with strain PMB in these experiments are exceptionally low. Most adult mouse-adapted horsesickness strains give a titre of $10^{-5.5}$ to $10^{-6.5}$ when titrated in adult mice and $10^{-6.5}$ to $10^{-7.0}$ in baby mice. From Table 7 it can be seen that adult mice infected with 69th baby mouse passage virus only started dying on the seventh day and the mortality in mice infected with the lower concentrations of virus continued until the twelfth day. This contrasts greatly with the disease produced by adult mouse passaged horsesickness virus in which deaths commence usually on the third or fourth day and rarely occur later than the eighth

day. None of the strains used in the neutralization tests had had more than 42 baby mouse passages and the mortality in these strains at these levels occurred between the fifth and tenth days after injection. Apparently with further baby mouse passage the difference between adult and baby mouse passaged virus becomes more marked. A further difference noted between infant and adult mouse passaged virus was that no mortality could be produced in guinea-pigs infected with baby mouse virus when injected intracerebrally. There is no evidence that the level of baby mouse passage to which the 1953 strains were taken had any effect on the immunological studies. While it is believed that even further passage would not effect such studies, for convenience, it is felt that where strains of horsesickness virus are to be adapted to mice for immunological studies in adult mice the number of baby mouse passages should be limited.

(c) *Effect of mouse passage level on the neutralizability of horsesickness virus strains*

It will have been noticed than many of the viruses investigated were at widely different passage levels. The earlier isolated strains usually were used at a level higher than the 100th and those more recently isolated at about the 30th. As the neutralization tests proceeded the results indicated that in general, higher serum titres were obtained with those strains at the higher levels of passage. Smithburn (1952) has reported that with Semliki Forest virus the neutralizability has also been noted to vary at different passage levels but in the case of this virus low passage level (fifth) virus is more neutralizable than high level (111th). Unfortunately no single horsesickness strain was available at its 100th and 30th passage levels for experiments which would be comparable with the older and recently isolated strains but in an attempt to demonstrate this difference in neutralizability, neutralization tests were carried out using strain 1100 at its third and 43rd passage levels. Virus at these levels was neutralized in a test by 1100 rabbit antiserum. This was the same antiserum which was used in the cross-neutralization series of experiments and was prepared by injections of 1100 virus at between the 30th and 40th passage levels. A similar experiment was carried out using strain Karen at the 24th and 51st passage levels against 1100 antiserum. In these two neutralizations virus dilutions against a constant serum concentration (one fifth) were used. The results are shown in Table 8 and are expressed as the amount of virus neutralized.

Result and discussion

The differences in the quantity of virus neutralized obtained with both strains at the high and low passage levels are too small to be significant. It must be concluded that a difference in neutralizability could not be demonstrated. It is possible that if the difference in passage levels had been greater a significant variation in neutralizability might have resulted.

TABLE 8

Neutralization of strains 1100 *and Karen at different passage levels by* 1100 *rabbit antiserum*

Strain	Passage level	Log. Virus neutralized
1100	3	3·0
1100	43	3·8
Karen	24	1·4
Karen	51	1·6

(d) Stability of mouse-adapted neurotropic virus in rabbit serum

At the commencement of the work with rabbit antisera, preliminary neutralization tests indicated that this animal's serum did not contain any non-specific virucidal components against horsesickness virus. For this reason it was considered unnecessary to obtain pre-inoculation sera from each rabbit before commencement of the hyperimmunization procedure. However, the following experiment was carried out to compare the stability of horsesickness virus in normal unheated rabbit serum with 10 per cent normal horse serum under the same conditions as the neutralization test. Two strains of virus, 1180 and Karen and a pooled serum specimen from three normal rabbits were used in the experiment. Aliquots of the virus strains in 10 per cent horse serum-saline were added to test-tubes containing the rabbit serum undiluted, diluted one fifth, and a control mixture containing 10 per cent horse serum-saline. The mixtures were incubated at 37° C for two hours, held in the refrigerator overnight, and the following day serial tenfold dilutions of each mixture were prepared and injected into mice. The results of these titrations are shown in Table 9.

TABLE 9

Stability of neurotropic virus in rabbit serum

Virus Diluent	Virus Titre	
	1180	Karen
Rabbit Serum, undiluted	4·8	4·8
Rabbit Serum, diluted 1/5	5·5	4·5
10 per cent Horse Serum-Saline	5·5	5·0

Result and discussion

While the control mixture gave the highest titres with both strains, the titres of the other mixtures are not related to the concentration of rabbit serum which indicated that the variations were probably the result of experimental error and did not indicate any detrimental effect on the virus by the rabbit serum. In support of these results, later experience in the neutralization tests revealed no evidence of the presence of non-specific virucidal components in rabbit serum. Also the use of serum dilutions in the neutralization tests would have tended to dilute out any non-specific components which might have been present.

(e) Reproducibility of the neutralizing antibody titre

For reasons which will be considered later, very little significance has been attached to the numerical value of the antibody titres obtained in the neutralization tests. However, it was thought desirable to determine to what degree these titres were reproducible. Four series of fivefold dilutions of L rabbit antiserum over the range 1/5, 1/25, 1/125, 1/625, and four series of similar dilutions of KA rabbit antiserum were prepared. KA antiserum was selected as it was known that KA and L strains were closely related antigenically. To these dilutions the estimated test dose of 100 LD50 of L virus was added. The mixtures were then held under the usual conditions of the neutralization test, viz. 2 hours at 37° C and overnight at 4° C, before injection into mice. The antibody titres obtained with each series of dilutions of each antiserum are shown in Table 10.

TABLE 10

Neutralization of strain L by L and KA antiserum

Serum	Serum Titre
L	416*
L	340
L	416
L	384
KA	201
KA	186
KA	278
KA	340

* Serum titre expressed as reciprocal of Serum dilution end-point.

Results and discussions

These results show that the variation in antibody titre was extremely small and the highest variation was less than a twofold difference. However, the protocols of neutralization tests with horsesickness virus show that the death or survival of mice is often very irregular. In many instances with positive sera there is a tendency for some mortality to occur in mice injected with the higher concentrations of serum resulting in some difficulty in the calculation of a 50 per cent serum dilution end-point with the Reed and Muench formula. This factor should be considered in the evaluation of neutralization experiments with horsesickness virus. For this reason the results of the present experiment cannot be applied too rigidly.

(*f*) *Effect of heating on neutralizing power of rabbit antisera*

Although it was known that heating had no effect on the ability of horse antisera to neutralize horsesickness virus (Alexander, 1935), it was considered advisable to determine whether or not any thermolabile components in rabbit sera enhanced the neutralizing power of these sera. Accordingly, neutralizations of L virus by unheated and heated L rabbit antiserum were carried out. The treated serum was heated at 56° C for half an hour. In this experiment, as well as the two following, the range of serum dilutions was increased to 1/3125 so as to be sure of obtaining a clear 50 per cent serum dilution end-point. The results are shown in Table 11.

TABLE 11

Effect of heating on neutralizing power of rabbit antiserum

Serum Treatment	Neut. Titre
Heated	625
Unheated	278

Result

Heating had no apparent effect on the ability of rabbit antiserum to neutralize horsesickness virus.

(*g*) *Effect of freezing and thawing on neutralizing antibodies*

With the intention of avoiding the repeated freezing and thawing of the antisera these were stored in 0·4 ml. amounts in a deep-freeze. This volume was just sufficient for one neutralization test. However, despite this precaution, some lots of sera, were unavoidably frozen and thawed three or four times and so the effect of this procedure on the antibodies was determined. A sample of 114 antiserum was frozen in a solid carbon dioxide-alcohol bath and then thawed in a water-bath held at 37° C. This procedure was repeated five times. Neutralization tests with this serum and an untreated control were then carried out against the homologous virus. Table 12 shows the results.

TABLE 12

Effect of freezing and thawing on neutralizing antibodies

Serum Treatment	Neut. Titre
Frozen and thawed	931
Untreated	1242

Result

Freezing and thawing five times had no apparent effect on the neutralizing antibodies of rabbit antisera.

(*h*) *Effect on the serum titre of the amount of virus used in a neutralization test with horsesickness virus and rabbit antisera*

Owing to the difficulty of arriving at the amount of the test dose of virus in the neutralization test with sufficient accuracy an experiment was conducted to observe the effect of this variable on the value of the antibody titre. The dilution of virus in each neutralization run was determined from the results of previous titrations and was calculated to provide from 50 to 100 LD50 of virus in each dose. However, the actual amount of virus in all the tests varied from 20 to 320 LD50. The effect of this variation was determined in a series of neutralization tests using graded doses of strain L against a series of identical dilutions of L antiserum. From a dilution of 1/10 of the L stock antigen two-fold dilutions were prepared up to 1/320 and quantities of virus from each dilution were added to one of the series of serum dilutions. The LD50 of virus present in the 1/80 dilution was determined by titration in mice and a value of 100 LD50 was obtained. The antibody titres obtained with each series of serum dilutions are shown in Table 13.

TABLE 13

Neutralization of graded doses of virus by an identical series of serum dilutions

Virus Dilutions	Virus LD50	Serum Titre
1/10	800	55
1/20	400	278
1/40	200	509
1/80	100*	522
1/160	50	1,125
1/320	25	744

* Value obtained by titration in mice.

These results are presented graphically in Fig. 1. The logarithm of each serum dilution end-point has been plotted against the logarithm of the quantity of virus neutralized. It has been assumed that a linear relationship exists between the two variables and a line has been drawn to fit the points (Horsfall, 1939; Horsfall and Lennette, 1941; Tyrrel and Horsfall, 1953).

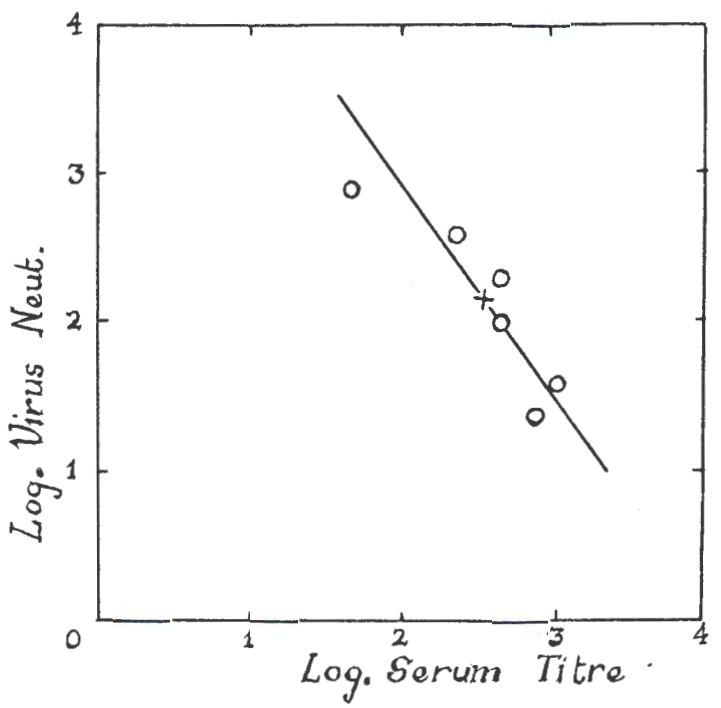

FIG. 1.—Slope of neutralization line with horsesickness virus.

Results and discussion

The slope of the neutralization line has been calculated by the method of least squares and a value of 1·4 was obtained. This means that a variation of 1·0 log. unit in the quantity of virus used results in a shift in the serum dilution end-point of 0·7 unit. From these results it is evident that a variation of between 20 and 320 LD50 of virus will result in at least a tenfold difference in serum titre. While it is not possible to determine accurately the slope of the neutralization line without numerous replicate experiments (Horsfall, 1939) the present experiment is sufficient to indicate that the slope with horsesickness virus is not steep and in comparison with many other viruses (Tyrrel and Horsfall, 1953) relatively small differences in virus concentration result in significant differences in serum titre.

(i) The cross-neutralization of the vaccine strains and sixteen recently isolated strains of horsesickness virus

As mentioned earlier, the eight vaccine strains had been selected for incorporation in the vaccine on the results of extensive neutralization tests in mice. These tests had involved numerous strains isolated and adapted to mice in this laboratory since 1933 and in the vast majority of cases the virus donors were immunized horses. As these eight strains had been selected mainly for their antigenic dissimilarity to each other it was evident that in cross-neutralization tests designed to classify new strains it would be advisable to include these eight strains in the series as a comparative group. The other strains included were sixteen strains isolated between 1949 and 1953 from breakdown cases in immunized horses. It was hoped that these tests would be sufficiently comprehensive to enable an evaluation to be made of the suitability of rabbit antisera in neutralization tests with horsesickness virus. As the antisera became available the neutralizations were carried out and the results are shown in Table 14. Those strains which were cross-reactive are shown in Table 15. Table 16 groups the strains which showed reciprocal neutralizations into antigenic types which have been designated with Arabic numerals as suggested by Dalldorf (1953) for the classification of immunological types of Coxsackie virus.

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TABLE 14.—*Results of cross-neutralizations, expressed as reciprocal of serum titre*

Year Isolated	Serum..... / Virus.	1180	KA	OD	VRY	VH	L	114	A501	RHOD	454	1513	1408	MFK	FR	ES	1100	1582	PMB	VR	P2	P1	1096	2627	ERM
1933	1180.....	625	—	6	—	—	—	—	229	—	—	152	32	—	—	—	—	—	—	—	—	—	—	340	—
1937	KA.....	—	625	—	—	—	625	—	—	—	—	—	—	—	—	—	7	—	—	—	—	—	—	—	—
1937	OD.....	7	—	213	—	—	—	—	5	—	—	—	—	—	—	—	—	—	—	—	—	—	—	5	—
1938	VRY.....	—	—	5	625	—	—	—	—	364	625	—	—	99	—	—	—	—	—	—	—	—	—	—	625
1940	VH.....	—	—	—	—	625	—	—	—	—	—	—	—	—	625	—	—	625	—	625	625	625	625	—	—
1940	L.....	—	453	—	—	—	625	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
1940	114.....	8	—	—	—	—	—	625	—	—	6	5	—	—	—	—	—	—	—	—	5	—	—	—	—
1948	A501.....	625	—	13	—	—	—	—	625	—	—	364	304	5	—	5	5	—	—	—	—	—	—	625	5
1949	RHOD.....	—	—	—	32	—	—	—	—	162	625	—	—	385	—	—	—	—	—	—	—	—	—	—	625
1949	454.....	—	—	—	21	—	—	—	—	156	416	—	—	186	—	—	—	—	—	—	—	—	—	—	625
1951	1513.....	416	—	—	—	—	—	—	625	—	5	278	55	—	—	—	—	—	—	—	—	—	—	327	—
1951	1408.....	156	—	—	—	—	—	—	106	—	—	201	13	—	—	—	—	—	—	—	—	—	—	31	—
1952	MFK.....	—	5	—	171	—	—	—	—	625	625	—	—	201	—	—	—	—	—	—	—	—	—	—	625
1952	FR.....	—	—	—	—	625	—	—	—	—	—	—	—	—	213	—	—	625	5	625	625	625	625	—	—
1953	ES.....	—	—	—	—	—	—	—	—	—	—	—	—	—	—	327	416	—	125	—	—	—	—	—	—
1953	1100.....	—	—	—	—	—	—	—	—	—	—	—	—	—	—	31	162	—	125	—	—	—	—	—	—
1953	1582.....	—	—	—	—	625	—	—	—	—	—	—	—	—	72	—	—	625	—	625	625	625	476	—	—
1953	PMB.....	—	—	—	—	—	—	—	—	—	—	—	—	—	—	327	625	—	291	—	—	—	—	—	—
1953	VR.....	—	—	—	—	65	—	—	—	—	—	—	—	—	303	—	—	495	—	364	213	625	625	—	—
1953	P2.....	—	—	—	—	19	—	—	—	—	—	—	—	—	186	—	—	451	—	625	50	364	125	—	—
1953	P1.....	—	—	—	—	625	—	—	—	—	—	—	—	—	625	—	—	625	—	625	625	625	625	—	—
1953	1096.....	—	—	—	—	625	—	—	—	—	—	—	—	—	625	—	—	625	—	625	625	625	625	—	—
9153	2627.....	156	—	—	—	—	—	—	125	—	—	45	14	—	—	—	—	—	—	—	—	—	—	107	—
1953	ERM.....	—	—	—	45	—	—	—	—	55	476	—	—	327	—	—	—	—	—	—	—	—	—	—	625

Strains included within the square in top left-hand corner are the present vaccine strains. All of them have been included in the vaccine since 1949.
— = Less than 5 or no neutralization.

TABLE 15.—*Cross neutralizations, indicating those strains which were cross-reactive*

	1180	KA	OD	VRY	VH	L	114	A501	RHOD	454	1513	1408	MFK	FR	ES	1100	1582	PMB	VR	P2	P1	1096	2627	ERM
1180...	H		X					X			X	X											X	
KA....		H				X																		
OD....			H					X																
VRY...				H					X	X			X											X
VH....					H									X			X		X	X	X	X		
L.....						H																		
114....							H																	
A501...								H			X	X											X	
RHOD.									H	X			X											X
454....										H			X											X
1513...											H	X											X	
1408...												H											X	
MFK..													H											X
FR.....														H			X		X	X	X	X		
ES.....															H	X		X						
1100...																H		X						
1582...																	H		X	X	X	X		
PMB...																		H						
VR.....																			H	X	X	X		
P2.....																				H	X	X		
P1.....																					H	X		
1096...																						H		
2627...																							H	
ERM...																								H

X = Reciprocal neutralizations.
X's within the top right-hand square show relationship of new strains with vaccine strains.
H = Virus X Homologous antiserum.

Results and discussion

It has been shown that a variation of between 20 LD50 and 320 LD50 in the test dose of virus used in a neutralization test can result in an approximate tenfold variation in serum titre. It was not possible to include all 24 antisera in one neutralization run with a single virus so that in some cases, even titres obtained with the various antisera against the same virus, are subject to this variation.

Other factors which might have resulted in differences in serum titre not related to antigenic structure are the passage level of the virus and variation in the immune response of the individual rabbits. While some variation in titre as a result of widely divergent passage levels might have occurred the experiment described above indicated that the degree of variation due to this cause was very slight and possibly may be ignored.

No experimental work was done on the uniformity of antigenic response of the rabbits to the hyperimmunization procedure used but it must be accepted that variable response accounted for some difference in serum titres. By the use of pooled sera from two rabbits and by keeping the method of hyperimmunization as uniform as possible an attempt was made to limit the effect of this variable. According to Sobey (1954) the use of an inbred strain of rabbits serves to increase the uniformity of antigenic response of rabbits. All the rabbits used for antisera production were from the Onderstepoort strain which has been inbred for many years. Most of the sera gave high titres against the homologous virus, except for 1408 antiserum which gave a titre of 13 against only 35 LD50 of the homologous virus. It is believed that this low value is mainly the result of poor antigenic response of the rabbits.

It was anticipated that a combination of weak antiserum, low neutralizability of the virus and a high test dose of virus might result in false negatives in tests between strains distantly related. To avoid such results a careful watch was kept on the survival time of mice injected with mixtures containing the higher concentrations of serum. For instance, neutralization of 134 LD50 of strain 1100 by ES antiserum gave only a trace of neutralization but on repetition of the test with 80 LD50 a titre of 31 was obtained. However, such poor and erratic neutralization was exceptional.

TABLE 16

Antigenic grouping as indicated by the cross-neutralizations

Type	Vaccine Strains	Recently Isolated Strains
1	1180, A501.....	1513, 1408, 2627.
2	OD.....	—
3	KA, L.....	—
4	VRY.....	RHOD, 454, MFK, ERM.
5	VH.....	FR, 1582, VR, P2, P1, 1096.
6	114.....	—
7	—	ES, 1100, PMB.

At an early stage in the cross-neutralization tests it became evident that a grouping of the viruses would be possible on a qualitative basis, if a reciprocal neutralization of two strains by their antisera, of any degree whatsoever, was accepted as an indication of antigenic relationship. This was fortunate, indeed, in view of the variables present in the test. This also enabled completion of the work with a minimum number of repetitions and made it unnecessary to determine the 50 per cent dilution end-point of sera in excess of 1/625.

The results of the cross-neutralizations shown in Table 14 may be summarized as follows:—

(1) Several strains showed complete reciprocal neutralization as a result of which the grouping of the twenty-four viruses into seven antigenic types is possible.

(2) In those groups with more than one strain all the strains within the group were cross-reactive with each other, with one exception, viz. strain OD was slightly cross-reactive with strains 1180 and A501 but not with the other strains within this group. As no antigenic relationship between the latter strains and OD was evident OD was grouped in a type by itself.

(3) It was not possible to assess the degree of antigenic relationship between strains within the same immunological type (homotypic). However, it is believed that such strains are not antigenically identical and the reasons for this opinion are given later. With several strains of virus the homologous titre was lower than titres obtained with heterologous antisera. It is obvious that these differences are not related to antigenic structure but are the result of the variables discussed above. On the other hand titre differences between homologous and heterologous antisera in some cases probably do represent genuine antigenic variation. For instance, it is fairly certain that the titres obtained with strains A501, 1180 and OD with their antisera are the result of a fairly marked antigenic difference between OD and the other two strains. Similarly, although VH and P2 are Type 5 strains, P2 was very poorly neutralized by VH antiserum. This result was reproducible and is possibly due to antigenic difference between the two strains.

(4) Of the sixteen recently isolated strains, thirteen are related to vaccine strains. If it is accepted that the virus donor animal had been successfully immunized it follows that severe infection can occur in immunized horses from strains of the same immunological type as the vaccine strains.

(5) The remaining recently isolated strains, ES, 1100 and PMB are related to each other but not to any vaccine strain.

(6) Antigenic types isolated as long ago as 1933 are still present in nature and apparently the presence of a large vaccine-immune equine population does not lead to a disappearance of the earlier isolated antigenic types. For instance, type 1 strains have been isolated in 1933, 1948, 1951 and 1953.

(7) In the same locality and during the same outbreak several antigenic types may be isolated from infected horses. Strains 2627, 1582 and 1100 were isolated from different horses on the Institute's farm Kaalplaas within a period of one month. This would indicate that in an enzootic area an outbreak in a restricted locality may originate more or less simultaneously from several different foci. On the other hand, strains P1 and P2 isolated from two horses sick at the same time on the same farm are related antigenically.

(8) During a widespread epizootic, in an enzootic area, such as occurred in Natal and the Transvaal during 1953, it is evident that several antigenic types of virus are implicated. Strains 2627, VR, ERM and ES are representative of different antigenic types (heterotypic) and were isolated during this epizootic from widely distant parts of these two provinces. In view of this, perhaps, it is justifiable to conclude that epizootics within the recognised areas of distribution of the disease originate from the development of conditions favourable to the virus reservoir or insect vector rather than from the appearance and propagation of a single virus strain possessing certain epizootic-producing characteristics. This is in agreement with the observations in Southern Africa where the disease annually makes its appearance simultaneously at widely scattered points over vast tracts of country with very little evidence of spread from one point to another.

(j) *Neutralization of* 18 *other horsesickness strains by selected type antisera*

At the conclusion of the cross-neutralization experiments the typing was extended to include certain other horsesickness strains which were available as mouse-adapted viruses. There were 18 of these viruses and the details of their origin have been given in Table 3. On the results of the cross-neutralizations it appeared that it would be unnecessary to prepare antisera against each of these viruses and the classification of these strains into antigenic types would be possible from neutralization of these strains by seven type antisera, each antiserum being representative of one of the seven immunological types. It was to be expected that each of the eighteen viruses would be neutralized by either one or none of the antisera. No neutralization by any of the seven sera would indicate, either that the virus consisted of a mixture of known types or was of a type not present amongst the viruses included in the cross-neutralization series. The results of these neutralizations are shown in Table 17. Except for type 7, which was not represented amongst the vaccine strains, the type antisera selected for this series of neutralization were antisera to various vaccine strains and were the same rabbit sera used in the cross-neutralizations.

TABLE 17
Neutralization of 18 horsesickness strains by selected type antisera

Virus	Selected Type Antisera						
	1180 1	OD 2	KA 3	VRY 4	VH 5	114 6	1100 7
1157	213	—	—	—	—	-	—
Cedara	—	—	384	—	—	—	—
449	186	—	—	—	—	—	-
O	5	—	5	—	—	625	—
Pirie	—	—	—	—	55	—	—
Galpin	—	—	—	—	224	—	—
Theiler	125	—	—	—	—	—	—
Mataffin	20	625	—	—	—	—	—
Keppel	—	—	—	55	—	—	—
Westerman	—	—	—	—	213	—	—
1397	—	—	—	—	—	364	—
1145	162	—	—	—	—	—	—
30B	476	—	—	—	—	—	—
CA	278	—	—	—	—	—	—
H409	60	—	—	—	—	—	—
Potch	95	—	—	—	—	—	—
1144	228	—	—	—	—	—	—
Karen	—	—	—	—	—	—	625

Results and discussion

Except for strain Mataffin, each strain was neutralized to a significant titre by only one of the seven type antisera, indicating that each of these strains falls within one of the seven antigenic types. That strain Mataffin was neutralized by 1180 antiserum as well as OD antiserum does not invalidate the previous antigenic grouping as it was shown in the cross-neutralization tests that some relationship exists between strains 1180, A501 and OD.

From this experiment it appears possible to classify new horsesickness strains without the necessity of preparing antisera against these strains. As the preparation of these sera is laborious and time-consuming this is a big advantage. Obviously, in those cases where no neutralization is obtained with any of the seven type antisera the preparation of an antiserum against the new strain would have to be undertaken. Table 18 shows the antigenic grouping of all the strains so far examined.

TABLE 18
Shows antigenic grouping of all strains examined

Type	Strains	No. of Strains
1	1180, A501, 1513, 1408, 2627, 1157, 449, Theiler, 1145, 30B, CA, H409, POTCH, 1144	14
2	OD, Mataffin	2
3	KA, L, Cedara	3
4	VRY, RHOD, 454, MFK, ERM, Keppel	6
5	VH, FR, 1582, VR, P2, P1, 1096, Pirie, Galpin, Westerman	10
6	114, O, 1397	3
7	ES, 1100, PMB, Karen	4

It is noteworthy that both strains 30B and CA isolated from *Culicoides* are shown to be horsesickness viruses in that they are both related to a type 1 strain, viz., 1180. Further it can be seen that O strain first encountered over 50 years ago by Theiler is related to strain 114 and 1397, isolated in 1940 and 1949 respectively.

(*k*) *Neutralization of certain horsesickness strains by antisera prepared in ferrets as a result of infection with viscerotropic horsesickness virus*

Although it has been shown by Alexander (1936) that there is a very close antigenic relationship between a mouse-adapted virus and its parent viscerotropic strain it was decided, at this stage, to investigate on a limited scale the validity of the antigenic grouping with rabbit antisera by neutralization tests using antisera prepared in ferrets infected with viscerotropic strains of virus. It was not the intention to prepare antisera against all the virus types and only three strains, viz. PMB, 1513 and FR were selected to infect ferrets. The ferrets were infected by injection of horse blood samples containing the viscerotropic form of these three strains. Pre-inoculation sera were collected from each ferret. The ferrets were bled for antiserum one month after the febrile reaction. Neutralization tests using these sera were then carried out with several selected mouse-adapted strains of virus. The strains were selected so as to include strains of all seven antigenic types. The results are shown in Table 19.

Results and discussion

Except for some slight neutralization of strain KA by PMB antiserum the results are in close agreement with those obtained with rabbit antisera and the indications are that ferret sera may be of some value in neutralization tests with horsesickness viruses. It should be noted that the antigenic relationship between KA and PMB revealed in this experiment, is evidence that the type 7 strains are horsesickness virus strains.

TABLE 19.

Neutralization of certain horsesickness strains by ferret antisera

Virus	Immunolog. Type of Virus	Serum		
		PMB	1513	FR
PMB	7	148	—	—
1513	1	—	222	—
FR	5	—	—	322
Karen	7	385	—	—
1180	1	—	625	—
VH	5	—	—	393
114	6	—	—	—
VRY	4	—	—	—
KA	3	12	—	—
OD	2	—	—	—

Within a few weeks of arrival of a virulent strain in the laboratory a ferret antiserum could be made available and this would mean a considerable saving in the time usually required to type a strain since neutralization tests with this serum against mouse-adapted strains of known antigenic type could be carried out.

Also it is evident that within the limits of the test these neutralizations indicate that there has been no apparent antigenic variation in strains PMB, 1513 and FR following neurotropic adaptation in the mouse. This result is in agreement with the earlier observations on the antigenic stability of the mouse-adapted horsesickness viruses when horses immunized with neurotropic virus were found to be solidly immune to the virulent parent strain.

Ferret antisera would be of value as a check on the identity of laboratory passaged strains.

(*l*) *Immune status of some immunized horses reacting to natural infection*

In order to investigate the immune status of immunized horses reacting to natural infection an effort was made to obtain acute phase sera from several breakdown cases. Six of these sera were obtained from immunized horses (Nos. 1513, 1408, 1100, 1582, 1096, 2627) on the Institute's farm Kaalplaas and two from Percherons on a farm near Pretoria. At the same time that blood was collected from these horses for serum a further blood sample was collected in O.C.G. for virus isolation and from these samples a virus was isolated in each case. These viruses were included in the cross-neutralization series and only strain 1100 proved to be antigenically different from the vaccine strains. The veterinary histories of these eight horses are shown in Table 20.

All these horses, except No. 2627, had been immunized several times with polyvalent neurotropic vaccine containing the eight vaccine strains. Horse 2627 was one of a group of susceptible horses introduced from Wakkerstroom and was immunized for the first time on 17.3.53 and died of horsesickness 22 days later.

Due to the short interval between immunization and infection this horse should not be considered as a true breakdown in immunity. Despite this, it was decided to include this serum in the experiment as a control serum. Horse 1582 in addition to two inoculations of vaccine had been challenged by inoculation with virulent viscerotropic strains FR and MFK and no reaction had resulted. Neutralization tests with these sera against the vaccine strains were carried out and in addition a neutralization test was done of each serum and the corresponding virus, e.g. Virus 1513 and Serum 1513. The results are given in Table 21.

TABLE 20

History of immunized horses reacting to natural infection

Horse.....	1513	1408	1100	1582	1096	2627	P2	P1
Locality.....	Kpl	Kpl	Kpl	Kpl	Kpl	Kpl	Pta	Pta
No. inoc. vaccine...	3*	4	5	2	8	1	3	3
Virulent strains.....	—	—	—	FR 18/7/52 MFK 7/8/52	—	—	—	—
Date of breakdown	12/3/51	30/4/51	9/3/53	25/3/53	28/3/53	8/4/53	16/3/53	16/3/53
Outcome of sickness	D	R	R	D	D	D	?	?

Kpl = Kaalplaas; Pta = Pretoria; D = Died; R = Recovered.
* Most inoculations were given at intervals of one year.

Results and discussion

The neutralization tests showed that all the horses, except 2627, possessed high titre antibody against each vaccine strain at the time of infection. As all the horses were exposed to possible natural infection during earlier horsesickness seasons it is impossible to ascertain to what extent this antibody was the result of the various inoculations of vaccine. Whatever produced this antibody the important fact is that the neutralization test in mice has shown that seven of these horses possessed antibody against all the vaccine strains as well as a variable amount against the infecting strains at the time of their illness.

Serum from horse 1100 gave a titre of only 8 against the aberrant strain 1100 although the horse recovered. Serum P2 also gave a low titre against the infecting virus despite the fact that P2 virus is closely related to the vaccine strain VH. Serum 2627 neutralized strains KA, L and 114 only. This antibody might have been the result of the injection of vaccine 22 days earlier. The absence of antibody against the other vaccine strains is probably due to the short interval between immunization and the collection of the serum. It is interesting to note that this serum neutralized only strains KA, and L to significant titre, two strains which were shown to be antigenically related to the rabbit sera.

Except for serum 2627, P2 and 1100, there was a strong neutralization by these horse sera of the virus which caused the breakdown reaction. Although this was to be expected in view of the antigenic relationship shown to exist between these strains and some vaccine strains the lack of immunity of each horse is not easily explained. As it is known that the immunity in horses to the homologous virus is always solid the probable explanation is that *not* all strains within the same antigenic type are immunologically identical. For instance,

TABLE 21

Neutralization tests using sera collected from breakdown cases in horses

Serum	1513	1408	1100	1582	1096	2627	P2	P1
Virus.								
A501	512	512	512	512	512	0	512	512
KA	512	405	512	512	255	26	405	512
VH	512	512	512	512	512	0	512	512
1180	512	512	222	512	222	0	512	512
OD	101	405	512	512	512	0	32	512
L	255	512	512	512	255	80	512	512
114	512	512	512	512	512	8	512	512
VRY	40	255	202	63	255	0	32	255
1513	512	—	—	—	—	—	—	—
1408	—	512	—	—	—	—	—	—
1100	—	—	8	—	—	—	—	—
1582	—	—	—	512	—	—	—	—
1096	—	—	—	—	512	—	—	—
2627	—	—	—	—	—	0	—	—
P2	—	—	—	—	—	—	16	—
P1	—	—	—	—	—	—	—	405

although it has been shown by means of the neutralization test using rabbit sera that strains A501, 1180, 1513, 1408 and 2627 are antigenically related and accordingly were grouped in the same antigenic type there are no grounds for concluding that these five strains are immunologically homogeneous. It is noteworthy that horse 1582 was solidy immune to the virulent viscerotropic form of strain FR and yet succumbed to infection a few months later with strain 1582. With rabbit antisera strains FR and 1582 were grouped as homotypic. It would appear that slight antigenic differences do exist between strains grouped within the same antigenic type and that these differences are sufficient to result in occasional breakdowns in immunity. This explanation is supported by the observations of Theiler (1915) who, as a result of his considerable experience with cross-immunity tests in horses with virulent viruses stated that after he had collected many different strains from all parts of the country he found that practically any virus could break down the immunity given by any other virus. In view of the large number of strains which Theiler worked with it is reasonable to assume that many of his strains would have been of the same antigenic type if classified by means of the neutralization test using rabbit sera.

The neutralization tests with these horse sera emphasize that the presence of neutralizing antibody in the serum of a horse against any particular mouse-adapted horsesickness virus does not mean that the horse is necessarily solidly immune to the virulent parent strain but can only signify that some degree of immunity exists. A somewhat similar relationship between serum antibodies and immunity has been reported by Salk, Menke and Francis, (1945) with influenza virus. These authors showed that infection sometimes occurs in persons with high antibody levels against the infecting virus.

IX. General discussion

From an analysis of the results of the neutralization tests with rabbit antisera it is evident that the value of horsesickness antisera prepared in rabbits lie in their group or antigenic type specificity. The degree of neutralization of strains by heterotypic antisera occurred only to an insignificant extent, while on the other hand there was complete cross-reactive neutralization between homotypic strains. It is believed that the methods used have enabled a valid immunological classification to be made of all the strains examined. A comparison between the results obtained with these rabbit antisera and certain neutralization tests carried out by Alexander (1949) in which he used horse antisera reveals a close similarity. For instance it was shown that the current horsesickness vaccine issued from Onderstepoort contains all antigenic types known to have been isolated before the present study was undertaken. As mentioned earlier these vaccine strains were selected on the basis of mouse neutralization tests with horse antisera.

A comparison between the antigenic grouping with the neutralization test using rabbit antisera and that based on the rate of virus inactivation by ultraviolet irradiation as reported by Polson and Dent (1950) shows certain discrepancies although some correlation is evident. Strains with markedly different rates of inactivation showed divergent antigenic structure with the neutralization test.

For immunization purposes it is important to know the degree of relationship between the immunological grouping of strains based on the mouse neutralization test and immunity in the horse and mule. The obvious answer lies in a series of cross-immunity tests in horses with virus strains of known immunological type. For such tests to be of any value large numbers of horses would have to be used. With the present facilities available this is impossible owing to the expense involved. Nevertheless, it would appear that a reasonably sound assessment of the relationship between the present grouping and immunity in the horse and mule can be made on the available evidence.

It is certain that strains classified in the present study as heterotypic possess a certain amount of common basic antigenic structure. This is evident from the small amount of heterotypic neutralization encountered in the tests with the rabbit antisera and more clearly from the results of neutralization tests with horse antisera. For instance, Alexander (1935) showed by means of neutralization tests using antisera obtained from horses which had received a single injection of neurotropic virus, that strains O and 449 are antigenically dissimilar, but further neutralization tests using a horse antiserum produced as a result of five injections of neurotropic strain 449 revealed a distinct antigenic relationship between these strains. With the rabbit antisera, strain O was grouped as a Type 6 strain and 449 as a Type 1.

It appears that the hyperimmunization of the horse with strain 449 revealed minor antigenic components common to O and 449 which were not evident with the rabbit antisera or with the horse antisera prepared from a single injection. It is interesting to note that strain 449 was isolated from a fatal case of horsesickness that occurred in a horse that had been hyperimmunized some months previously against strain O (Alexander and du Toit, 1934). That a certain amount of antigenic overlap existed between strain 449 and O was shown later by Alexander and du Toit (*loc. cit.*) from cross-immunity experiments in mules in which immunization against the one strain produced an undoubted but not solid immunity against the other. Furthermore, it is doubtful whether the relative success achieved by Theiler with the serum-virus method of immunization, in which only one immunizing strain was used in mules and two in horses, would have been possible without this antigenic relationship between heterotypic strains.

The results obtained with the rabbit antisera have not elucidated the degree of antigenic relationship between strains classified as homotype but the evidence indicates that these strains are not antigenically identical.

It has been shown that in immunized horses, horsesickness sometimes occurs due to infection with strains of virus which are homotypic to one or more of the vaccine strains. While it may be argued that these infections occur only in horses inadequately immunized with poor quality vaccine, this contention has been shown to be unsatisfactory in many cases, for examination of acute phase sera obtained from several immunized horses during infection with virus strains shown to be homotypic to vaccine strains showed that many of these horses possess high titre antibody against all vaccine strains at the time of infection. From this it would appear that the most logical explanation of failures in immunity as a result of infection with homotypic strains is that antigenic differences exist between such strains. In other words the neutralization test does not reflect the true state of immunity in the horse. This species is apparently a more sensitive test animal than the mouse for detecting antigenic differences between strains within a single type. This sensitivity of the horse is evidenced also by the fact that Theiler's cross-immunity tests showed that a solid immunity in the horse develops only to the homologous virus at more or less the same equine passage level whereas neutralization tests in mice with neurotropic virus reveal numerous strains with an apparent indistinguishable antigenic relationship. From all the evidence it is reasonable to assume that slight antigenic differences exist between strains within a single type and these differences are sufficient to result in occasional deaths in immunized horses.

While this explanation is the most likely one to account for breakdowns with homotypic strains in immunized horses two further possibilities should be considered. These are:—

(*a*) With mouse-adaptation antigenic changes occurred in the virus, which resulted in the appearance of a strain similar to one of the vaccine strains;

(*b*) the horse was infected with more than one strain and only one of the strains became mouse-adapted.

In considering the first point, it is obvious that if results of antigenic studies with laboratory propagated viruses are to have any practical value, it is important that there should be no tendency for spontaneous antigenic variation to occur at the time of isolation or during the various laboratory manipulations to which the viruses are subjected. The antigenic variation in laboratory propagated influenza viruses (Hirst, 1947; Francis, 1947; Chu, Andrews and Gledhill, 1950) indicate that such an occurrence is more than a theoretical possibility. Chu, Andrews and Gledhill (*loc. cit.*) state that although there is no doubt that different antigenic varieties of influenza virus do occur in nature, it is necessary for a proper evaluation of these differences, that as few egg passages as possible be made and in no case should a virus passed in mice or ferrets be used for antigenic studies. According to Röhrer (1953) certain field and laboratory observations on foot and mouth disease virus by Ramon in France, Geiger and Demnitz in Germany and Kindjokaw, Bajndinaw, Fillipowitsch and Nikonowa in Russia have shown that one antigenic type may change into another type. Apparently the infection or passage of virus in immune animals favours the appearance of antigenic changes in this virus.

Whether horsesickness viruses also possess this antigenic lability is a question which should receive attention in any evaluation of antigenic studies with these viruses in the laboratory. According to Theiler (1915) some modification occurred in two virulent horsesickness virus strains he passaged in horses. When discussing these changes Theiler states " of Tzaneen strain we possess two varieties, a virulent one and an attenuated one. Both qualities have been obtained by the same process viz., by passage from one animal to another . . . . in the case of Tzaneen strain a lower generation (passage level) will break the immunity conveyed by a higher generation and in the case of the ordinary 'O' strain the higher generation will break that conveyed by a lower ". Elsewhere in the same report Theiler states, " a horse that reacted to the minimum test dose of at least 2,000 fatal doses of ordinary virus cannot be infected with horsesickness if we use the same virus at a dose of a million times or even more. The horse will not show the slightest reaction . . . Notwithstanding the presence of antibodies in the bloodstream, the horse can contract the disease again, when a virus of a higher generation of the same strain is used." From these statements it appears that some antigenic variation may occur in virulent viscerotropic horsesickness virus when passaged in horses. While it may be contended that these changes did not involve mutations since it is possible that the original isolates consisted of a mixture of two or more strains, one of which was lost during serial passage, this contention is not in agreement with the observation that low passage O did not give solid protection to the high passage virus. However, it is doubtful whether such antigenic changes, if they did occur, can be considered purely as mutations. For it appears equally possible that changes in antigenic pattern may be the result of selection or re-arrangement and recombination processes brought about by changes in the environment such as passage in partially immune horses, as has been suggested might occur sometimes with influenza virus variations (van Magnus, 1953). A factor which may have facilitated antigenic changes in Theiler's viruses was that he had no means of determining the susceptibility of the horses he used for passage; it is not unlikely that some of his horses possessed a partial immunity against the strains passaged. Further evidence pointing to the antigenic lability of horsesickness virus is the fact that so many antigenically different virus strains occur in nature. Discussing these changes Theiler (1921) states that " under natural conditions some such process must be expected to take place; it is otherwise difficult to understand how there can exist so many different strains of horsesickness virus breaking the various immunities."

Against this evidence of possible antigenic change in viscerotropic horsesickness virus considerable past experience with neurotropic mouse-adapted horsesickness strains indicates that the neurotropic viruses are antigenically stable as horses immunized with them have always been solidly immune to the virulent parent strains by challenge-inoculation (Alexander, 1954*d*).

Also, an attempt to produce an antigenic variant of a mouse-adapted strain by serial mouse passage in the presence of homologous immune serum was unsuccessful (Polson and van Rooy, 1951). Furthermore, the results of the neutralization tests with ferret antisera obtained as a result of infection with viscerotropic strains, indicate that within the limits of the test used the mouse-adapted strains, PMB, 1513 and FR are antigenically identical to their virulent parent strains. Additional evidence of the antigenic stability of horsesickness virus is that in the few instances in which horses have been immunized with egg-adapted horsesickness viruses these horses have been solidly immune to the homologous virulent strain when challenged (Alexander, 1949; McIntosh, 1954*b*).

It is possible that the difference observed in the behaviour of viscerotropic and neurotropic forms of horsesickness virus is genuine and that the viscerotropic virus is antigenically labile and the neurotropic virus stable. Alternatively, the passage of the viscerotropic form in partially immune horses possibly provided a more favourable environment for the appearance of variants. While antigenic change in influenza virus has occurred during passage in eggs as well as in ferrets and mice these changes are apparently more liable to occur in the latter two species than in eggs (Chu, Andrews and Gledhill, 1950). It has been postulated by Gordon (1950) that the undeveloped capacity for immune response in the embryonic egg tissue is a factor in the apparently greater genetic stability of egg-cultured influenza virus. Whatever the position may be in regard to viscerotropic horsesickness virus it is extremely unlikely that antigenic variation of neurotropic virus is an explanation for breakdowns in immunized horses as a result of infection with virus strains antigenically similar to the vaccine strains. Even assuming that antigenic changes had occurred during mouse-adaptation with some strains it is likely that these changes would have been slight and hence not detectable with the neutralization test.

The second point to be considered is whether exposed horses become infected at the same time with more than one strain of horsesickness virus. In an evaluation of antigenic studies on strains isolated from breakdown cases in immunized horses it is important to know that the particular strain isolated was the cause of the symptoms, for it is possible that where horses are exposed to massive infection with more than one strain such as occurred on the farm Kaalplaas in 1953 a horse may be infected simultaneously with several different strains.

While it is known that injection of horses with several neurotropically attenuated strains probably results in infection with each strain in that neutralizing antibody against each strain develops later, there is very little positive evidence to suggest that horses in nature experience concurrent infection with more than one virulent strain. When Theiler (1909) attempted the immunization of mules with what he called " Composite virus " with a view to the production of a wide polyvalent immunity he found that a percentage of animals reacted later to challenge inoculation with the same "composite virus ". This virus consisted of a mixture of strains, usually three, and they were injected simultaneously. As we know that the immunity in horsesickness to homologous viruses is always solid, these breakdowns indicate that certainly not all the injected strains succeeded in infecting all the mules. On the other hand it is not possible to conclude that infection resulted with only one of these strains.

Furthermore, the fact that it has been observed that neurotropic strains always confer a solid immunity to the viscerotropic parent strains indicated that the virulent strains were single antigenic entities; if this were not the case, continued passage in the mouse would very likely have resulted in the eventual emergence of a single antigenic type which would not have conveyed solid protection to the original virulent mixture.

That horses may be infected artificially with more than one strain at the same time has been reported by Alexander (1935). He injected a mixture of six viscerotropic strains into a susceptible horse and from the blood of this horse a virus was isolated in mice. From this fixation two strains emerged viz., 464A and 464B, which were characterized by a slightly different period of incubation and course.

It is perhaps significant that all the viruses examined in the present investigation gave no indication that they consisted of a mixture of viral types. If mixtures had been present, instances of unilateral neutralization would have occurred. For example, if a mouse-adapted horsesickness strain consists of a mixture of two heterotypic strains " A " and " B ", its antiserum would neutralize viruses " C " and " D " from two different antigenic types, but neither antiserum of " C " or " D " strains alone would neutralize the virus " AB ".

The neutralization tests with the three ferret antisera indicate that the horse blood specimens, containing the viscerotropic virus strains, which were used to infect these ferrets contained only one strain. For if this had not been the case these antisera would have neutralized strains from different antigenic types.

In regard to isolates consisting of viral mixtures, the work of Contreras, Barnett and Melnick (1952) on the typing of Coxsackie viruses may be cited. These authors typed 232 strains of Coxsackie virus isolated from patients, flies and sewage. In all isolates from patients, which totalled 95 cases, only single antigenic types were present in contrast to isolates from flies and sewage in which multiple antigenic types were often present. From this it would seem that although several antigenic types of Coxsackie virus may be prevalent in a community, individuals are infected with only one strain at a time. However, it is obvious from this work that multiple infection of mice is possible since in the isolates from sewage and flies antigenically distinct strains were carried for a limited number of passages in the same mice.

Dual infections by two viruses causing separate diseases are well-known and such infections have been reviewed by Henle (1950). But as this author points out, with one possible exception, the viruses infected " two different organs or tissues, two different cells within one organ or tissue, or two different structures within one cell ". The exception was the work of Syverton and Berry (1947) which showed that individual cells of the Shope rabbit papilloma which presumably contain the papilloma virus, may be superinfected with B virus and myxoma virus. A search through the literature has revealed only one instance of a natural dual infection with two distinct antigenic types of the same virus. This was a case of infection with two antigenic types of Coxsackie virus reported by Beeman, Huebner and Cole (1952) who investigated isolates from 106 persons. Experimentally induced dual infections with different antigenic types of influenza virus in the egg embryo has been possible (Sugg and Magill, 1948; Hirst and Gottlieb, 1953) and Burnet and Lind, (1951) studied dual infections with influenza strains in mice. With some experimental dual infections a recombined form of virus appeared which contained certain properties of both parent strains.

As dual infections in nature with different antigenic types of the same virus have been encountered so seldom it must be assumed that they are not of frequent occurrence. While it would appear that these dual infections are possible, perhaps the special conditions necessary for such infections to succeed seldom occur coincidentally in nature and so explain their apparent rarity.

It is believed that breakdowns in immunized horses resulting from infection with strains shown to be homotypic to vaccine strains occur too frequently to be accounted for by dual infections and a failure to isolate the aberrant strain which was in fact the cause of the overt disease. In any case, that the particular homotypic strain isolated was present in the peripheral blood is strong evidence that a solid immunity against this strain was not present.

It must be concluded that neither antigenic change of the virus during mouse-adaptation nor dual infections can account for failures in immunity by strains of virus shown later to be antigenically related to vaccine strains. It appears rather that strains classified in the same antigenic type are not immunologically identical and that it is these antigenic differences which are the cause of the breakdowns in immunity.

In summarizing, the immunological status of the horsesickness viruses would appear to be as follows:—

There occurs in nature a limited number of main antigenic types of virus with a minor antigenic differentiation of strains within a single type. The nature of the immunity in the horse indicates that there is a basic antigenic relationship between all strains, extending even to strains of the various types. On the other hand, neutralization tests in mice with rabbit antisera reveal practically no serological relationship between strains of different types. Although direct experimental proof is lacking, it may be assumed that the immunity in the horse produced by any strain within a single type will be greater to other strains of the same type than to strains of different antigenic types. Although further types may exist or may arise in future as a result of antigenic variation the fact that the strains investigated had been isolated at various times over the past two decades from parts widely separated geographically, suggests that the seven antigenic types shown to exist comprise the main present-day antigenic variations of the horsesickness viruses. The number of different strains within any one type is unknown and the indications are that either their number is large or that new variants appear continually. If the latter alternative occurs, which appears likely, it is probable that the degree of variation is slight and very rarely leads to the appearance of markedly different immunological types. It is fairly certain that the antigenic diversity of strains within a single type is such that very few strains isolated are probably identical. This antigenic multiplicity together with the sensitivity of some horses to small antigenic differences are potent factors in the limitation of the efficacy of prophylactic immunization against horsesickness.

Immunologically, the behaviour of horsesickness virus in the horse appears to be very similar to bluetongue virus in sheep. Neitz (1948) carried out extensive cross-immunity experiments with bluetongue virus in sheep. These tests showed that while a solid immunity exists only against the homologous virus there is a variable degree of common or basic immunity between all strains. Although certain strains appeared to be very similar and justified grouping there was definite evidence that they were not identical. Just as Theiler's cross-immunity tests in horses indicated that all horsesickness strains isolated differed antigenically so did these tests in sheep suggest that every bluetongue strain differed from all others.

For the immunization of horses and mules against horsesickness it is believed that it is possible to produce an adequate immunity with a vaccine composed of strains representative of each antigenic type. If for any reason it should be decided to reduce the number of strains in the vaccine, the strain to be eliminated should be selected from either Type 1 or 2 owing to the close antigenic relationship shown to exist between these two types. Unfortunately, due to the apparent large number of antigenically different strains within a single type it is obvious that absolute protection of all animals will not be achieved by a vaccine consisting of seven strains.

Which particular strain of each immunological type should be selected for inclusion in the vaccine presents some difficulty. The ease with which the strain can be utilized for mass-production of vaccine as well as its immunogenicity in the horse should be considered, and it would be logical to select a strain recently isolated rather than an older strain. Perhaps the wide basic immunity in horsesickness between homotypic strains makes the need for the accurate immunological differentiation of these strains less important than might be the case with such viruses of foot-and-mouth disease virus and influenza virus. The isolation of the antigenically distinct Type 7 strains in 1953 emphasizes the fact that in any long term policy of horsesickness control by means of immunization, facilities should be available for the continual isolation and typing of the prevalent strains if failures in immunity are to be kept to the lowest possible minimum.

Due to the large number of different strains which may be isolated in the same locality during the same outbreak in enzootic areas, it is evident that for the control of the disease in such areas a polyvalent vaccine is necessary, whereas in areas usually considered as horsesickness-free a monovalent vaccine may possibly be used with success. In a horsesickness-free area an epizootic may originate from a single focus of infection from where the disease would be maintained by a spread of the same strain from horse to horse by the insect vector. For instance, Alexander (1948) showed that there was a very close antigenic relationship between all four strains of virus which he isolated in Egypt and Palestine during the 1944 epizootic.

However, due to the time required for the typing of a strain at present, it is doubtful whether a monovalent vaccine would be a practical procedure. Although the use of ferret or, possibly, donkey antisera prepared from inoculation of these animals with viscerotropic virus could be used to facilitate the early recognition of the antigenic type of virus implicated in the outbreak, several isolates would have to be examined to justify the use of a monovalent vaccine. Possibly, too few isolates have been studied from epizootics in horsesickness-free areas for dogmatic statements to be made in regard to the type of vaccine best suited for the control of these outbreaks and some caution would be indicated in the use of monovalent vaccines. It should not be overlooked that conditions which lead to the introduction of a single strain of virus from an adjoining enzootic area into a horsesickness-free area may also result in further introductions of strains antigenically different from the original strain. Evidence is produced by Alexander (1948) which suggests that the Egyptian and Palestine outbreaks during 1944 did not have a common origin despite the antigenic similarity of the strains from the two countries.

Antigenic studies on different isolates have been too few to enable any conclusion to be made from the isolation of the aberrant Type 7 strains from three widely separated localities during the 1953 epizootic in the Union and once in Kenya in 1952. It is not possible to say whether their isolation is indicative of the appearance of an antigenic variant whose propagation was favoured by the existence of a dominantly vaccine-immune equine host or whether chance has been a factor in the failure to isolate this type earlier. It is clear from the results of the present investigation that immunization certainly has not resulted in the disappearance of the earlier encountered antigenic types. When it is realized that horsesickness virus can be maintained in nature by a host-cycle not dependent upon equidae, this possibly, is only to be expected. That the aberrant type of virus was present in three out of ten isolates from the 1953 epizootic points to the prevalence of this type throughout the Union at this time. It is possible that many failures in immunity in immunized horses during this epizootic can be attributed to this type of virus. However, it is of interest to note, that although this type was present on Kaalplaas, viz. strain 1100, there was no evidence that it infected horses to a greater extent than strains nonaberrant to vaccine strains. A possible explanation is that in an enzootic area the main source of infection for the insect vector is the virus reservoir rather than sick horses, in which case there would be no tendency for the immune horse to favour the spread of strains different from vaccine strains. Furthermore, as the horses on Kaalplaas were immunized, it is possible that the presence of high level antibody in their blood prevented infection of the vector as has been shown to be the case with yellow fever virus and the *Aëdes aegypti* mosquito (Hindle, 1932). Hence a horse to horse spread would not have been possible.

Whether immunized horses which later contract horsesickness are able to infect the horsesickness vector has an important bearing on the prevention of the spread of the disease into horsesickness-free areas from adjoining enzootic areas. If infection of the vector is possible from these horses, it is obvious that the movement of even immunized horses into horsesickness-free areas from enzootic areas would involve some risk.

At the moment it does not appear possible to correlate the antigenic grouping of the horsesickness viruses with other viral properties. Theiler noticed that the pathogenicity for horses of different strains varied and in this connection mentioned (Theiler, 1921) the virulent and highly fatal strain O in comparison with the less virulent strain Tzaneen which showed a slightly longer incubation period, with a prolonged course and leading to recovery in fifty per cent of cases. Unfortunately the only strain of Theiler's which is mouse-adapted is O. Hence the comparisons which are possible between his observations in horses with virulent strains and later experience with mouse-adapted strains are limited. Apart from this difference in pathogenicity, horsesickness strains appear to be remarkably uniform in their behaviour in the various susceptible hosts. The classic differentiation of the symptomatology into "dikkop" and "dunkop" forms appears to be related to variation in susceptibility as a result of immunity from previous infection rather than to any viral property. This is evident from the higher incidence of "dikkop" in immunized as against susceptible horses (Theiler, 1921). Furthermore, there is no evidence to connect antigenic structure of strains with epizootiological behaviour.

In conclusion it should be stated that while the type of mouse neutralization test applied in the present study is able to identify the main antigenic structure of horsesickness strains, which is probably sufficient from the point of view of practical prophylactic immunization, for more exact antigenic differentiation other methods will be necessary. The nature of antigenic relationship between strains of one type is still obscure, as also is the reason why horses, possessing apparent equal immunity, react differently to infection with the same virus. It is difficult to see how progress in this direction is possible without more precise methods than are available at present.

X. Summary

1. A brief review is given of the epizootiology of horsesickness, the antigenic plurality of the virus strains, and the history of the control of the disease by immunization.

2. It is often not possible to isolate virus directly in mice from cases of horsesickness in immunized horses.

3. The usefulness of the ferret, and possibly the dog, for the isolation of virus from such horses was demonstrated.

4. Immunological studies on 42 mouse-adapted horsesickness virus strains were conducted.

5. For these studies an intracerebral neutralization test in mice was used. Hyperimmune sera from rabbits were mainly used in the tests.

6. Cross-neutralization tests with rabbit antisera were carried out on the eight virus strains included in the present vaccine issued from Onderstepoort and 16 strains recently isolated from cases of horsesickness in immunized horses.

7. This series of tests showed that the virus strains could be grouped into seven immunological types. It was also evident that some immunized horses become infected with strains of the same immunological type as the vaccine strains.

8. Using type rabbit antisera a further series of neutralization tests was conducted on 18 other horsesickness virus strains. These tests showed that these strains also could be grouped within the same immunological types.

9. It was shown that four recently isolated strains belonging to the same immunological type were not represented in the present Onderstepoort vaccine.

10. A limited number of neutralization tests with ferret antisera supported the antigenic grouping as revealed by the tests with rabbit antisera.

11. A serological study of sera obtained from eight immunized horses reacting to horsesickness was made.

12. Viruses isolated from each of these horses were included in the cross-neutralization tests which showed that seven of these viruses are of the same immunological type as the vaccine strains.

13. High level antibody against all the vaccine strains was shown to be present in sera from seven horses.

14. It was concluded that these particular failures in immunity were not due to inadequate immunization but were apparently the result of slight antigenic differences between the infecting virus and the vaccine strains.

15. The significance of the antigenic grouping based on the mouse neutralization test to immunity in equidae is discussed.

16. It is believed that this antigenic grouping has a definite relationship to immunity in these animals.

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APPENDIX No. 1

CROSS-NEUTRALIZATIONS USING RABBIT ANTI-SERA

VIRUS 1180

	Serum Dilut.					Virus Dilut.								
Serum.....	5	25	125	625	Tit.	U.D	5	10	20	40	80	160	320	LD50
VRY.....	—	—	—	—	0	—	—	—	—	2*	—	3	3	102
VH.....	—	—	1†	—	0									
KA.....	1	—	—	—	0									
114.....	—	—	—	—	0									
1180.....	6	6	6	3	625									
L.....	—	—	—	—	0									
OD.....	3	1	—	—	6									
A501.....	5/5	5/5	5	—	229									
RHOD.....	1	—	1	—	0	—	—	—	—	—	—	2	4	160
454.....	—	—	—	—	0									
1513.....	6	5	4	—	152									
1408.....	4	3	2	1	32									
MFK.....	—	—	—	—	0									
FR.....	—	—	—	—	0									
ES.....	—	—	—	—	0	—	—	—	—	1	1	3	3	113
1100.....	—	—	—	—	0									
1582.....	—	—	—	—	0									
PMB.....	1	—	—	—	0									
VR.....	—	—	—	—	0									
P2.....	1	—	—	—	0									
2627.....	6	6	5/5	1	340	—	—	—	—	—	—	2	4	160
ERM.....	—	—	—	—	0									
1096.....	—	—	—	—	0									
P1.....	1	—	—	—	0									

* Virus dilutions 4 mice injected, 2 survivors.
† Serum-Virus Mixtures 6 mice injected, 1 survivor.
N.B.—All figures denote surviving mice.
5/5 = Of 5 mice injected, 5 survived.

VIRUS KA

RHOD.....	—	—	—	—	0	—	—	1	2	1	3	3	4	44
454.....	—	—	—	—	0									
1513.....	1	—	—	—	0									
1408.....	—	—	—	—	0									
MFK.....	—	—	1	—	0									
FR.....	—	—	—	—	0									
ES.....	1	1	—	—	0	—	—	—	—	1	2	3	1	127
1100.....	4	—	—	—	7									
1582.....	—	—	—	—	0									
PMB.....	2	—	—	—	0									
VR.....	—	—	—	—	0									
P2.....	—	—	—	—	0									
2627.....	—	—	—	—	0	—	—	—	2	1	2	4	4	56
ERM.....	—	—	—	—	0									
1096.....	—	—	—	—	0									
P1.....	—	—	1	—	0									

APPENDIX No. 1 (*continued*)

VIRUS KA (*continued*)

	Serum Dilut.					Virus Dilut.								
Serum.....	5	25	125	625	Tit.	U.D	5	10	20	40	80	160	320	LD50
A501.....	—	2	—	—	0	—	—	—	1	1	2	2	3	95
VH.....	—	—	—	—	0									
VRY.....	—	—	—	—	0									
OD.....	—	—	—	—	0									
1180.....	—	—	—	—	0									
114.....	—	—	—	—	0									
L.....	5	6	6	5	625									
KA.....	6	6	5	5	625									
VIRUS OD														
VRY.....	—	—	—	—	0	—	—	—	—	2	3	2	1	100
VH.....	—	1	—	—	0									
KA.....	—	—	—	—	0									
114.....	—	—	—	—	0									
1180.....	3	1	—	—	7									
L.....	—	—	—	—	0									
OD.....	5	5	5	1	213									
A501.....	2	1	1	—	5									
RHOD.....	—	—	—	—	0	—	—	—	—	—	—	3	4	126
454.....	—	—	—	—	0									
1513.....	—	1	—	—	0									
1408.....	—	—	—	—	0									
MFK.....	—	—	—	—	0									
FR.....	—	—	—	—	0									
ES.....	2	—	—	—	0	—	—	1	1	3	3	4	4	28
1100.....	—	—	—	—	0									
1582.....	—	—	—	—	0									
PMB.....	—	—	1	—	0									
VR.....	—	1	—	—	0									
P2.....	—	—	—	—	0									
2627.....	3	—	—	—	5	—	1	—	1	1	2	4	3	33
ERM.....	—	—	—	—	0									
1096.....	1	—	—	—	0									
P1.....	—	—	1	—	0									
VIRUS VRY														
VRY.....	6	4	5/5	6	625	—	—	—	2	2	3	4	3	40
VH.....	—	—	—	—	0									
KA.....	—	—	—	—	0									
114.....	1	2	—	—	0									
1180.....	—	1	—	—	0									
L.....	—	—	—	—	0									
OD.....	3	—	—	—	5									
A501.....	—	—	—	1	0									

APPENDIX No. 1 (*continued*)

VIRUS VRY (*continued*)

	Serum Dilut.					Virus Dilut.								
Serum.....	5	25	125	625	Tit.	U.D	5	10	20	40	80	160	320	LD50
RHOD.....	6	6	5	2	364	—	1	—	1	1	2	2	4	71
454.....	6	6	6	3	625									
1513.....	—	—	—	—	0									
1408.....	1	—	—	—	0									
MFK.....	6	4	3	1	99									
FR.....	—	—	—	—	0									
ES.....	—	—	1	1	0	—	—	—	2	1	1	4	3	63
1100.....	—	—	—	2	0									
VR.....	1	1	—	—	0									
1582.....	—	—	—	—	0									
PMB.....	—	—	—	—	0									
P2.....	—	—	—	—	0									
2627.....	—	—	—	—	0	—	—	—	—	2	2	3	4	67
ERM.....	6	6	6	6	625									
1096.....	—	—	—	—	0									
P1.....	—	—	—	—	0									

VIRUS VH

VRY.....	—	1	—	1	0	—	—	1	2	3	2	2	4	31
KA.....	—	—	—	—	0									
VH.....	6	6	5	5	625									
114.....	2	1	—	—	0									
1180.....	—	—	—	—	0									
L.....	—	—	—	—	0									
OD.....	1	—	—	—	0									
A501.....	—	—	—	—	0									
RHOD.....	—	—	—	1	0	—	—	—	1	3	3	3	4	35
454.....	1	2	—	—	0									
1513.....	—	—	—	—	0									
1408.....	—	—	—	—	0									
MFK.....	—	—	—	—	0									
FR.....	5	6	6	4	625									
ES.....	1	1	—	—	0	—	—	—	1	4	1	3	4	36
1100.....	1	1	—	—	0									
1582.....	6	3	5	5	625									
PMB.....	—	—	—	—	0									
VR.....	4	6	6	4	625									
P2.....	6	5	5	5	625									
2627.....	—	1	—	—	0	—	—	—	—	1	1	2	4	113
ERM.....	—	—	—	—	0									
1096.....	6	6	5	4	625									
P1.....	5/5	4/5	6	6	625									

APPENDIX No. 1 (*continued*)

VIRUS L

	Serum Dilut.					Virus Dilut.								
Serum.....	5	25	125	625	Tit.	U.D	5	10	20	40	80	160	320	LD50
L.....	6	6	6	6	625	—	—	1	—	2	2	4	4	47
KA.....	5/5	6	5	3	453									
RHOD.....	—	—	—	—	0									
454.....	—	—	—	—	0									
1513.....	—	—	—	—	0									
1408.....	—	—	—	—	0									
MFK.....	1	—	—	—	0									
FR.....	—	—	—	—	0									
ES.....	2	—	—	—	0	—	1	—	2	1	1	2	4	80
1100.....	1	—	—	—	0									
1582.....	1	2	—	—	0									
PMB.....	2	—	—	—	0									
VR.....	—	—	—	1	0									
P2.....	—	—	—	—	0									
2627.....	—	—	—	—	0	—	—	—	—	3	1	4	2	89
ERM.....	1	1	—	—	0									
1096.....	—	—	—	—	0									
P1.....	—	—	—	—	0									
A501.....	—	—	—	—	0	—	—	—	—	1	4	1	3	72
VRY.....	—	—	—	—	0									
1180.....	—	—	—	—	0									
OD.....	—	—	—	—	0									
VH.....	—	—	—	—	0									
114.....	—	—	—	—	0									
VIRUS 114														
VRY.....	1	—	—	—	0	—	—	1	—	1	3	1	4	42
VH.....	—	—	—	—	0									
KA.....	—	—	—	—	0									
114.....	6	6	6	6	625									
1180.....	4	1	—	—	8									
L.....	—	—	—	—	0									
OD.....	—	—	—	—	0									
A501.....	—	—	—	—	0									
RHOD.....	—	—	—	—	0	—	—	—	—	3	2	4	4	40
454.....	2	2	1	—	6									
1513.....	2	2	—	—	5									
1408.....	—	—	—	—	0									
MFK.....	1	2	—	—	0									
FR.....	—	—	1	2	0									
ES.....	—	—	—	—	0	—	—	—	—	—	3	3	3	80
1100.....	—	—	1	—	0									
VR.....	1	—	—	—	0									
1582.....	1	—	1	—	0									
PMB.....	—	—	—	—	0									
P2.....	1	2	—	—	5									
2627.....	—	—	—	—	0	—	—	2	1	1	3	2	2	63
ERM.....	—	—	—	1	0									
1096.....	1	—	—	—	0									
P1.....	1	—	—	—	0									

APPENDIX No. 1 (*continued*)

VIRUS A501

	Serum Dilut.					Virus Dilut.								
Serum.....	5	25	125	625	Tit.	U.D	5	10	20	40	80	160	320	LD50
A501.....	6	6	6	4	625	—	—	—	1	—	1	3	4	100
1180.....	6	5	6	4	625									
VH.....	—	1	—	—	0	—	—	—	2	1	2	4	4	56
VRY.....	—	—	—	—	0									
OD.....	5	1	—	1	13									
114.....	1	—	—	1	0									
L.....	—	1	1	0	0									
KA.....	2	—	—	—	0									
RHOD.....	—	1	3	1	0	—	—	—	2	4	3	4	4	22
454.....	2	—	2	—	0									
1513.....	6	6	4	3	364									
1408.....	5	6	4	3	304									
MFK.....	2	1	—	—	5									
FR.....	—	1	—	—	0									
ES.....	3	1	—	—	5	—	—	—	2	3	3	4	4	28
1100.....	3	—	—	—	5									
1582.....	1	2	1	—	0									
PMB.....	1	—	1	—	0									
VR.....	2	—	—	—	0									
P2.....	—	—	—	—	0									
2627.....	6	6	6	4	625	—	—	—	2	2	3	4	3	40
ERM.....	3	—	—	—	5									
1096.....	—	—	—	—	0									
P1.....	1	1	—	—	0									
VIRUS RHOD														
KA.....	—	—	—	—	0	—	—	—	1	—	—	1	2	270
L.....	—	—	—	—	0									
1180.....	—	—	—	—	0									
114.....	—	—	—	—	0									
A501.....	—	—	—	—	0									
VH.....	—	—	—	—	0									
RHOD.....	5	6	3	2	162	—	1	—	1	1	2	3	4	56
VRY.....	4	2/5	3	1	32									
OD.....	—	—	1	—	0	—	—	—	1	—	—	4	2/3	113
454.....	6	5/5	6	6	625									
1513.....	—	—	—	—	0									
1408.....	—	—	—	—	0									
MFK.....	6	6	4	3	385									
FR.....	—	—	—	—	0									
VR.....	—	1	—	—	0	—	—	—	—	2	2	3	1	113
PMB.....	—	—	—	—	0									
1582.....	1	—	—	—	0									
ES.....	—	—	—	—	0									
P2.....	—	—	—	—	0									
1100.....	1	—	—	—	0									
2627.....	—	—	—	—	0	—	—	1	1	2	3	4	3	40
ERM.....	6	6	6	6	625									
1096.....	2	1	—	—	0									
P1.....	—	—	1	—	0									

APPENDIX No. 1 (*continued*)

VIRUS 454

	Serum Dilut.					Virus Dilut.								
Serum.....	5	25	125	625	Tit.	U.D	5	10	20	40	80	160	320	LD50
1180.....	—	—	—	—	0	—	—	—	—	—	—	1	2	268
A501.....	—	—	—	1	0									
OD.....	—	—	—	—	0									
KA.....	—	—	—	—	0									
114.....	—	—	—	—	0									
L.....	—	—	—	1	0									
454.....	6	6	6	2	416	—	—	—	1	1	—	2/2	1	133
VRY.....	5	3	—	—	21									
VH.....	1	—	—	—	0	—	—	2	1	2	3	4	3	35
RHOD.....	5	5	4	1	156									
1408.....	—	—	—	—	0									
1513.....	—	—	—	—	0									
MFK.....	5	2	5	3	186									
FR.....	—	—	—	—	0									
ES.....	—	—	—	—	0	—	—	—	—	1	3	4	4	56
1100.....	—	—	—	—	0									
1582.....	—	—	—	—	0									
PMB.....	—	—	—	—	0									
VR.....	—	—	—	—	0									
P2.....	—	—	—	—	0									
2627.....	—	—	—	—	0	—	—	1	1	2	3	4	3	40
ERM.....	6	6	6	5/5	625									
1096.....	—	—	—	—	0									
P1.....	—	—	—	—	0									

VIRUS 1513

FR.....	—	—	—	—	0	—	—	—	1	1	3	4	4	50
KA.....	—	—	—	—	0									
L.....	—	—	—	—	0									
VRY.....	—	—	—	—	0									
114.....	1	—	—	—	0									
OD.....	1	—	—	—	0									
VH.....	—	—	—	—	0									
A501.....	6	6	4	4	625	—	1	—	—	3	4	4	4	28
1180.....	6	6	5/5	2	416									
RHOD.....	—	1	—	—	0									
454.....	1	2	2	—	5									
1513.....	5	6	5	2	278									
1408.....	5	3	2	2	55									
MFK.....	1	—	—	—	0									
ES.....	—	—	—	—	0	—	—	2	2	1	2	1	2	80
1100.....	—	—	1	—	0									
1582.....	—	—	—	—	0									
PMB.....	—	—	2	—	0									
VR.....	—	—	—	—	0									
P2.....	—	—	—	—	0									
2627.....	6	4	5	3	327	—	—	—	—	4	3	4	4	36
ERM.....	—	—	—	—	0									
1096.....	1	—	1	—	0									
P1.....	—	—	—	—	0									

APPENDIX No. 1 (*continued*)

VIRUS 1408

	Serum Dilut.					Virus Dilut.								
Serum.....	5	25	125	625	Tit.	U.D	5	10	20	40	80	160	320	LD50
VH.....	1	2	1	—	0	—	—	—	1	4	3	3	3	35
L.....	—	2	—	—	0									
OD.....	1	1	—	2	0									
FR.....	1	1	2	—	0									
114.....	1	1	—	1	0									
VRY.....	—	1	—	—	0									
KA.....	—	1	—	—	0									
A501.....	4	6	3	1	106	—	—	1	2	3	2	3	3	35
1180.....	5	3	4	3	156									
RHOD.....	—	—	—	—	0									
454.....	—	1	—	—	0									
1513.....	5	4	4	3	201									
1408.....	4	2	1	—	13									
MFK.....	—	1	—	—	0									
ES.....	—	—	—	—	0	—	1	—	1	1	2	3	3	57
1100.....	—	1	—	—	0									
1582.....	—	—	—	—	0									
PMB.....	—	—	—	—	0									
VR.....	—	—	—	—	0									
P2.....	—	—	—	—	0									
2627.....	5	4	—	—	41	—	—	1	1	—	3	4	4	51
ERM.....	1	—	1	—	0									
1096.....	—	—	—	—	0									
P1.....	2	—	—	—	0									

VIRUS MFK

A501.....	—	—	—	—	0	—	1	2	2	4	3	2	2	25
OD.....	1	—	1	—	0									
1180.....	—	—	—	—	0									
KA.....	—	6	—	—	5									
VH.....	2	—	1	—	0									
L.....	—	1	—	—	0									
114.....	1	1	—	—	0									
VRY.....	5	4	5	1	171	—	1	1	—	2	3	4	3	40
RHOD.....	5	5	6	5	625									
454.....	5	5	6	5	625									
1513.....	—	—	1	—	0									
1408.....	—	—	—	—	0									
MFK.....	5	4	4	3	201									
FR.....	—	—	—	—	0									
ES.....	—	—	1	—	0	—	—	—	—	—	3	2	4	80
1100.....	—	—	—	—	0									
1582.....	—	—	—	—	0									
PMB.....	—	—	—	—	0									
VR.....	—	—	1	—	0									
P2.....	—	—	—	—	0									
2627.....	—	—	—	—	0	—	—	1	—	2	2	4	4	46
ERM.....	5/5	6	6	4	625									
1096.....	1	—	—	—	0									
P1.....	—	—	—	—	0									

APPENDIX No. 1 (*continued*)

VIRUS FR

	Serum Dilut.					Virus Dilut.								
Serum.....	5	25	125	625	Tit.	U.D	5	10	20	40	80	160	320	LD50
VRY.....	2	—	—	—	0	—	—	—	—	—	1	3	3	121
VH.....	6	6	4	4	625									
KA.....	—	—	—	—	0									
L.....	—	—	1	1	0									
114.....	1	—	—	—	0									
OD.....	1	—	—	—	0									
FR.....	5	6	4	2	213									
A501.....	—	—	—	—	0	—	—	1	—	1	3	2	3	67
1180.....	—	—	1	—	0									
RHOD.....	—	—	—	—	0									
454.....	—	—	—	—	0									
1513.....	1	—	—	—	0									
1408.....	—	—	—	1	0									
MFK.....	—	—	—	—	0									
ES.....	—	—	1	—	0	—	—	—	2	3	2	3	4	33
1100.....	—	—	—	—	0									
1582.....	6	6	6	5	625									
PMB.....	3	—	—	—	5									
VR.....	6	6	5/5	6	625									
P2.....	6	6	5/5	6	625									
2627.....	1	—	—	—	0	—	—	—	3	2	2	4	4	28
ERM.....	—	1	—	—	0									
1096.....	5	6	6	3	625									
P1.....	6	6	5	5	625									

VIRUS ES

VRY.....	—	—	—	—	0	—	—	—	—	1	2	3	1	113
OD.....	—	—	—	—	0									
VH.....	—	—	—	—	0									
A501.....	—	—	—	—	0									
L.....	—	—	—	2	0									
KA.....	—	—	—	—	0									
114.....	—	—	—	—	0									
ES.....	5	5	5	3	327									
1180.....	—	—	—	—	0									
MFK.....	—	—	—	—	0	—	—	—	—	1	1	2	3	134
RHOD.....	—	—	—	—	0									
1513.....	—	—	—	—	0									
1408.....	—	—	—	—	0									
454.....	—	—	—	—	0									
1100.....	6	4	6	3	416	—	—	—	1	—	2	1	1	284
1582.....	1	—	—	—	0									
PMB.....	6	4	3	2	125									
VR.....	—	—	—	—	0									
P2.....	—	—	—	—	0									
2627.....	—	—	—	—	0	—	—	—	—	2	2	4	4	56
ERM.....	—	—	—	—	0									
1096.....	—	—	—	—	0									
P1.....	—	—	—	1	0									
FR.....	—	—	—	—	0									

APPENDIX No. 1 (*continued*)

VIRUS 1100

	Serum Dilut.					Virus Dilut.								
Serum.....	5	25	125	625	Tit.	U.D	5	10	20	40	80	160	320	LD50
OD.....	—	—	—	—	0	—	—	—	—	—	—	—	3	253
1180.....	—	—	—	1	0									
114.....	—	—	—	—	0									
KA.....	—	—	—	—	0									
MFK.....	2	—	—	—	0	—	—	—	2	—	3	4	4	52
1408.....	1	—	—	1	0									
VRY.....	—	—	2	—	0									
FR.....	1	—	—	—	0									
RHOD.....	—	1	—	—	0									
A501.....	—	—	—	—	0									
1513.....	—	—	—	—	0									
1582.....	—	—	—	—	0	—	—	—	—	—	2	2	3	134
VR.....	—	—	—	—	0									
P2.....	—	—	—	—	0									
VH.....	1	—	—	—	0									
ES.....	2	4	3	—	31	—	—	—	1	3	2	1	3	80
1100.....	5	5	3/5	2	162									
PMB.....	6	6	3	—	125									
2627.....	—	—	—	—	0	—	—	—	—	—	3	4	2	80
ERM.....	2	1	—	—	0									
1096.....	1	1	—	—	0									
P1.....	—	—	—	—	0									
L.....	—	—	—	—	0	—	—	—	—	—	—	2	3	190
454.....	—	—	—	—	0									

VIRUS 1582

1582.....	6	5/5	6	5	625	1	—	2	3	3	3	3	4	20
ES.....	—	—	—	2	0									
1100.....	—	—	2	—	0									
PMB.....	—	—	1	1	0									
VR.....	6	4	6	5	625									
P2.....	6	5	5	4	625									
A501.....	—	—	1	—	0	—	1	—	1	—	3	3	4	63
KA.....	—	—	—	—	0									
VH.....	6	5	5	5	625									
1180.....	—	—	—	—	0									
OD.....	1	—	—	—	0									
L.....	—	—	—	—	0									
114.....	—	—	—	—	0									
VRY.....	—	—	—	—	0	—	—	—	—	1	3	4	2	71
RHOD.....	—	—	—	—	0									
454.....	—	—	—	—	0									
1513.....	—	—	—	—	0									
1408.....	1	—	—	—	0									
MFK.....	—	—	—	—	0									
FR.....	5	3	2	4	72									
2627.....	—	—	—	—	0	—	—	—	2	3	4	3	4	26
ERM.....	2	1	—	—	0									
1096.....	6	6	5	3	476									
P1.....	6	6	6	6	625									

APPENDIX No. 1 (*continued*)

VIRUS PMB

Serum Dilut.						Virus Dilut.								
Serum.....	5	25	125	625	Tit.	U.D	5	10	20	40	80	160	320	LD50
PMB.....	5	5	5	3	291	—	—	2	1	2	2	3	4	35
VRY.....	2	1	—	—	0									
L.....	—	—	—	—	0									
OD.....	—	—	1	2	0									
A501.....	—	—	—	—	0									
KA.....	1	1	—	—	0	—	—	—	1	1	2	2	4	80
VH.....	—	1	—	—	0									
1180.....	—	—	—	—	0									
RHOD.....	—	—	—	—	0									
454.....	—	—	—	—	0									
1513.....	—	—	—	—	0									
1408.....	—	—	—	—	0									
114.....	1	—	—	—	0	—	—	—	1	1	2	3	4	65
MFK.....	—	—	—	—	0									
FR.....	—	—	—	—	0									
ES.....	5	4	6	3	327									
1100.....	6	5	5	5	625									
1582.....	—	—	—	—	0									
VR.....	—	—	—	—	0									
P2.....	—	—	—	—	0									
2627.....	—	—	1	—	0	—	—	—	—	—	3	2	4	80
ERM.....	2	—	—	—	0									
1096.....	—	—	—	—	0									
P1.....	—	—	—	—	0									

VIRUS VR

VRY.....	1	—	—	—	0	—	—	—	—	—	1	1	1	320
A501.....	1	—	—	1	0									
KA.....	—	—	—	1	0									
L.....	2	—	—	1	0									
FR.....	6	5	4	3	303	—	—	—	1	2	2	4	3	56
ES.....	—	—	—	—	0									
1100.....	—	—	—	—	0									
PMB.....	—	—	1	1	0									
P2.....	6	5	3	3	213									
1180.....	1	—	—	—	0	—	—	—	—	1	2	—	4	174
OD.....	1	1	—	—	0									
114.....	—	—	—	—	0									
RHOD.....	—	—	1	—	0									
454.....	—	—	—	—	0									
1513.....	—	1	—	—	0									
1408.....	—	—	1	1	0									
2627.....	1	—	—	1	0	—	—	—	2	2	2	2	4	56
ERM.....	1	1	—	—	0									
1096.....	6	6	4	5	625									
P1.....	6	6	5	5	625									
VH.....	2	4	4	2	65	—	—	—	2	3	1	2	4	56
VR.....	5	5	4	4	364									
MFK.....	—	1	—	—	0									
1582.....	6	5	4	4	495									

APPENDIX No. 1 (*continued*)

VIRUS P2

	Serum Dilut.					Virus Dilut.								
Serum.....	5	25	125	625	Tit.	U.D	5	10	20	40	80	160	320	LD50
P2.....	4	4	2	1	50	—	—	—	—	—	2	3	3	108
A501.....	—	—	1	—	0									
1180.....	—	—	—	—	0									
OD.....	—	—	—	—	0									
L.....	—	—	—	—	0									
KA.....	—	—	—	—	0									
MFK.....	—	1	—	—	0	—	—	—	—	1	—	1	4	179
FR.....	5	5	4	2	186									
1100.....	—	1	—	—	0									
ES.....	—	—	—	2	0									
1582.....	6	6	3	4	451									
PMB.....	—	—	—	—	0									
VR.....	5	6	4	5	625									
114.....	1	—	—	1	0	—	—	2	—	1	1	4	2	88
VRY.....	—	—	—	—	0									
RHOD.....	—	—	—	—	0									
454.....	—	—	1	—	0									
1513.....	—	1	—	—	0									
1408.....	—	—	—	—	0									
2627.....	—	—	1	—	0	—	—	—	—	2	1	2	4	113
P1.....	6	5	6	2	364									
1096.....	5	3	4	2	125									
ERM.....	—	—	—	—	0									
VH.....	6	2	1	—	19									

VIRUS P1

1513.....	—	—	—	—	0	—	—	—	1	3	3	2	4	40
KA.....	—	—	—	—	0									
454.....	—	—	—	—	0									
PMB.....	1	—	1	—	0									
FR.....	6	6	6	4	625									
P2.....	5	6	4	6	625									
1408.....	1	—	—	—	0									
MFK.....	—	—	—	—	0									
L.....	—	—	—	—	0									
1582.....	6	6	6	5	625									
114.....	—	—	—	—	0									
ES.....	—	—	—	—	0									
VRY.....	—	—	—	1	0	—	1	—	—	4	2	2	3	40
A501.....	1	—	—	—	0									
OD.....	—	—	—	—	0									
1180.....	—	2	—	—	0									
RHOD.....	1	—	—	—	0									
VH.....	5	6	6	3	625									
ERM.....	2	—	—	—	0									
2627.....	—	—	—	—	0									
P1.....	6	6	6	6	625									
1096.....	6	6	4	6	625									
VR.....	5	6	6	3	625									
1100.....	—	—	—	—	0									

APPENDIX No. 1 (*continued*)

VIRUS 1096

	Serum Dilut.					Virus Dilut.								
Serum.....	5	25	125	625	Tit.	U.D	5	10	20	40	80	160	320	LD50
1513.....	—	—	—	1	0	—	—	2	2	2	3	3	2	40
KA.....	—	1	—	—	0									
454.....	1	—	2	—	0									
PMB.....	—	—	—	1	0									
FR.....	5	3	5	6	625									
P2.....	6	6	5/5	5	625									
1408.....	1	—	2	—	0									
MFK.....	—	1	1	—	0									
L.....	—	—	—	—	0									
1582.....	6	5	5/5	5	625									
114.....	—	—	2	—	0									
ES.....	—	—	1	—	0									
VRY.....	1	1	—	—	0	—	—	—	—	2	3	3	4	59
A501.....	—	—	—	—	0									
OD.....	1	—	—	—	0									
1180.....	—	—	—	—	0									
RHOD.....	—	—	—	1	0									
VH.....	6	6	6	4	625									
ERM.....	2	—	—	—	0									
P1.....	6	6	6	6	625									
1096.....	6	6	6	6	625									
VR.....	6	5	5	6	625									
1100.....	—	—	—	—	0									
2627.....	—	—	—	—	0	—	—	—	3	1	1	2	3	89

VIRUS 2627

OD.....	—	—	—	2	0	—	—	—	1	1	2	4	4	56
P2.....	—	—	—	—	0									
VRY.....	—	—	—	—	0									
ES.....	—	—	—	—	0									
RHOD.....	—	—	—	—	0									
1180.....	4/5	4	4	2	156									
VH.....	—	—	—	—	0									
454.....	—	1	1	—	0									
1100.....	—	—	—	—	0									
FR.....	—	—	—	—	0									
PMB.....	1	—	—	—	0									
A501.....	5	4	4	1	125	—	—	—	—	—	2	2	4	113
KA.....	—	—	—	—	0									
1408.....	5	2	—	—	14									
1513.....	5	5	—	1	45									
114.....	—	—	—	—	0									
MFK.....	1	—	—	—	0									
VR.....	—	—	—	—	0									
1582.....	—	—	—	—	0									
P1.....	—	—	—	—	0									
1096.....	—	—	—	—	0									
2627.....	6	6	1	3	107									
ERM.....	1	2	—	—	0									
L.....	—	—	—	—	0									

APPENDIX No. 1 (*continued*)
VIRUS ERM

	Serum Dilut.					Virus Dilut.								
Serum.....	5	25	125	625	Tit.	U.D	5	10	20	40	80	160	320	LD50
A501.....	—	—	—	—	0	—	—	1	—	3	—	1	4	113
KA.....	—	—	—	—	0									
VH.....	—	—	—	—	0									
1180.....	—	—	—	—	0									
OD.....	—	—	—	—	0									
L.....	—	—	—	—	0									
114.....	—	—	—	—	0									
VRY.....	5	4	1	1	54									
RHOD.....	5	4	2	1	55									
454.....	5	4	4	5	476									
1513.....	—	—	—	—	0	—	—	—	—	2	3	2	4	113
1408.....	—	—	—	—	0									
MFK.....	6	4	5	3	327									
FR.....	—	—	—	—	0									
ES.....	—	—	—	—	0									
1100.....	—	—	—	—	0									
1582.....	—	—	—	—	0									
PMB.....	—	—	—	—	0									
VR.....	—	—	—	—	0									
P2.....	—	—	—	—	0									
ERM.....	6	5/5	6	6	625	—	—	—	—	—	1	1	4	179
1096.....	—	—	—	—	0									
2627.....	—	—	—	—	0									
P1.....	—	—	—	—	0									

APPENDIX No. 2

NEUTRALIZATIONS OF CERTAIN STRAINS BY RABBIT TYPE ANTI-SERA

Virus Karen

	Serum Dilut.					Virus Dilut.								
Serum.....	5	25	125	625	Tit.	U.D	5	10	20	40	80	160	320	LD50
1180.....	—	1	—	—	0	—	—	—	—	2	3	3	3	56
OD.....	—	—	—	—	0									
VH.....	—	—	—	—	0									
VRY.....	—	—	—	—	0									
114.....	—	—	—	—	0									
KA.....	—	1	—	—	0									
1100.....	5	4	5	6	625									

Virus 1157

1180.....	5	6	5	—	213	—	—	—	1	3	4	2	2	36
OD.....	1	1	—	—	0									
VH.....	1	—	1	—	0									
VRY.....	1	—	—	—	0									
114.....	1	—	—	—	0									
KA.....	—	—	—	1	0									
1100.....	—	—	—	—	0									

Virus Cedara

1180.....	—	1	1	—	0	—	—	—	—	—	3	2	2	113
OD.....	—	—	—	—	0									
VH.....	1	—	—	—	0									
VRY.....	—	—	—	—	0									
114.....	—	1	—	—	0									
KA.....	6	6	4	3	384									
1100.....	1	—	—	—	0									

Virus 449

1180.....	5	5	4	2	186	—	—	—	1	1	2	3	3	80
OD.....	—	—	—	—	0									
VH.....	—	3	—	—	0									
VRY.....	1	3	—	—	0									
114.....	—	—	—	—	0									
KA.....	1	—	—	—	0									
1100.....	1	—	—	2	0									

APPENDIX No. 2 (*continued*)

VIRUS O

	Serum Dilut.					Virus Dilut.								
Serum.....	5	25	125	625	Tit.	U.D	5	10	20	40	80	160	320	LD50
1180.....	3	—	—	—	5	—	1	1	—	3	3	4	4	28
OD.....	—	3	—	—	0									
VH.....	—	—	—	—	0									
VRY.....	1	—	—	—	0									
114.....	6	6	6	3	625									
KA.....	3	—	—	—	5									
1100.....	—	—	—	—	0									

VIRUS PIRIE

1180.....	—	—	—	—	0	—	—	—	—	2/3	—	3	1	160
OD.....	—	—	—	—	0									
VH.....	3	5	3	—	55									
VRY.....	—	—	—	—	0									
114.....	—	—	—	—	0									
KA.....	—	—	—	—	0									
1100.....	—	—	—	—	0									

VIRUS GALPIN

1180.....	—	—	—	—	0	—	—	1	—	—	2	3	3	91
OD.....	—	—	—	—	0									
VH.....	5	5	6	3	224									
VRY.....	—	—	—	—	0									
114.....	—	—	—	—	0									
KA.....	—	—	—	—	0									
1100.....	1	—	—	—	0									

VIRUS THEILER

1180.....	4	6	4	—	125	—	—	—	—	—	—	4	4	113
OD.....	—	—	—	—	0									
VH.....	—	—	—	—	0									
VRY.....	—	—	—	—	0									
114.....	—	—	—	—	0									
KA.....	—	—	—	—	0									
1100.....	—	—	—	—	0									

APPENDIX No. 2 (*continued*)

VIRUS MATAFFIN

	Serum Dilut.					Virus Dilut.								
Serum.....	5	25	125	625	Tit.	U.D	5	10	20	40	80	160	320	LD50
1180.....	5	2	2	—	20	—	1	—	2	1	4	3	4	40
OD.....	6	6	3	5	625									
VH.....	—	—	—	—	0									
VRY.....	—	—	—	—	0									
114.....	—	—	—	—	0									
KA.....	—	—	—	—	0									
1100.....	—	—	—	—	0									

VIRUS KEPPEL

1180.....	—	—	—	—	0	—	—	—	—	—	—	—	2	320
OD.....	—	—	—	—	0									
VH.....	—	—	—	—	0									
VRY.....	6	2	4	—	55									
114.....	—	—	—	—	0									
KA.....	—	—	—	—	0									
1100.....	—	—	—	—	0									

VIRUS WESTERMAN

1180.....	—	—	—	—	0	—	—	—	1	1	—	1	1	253
OD.....	—	—	—	—	0									
VH.....	4	4	5	3	213									
VRY.....	—	—	—	—	0									
114.....	—	—	—	—	0									
KA.....	—	—	—	—	0									
1100.....	—	—	—	—	0									

VIRUS 1397

1180.....	—	—	—	—	0	—	—	—	—	—	1	—	2	235
OD.....	—	—	—	—	0									
VH.....	—	—	—	—	0									
VRY.....	—	—	—	—	0									
114.....	6	6	5	2	364									
KA.....	—	—	—	—	0									
1100.....	—	—	—	—	0									

VIRUS 1145

1180.....	5	6	3	2	162	—	—	—	—	—	—	1	4	201
OD.....	1	1	—	—	0									
VH.....	—	—	1	1	0									
VRY.....	—	1	—	—	0									
114.....	—	—	—	—	0									
KA.....	—	—	—	—	0									
1100.....	—	—	—	—	0									

APPENDIX No. 2 (*continued*)

VIRUS 30B

	Serum Dilut.					Virus Dilut.								
Serum.....	5	25	125	625	Tit.	U.D	5	10	20	40	80	160	320	LD50
1180.....	6	6	5	3	476	—	—	—	1	1	2	3	3	80
OD.....	—	1	—	1	0									
VH.....	—	—	—	—	0									
VRY.....	—	—	1	—	0									
114.....	—	—	—	—	0									
KA.....	—	—	1	—	0									
1100.....	—	—	—	—	0									

VIRUS CA

OD.....	—	—	—	—	0	—	—	—	3	2	4	2	4	45
1180.....	6	3	5	3	278									
VH.....	—	—	—	—	0									
VRY.....	—	—	—	—	0									
114.....	—	1	—	—	0									
KA.....	—	1	—	—	0									
1100.....	—	—	—	—	0									

VIRUS H409

1180.....	5	4	3	—	60	—	—	—	—	—	—	1	2	320
OD.....	1	—	—	—	0									
VH.....	—	—	—	—	0									
VRY.....	—	—	—	—	0									
114.....	—	—	—	—	0									
KA.....	—	—	—	—	0									
1100.....	—	—	—	1	0									

VIRUS POTCH

1180.....	4	4	4	1	95	—	—	—	—	1	—	2	4	137
OD.....	1	—	—	—	0									
VH.....	—	—	—	—	0									
VRY.....	—	—	—	—	0									
114.....	1	—	—	—	0									
KA.....	1	—	—	—	0									
1100.....	—	—	—	2	0									

VIRUS 1144

1180.....	6	6	5	1	228	—	—	—	2	3	3	4	3	28
OD.....	—	—	—	—	0									
VH.....	2	—	—	—	0									
VRY.....	—	—	—	—	0									
114.....	2	—	—	—	0									
KA.....	1	1	—	—	0									
1100.....	2	—	—	—	0									

APPENDIX No. 3

NEUTRALIZATIONS WITH FERRET ANTISERA

VIRUS PMB

	Serum Dilut.						Virus Dilut								
Serum.....	2	8	32	128	512	Tit.	U.D	5	10	20	40	80	160	320	LD_{50}
PMB.....	5	5	5	5	—	148	—	—	—	—	1	—	3	4	113
PMB(PI)* ..	—	—													
1513.....	—	—	—	—	—	0									
1513(PI)....	—	—													
FR.....	—	—	—	—	—	0									
FR(PI).....	—	—													

* (PI) = Pre-inoculation serum.

VIRUS FR

PMB.....	—	—	—	—	—	0	—	—	—	—	1	—	2	3	160
PMB(PI)...	—	—													
1513.....	—	—	—	—	—	0									
1513(PI)....	—	—													
FR.....	5	6	5/5	6	2	322									
FR(PI).....	—	—													

VIRUS 1513

PMB.....	—	—	—	1	—	0	—	—	—	1	1	3	4	3	56
PMB(PI)...	—	—													
1513.....	6	6	6	5	—	222									
1513(PI)....	—	—													
FR.....	—	—	—	—	—	0									
FR(PI).....	—	—													

VIRUS 1180

	Serum Dilut.					Virus Dilut.								
Serum.....	5	25	125	625	Tit.	U.D	5	10	20	40	80	160	320	LD50
PMB.....	—	2	1	1	0	—	—	—	—	2	2	2	2	113
1513.....	4/5	5	6	4	625									
FR.....	3	—	—	1	0									

VIRUS VH

PMB.....	—	—	1	—	0	—	1	1	1	3	4	2	4	28
1513.....	1	2	—	—	0									
FR.....	5	6	5	3	393									

APPENDIX No. 3 (*continued*)

VIRUS KAREN

	Serum Dilut.					Virus Dilut.								
Serum.....	5	25	125	625	Tit.	U.D	5	10	20	40	80	160	320	LD50
PMB.....	6	6	4	3	385	—	—	—	1	3	1	4	3	56
1513.....	—	—	—	—	0									
FR.....	—	—	—	—	0									

VIRUS 114

PMB.....	—	—	—	—	0	—	—	—	—	4	4	1	4	28
1513.....	—	—	—	—	0									
FR.....	—	—	—	—	0									

VIRUS VRY

PMB.....	—	1	—	—	0	—	1	3	1	3	4	4	4	14
1513.....	1	—	—	—	0									
FR.....	1	—	—	—	0									

VIRUS KA

PMB.....	6	1	—	—	12	—	—	—	1	3	—	1	3	130
1513.....	—	—	—	—	0									
FR.....	—	—	—	—	0									

VIRUS OD

PMB.....	—	1	—	—	0	—	—	—	—	4	3	4	3	30
1513.....	1	—	—	—	0									
FR.....	2	1	—	—	0									

APPENDIX No. 4

NEUTRALIZATIONS OF VACCINE STRAINS AND CERTAIN BREAKDOWN STRAINS BY SERA OBTAINED FROM BREAKDOWN CASES IN IMMUNIZED HORSES

VIRUS A 501

	Serum Dilut.						Virus Dilut.								
Serum.....	2	8	32	128	512	Tit.	U.D	5	10	20	40	80	160	320	LD50
P1.....	6	6	6	6	6	512	—	—	—	2	2	3	4	4	40
1100.....	6	6	5	6	6	512									
1408.....	6	5/5	6	6	6	512									
1513.....	6	6	6	5	6	512									
P2.....	6	6	6	6	6	512									
2627.....		1	1	—	—	0									
1096.....	6	6	6	5	6	512	—	—	—	—	3	3	4	4	35
1582.....	6	6	6	6	6	512									

VIRUS KA

1096.....	6	6	6	5	1	255	—	—	1	2	2	3	4	3	40
1582.....	6	6	5	5	4	512									
P1.....	6	6	6	5	5	512	—	—	1	1	3	2	2	2	63
1100.....	6	6	6	6	6	512									
1408.....	6	6	5	6	3	405									
1513.....	6	5	6	6	5	512									
P2.....	6	6	6	5	3	405									
2627.....		4	3	1	—	26									

VIRUS VH

1096.....	6	6	6	6	6	512	—	1	1	3	3	3	4	4	16
1582.....	6	5/5	6	5/5	5	512									
P1.....	6	6	6	6	6	512	—	—	—	2	1	4	4	3	44
1100.....	5/5	5	6	6	4	512									
1408.....	6	5	5/5	5/5	4	512									
1513.....	6	6	6	6	5	512									
P2.....	6	6	6	6	4	512									
2627.....		—	—	—	—	0									

VIRUS 1180

1096.....	6	6	5	4	2	222	—	—	—	4	2	4	3	4	20
1582.....	6	6	6	5	4/5	512									
P1.....	6	6	6	5	5	512	—	—	—	—	—	2	3	3	139
1100.....	6	5/5	5	5	1	222									
1408.....	6	6	6	6	6	512									
1513.....	6	6	6	6	5/5	512									
P2.....	6	6	6	6	5	512									
2627.....		—	—	—	—	0									

APPENDIX No. 4 (*continued*)

VIRUS OD

	Serum Dilut.						Virus Dilut.								
Serum.....	2	8	32	128	512	Tit.	U.D	5	10	20	40	80	160	320	LD50
1096.....	6	6	6	5	4	512	—	—	1	1	3	3	4	3	28
1582.....	6	6	5	6	5	512									
P1.....	6	6	6	5/5	5	512	—	—	—	1	1	1	4	4	89
1100.....	6	6	6	6	3	512									
1408.....	6	5	5	5	4	405									
1513.....	6	5	6	3	—	101									
P2.....	6	6	1	1	3	32									
2627.....		1	—	1	—	0									

VIRUS L

1096.....	6	6	5	5	2	255	—	—	—	—	1	2	2	3	113
1582.....	6	6	6	6	6	512									
P1.....	6	5	6	6	4	512	—	1	—	—	1	2	1	1	91
1100.....	6	6	6	5	6	512									
1408.....	6	6	6	6	4	512									
1513.....	5	5	5	6	2/5	255									
P2.....	6	6	5	6	5	512									
2627.....		5	2/5	3	2	80									

VIRUS 114

1096.....	5/5	6	6	6	5	512	—	—	—	3	3	3	3	3	56
1582.....	6	6	5	6	4	512									
P1.....	6	6	6	6	6	512	—	1	1	1	3	2	3	4	31
1100.....	6	6	6	6	5	512									
1408.....	6	6	6	6	6	512									
1513.....	6	6	6	6	4	512									
P2.....	6	6	6	6	6	512									
2627.....		1	4	—	1	8									

VIRUS VRY

1096.....	6	6	6	6	—	255	—	—	—	—	1	2	2	3	113
1582.....	5	6	6	1	—	63									
P1.....	6	6	5	5	2	255	—	—	1	1	2	2	4	4	40
1100.....	6	6	4	6	—	202									
1408.....	6	6	6	4	2	255									
1513.....	6	6	2	3	—	40									
P2.....	5	6	3	—	1	32									
2627.....	1	—	—	—	—	0									

APPENDIX No. 4 (*continued*)

VIRUS 1513

	Serum Dilut.						Virus Dilut.								
Serum.....	2	8	32	128	512	Tit.	U.D	5	10	20	40	80	160	320	LD50
1513.....	6	6	6	6	6	512	—	1	—	—	3	4	4	4	28

VIRUS 1408

1408.....	6	6	6	6	6	512	—	—	—	—	1	3	2	4	71

VIRUS 1100

1100.....	1	3	3	2	—	8	—	—	—	—	—	—	2	3	190

VIRUS 1582

1582.....	6	6	6	5/5	6	512	—	—	—	—	2	4	2	3	58

VIRUS 1096

1096.....	6	6	6	4	5	512	—	—	—	3	1	1	2	3	89

VIRUS 2627

2627.....		—	—	—	—	0	—	—	—	—	—	2	2	4	113

VIRUS P2

P2.....	5	3	2	2	—	16	—	—	—	—	—	2	3	3	108

VIRUS P1

P1.....	6	6	5	6	3	403	—	—	—	1	3	3	2	4	40