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IDENTIFICATION AND TYPING OF HORSE-SICKNESS VIRUS STRAINS ISOLATED IN THE RECENT EPIZOOTIC OF THE DISEASE IN MOROCCO, TUNISIA, AND ALGERIA

By

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Summary

Several virus strains isolated during the recent epizootic of African horse-sickness in North Africa were studied. These strains were either received for confirmation and typing or were isolated from samples submitted to the Razi Institute, Iran.

The virus strains were identified as African horse-sickness virus because of their pathogenicity for suckling mice, adult mice, guinea pigs and horses (in one case) as well as their ability to fix complement in the presence of horse-sickness antiserum when tested by the complement fixation test.

In serum neutralization tests in mice, using antisera prepared against all known types of horse-sickness virus, the virus strains were most strongly neutralized by type 9 antiserum and, to some extent, by type 6 antiserum. The other antisera did not neutralize the virus even at 1/5 dilutions.

Precise specific neutralization tests in monkey kidney stable (MS) cell cultures showed that the strains should be considered as type 9 African horse-sickness virus.

Introduction

In 1965 a fatal disease clinically diagnosed as African horse-sickness was reported from Morocco and Algeria. The disease spread rapidly over the infected

area and caused a high mortality among soliped population of both countries.

In spite of all sanitary control measures and a mass vaccination, the disease progressed gradually northward and appeared in Tunisia (June 1966) and the southern part of Spain (Oct. 1966).

The virus has been isolated in Onderstepoort Laboratory, Razi Institute, Algerian Pasteur Institute, and Institut d'Elevage et de Médécine Vétérinaire, Alfort, and identified as type 9 horse-sickness virus.

This communication presents the results of studies on several horse-sickness virus strains isolated during this outbreak.

Methods and Materials

Tissue Cultures: Monkey kidney stable (MS) cells were used throughout the experiment. The source of the cells, the method of its cultivation and its susceptibility to horse-sickness virus have been described in a previous paper. (9)

Animals: 3 to 5 day-old Suckling mice, 30 to 35 day-old, adult mice, and guinea pigs were supplied by the animal breeding section of the Razi Institute.

Nonvaccinated healthy horses were bought from a hitherto horse-sickness free area in the northern part of Iran and were kept in a mosquito proof stable for at least one month before and during the experiment. The horses were shown to be free of horse-sickness antibodies before use.

Virus strains: Seven antigenically different neurotropic vaccine strains, namely A501, OD, L, Vryheid, VH, 114, and Karen, as well as viscerotropic strain 18/60 were used as representatives of types 1 to 8 of horse-sickness virus, respectively. They were obtained from the Onderstepoort Veterinary Laboratory, South Africa. Neurotropic strain S2 was used as type 9 virus. This was originally isolated in Iran and was attenuated through more than 100 intracerebral passages in adult mice. (4)

The origin of newly isolated virus strain, namely strains FA 1/66, Alg 2/66, M 3/66, Alg 5/66, T 6/66, Alg 12/66, Alg 13/66, Alg 14/66, Alg 15/66, Alg 16/66, and Alg 17/66, isolated during the recent outbreak of horse-sickness in Algeria, Morocco, and Tunisia, are presented as follows:

Strain FA 1/66 was isolated from a horse in Morocco by Institut d'Elevage et de Médécine des Pays Tropicaux, Alfort, and submitted to this Institute for typing at the first passage level in mouse brain. Strain M 3/66 and T 6/66 were isolated from horse bloods received from Morocco and Tunisia, respectively. Alg 2/66, Alg 5/66, Alg 12/66, Alg 13/66, Alg 14/66, Alg 15/66, Alg 16/66, and Alg 17/66 were received at different mouse passage levels through courtesy of Dr. Pilo-Moron of the Algerian Pasteur Institute. These strains were designated, by the Pasteur Institute, as ELOUED 1/65, SAIDA 1/65, COLOMB BECHAR 1/65 TADJMOUT 1/65, GOURAYA 1/66, LEMNAH 1/66, AISSAI 1/66, BARIKA 1/66, respectively. (10)

A few additional passages of each strain were made in mice to prepare fresh seed virus to use in the experiments and to adapt them to MS cell cultures. The technique for isolation and passage of virus in mouse brain and adaptation

to MS cell cultures were previously described. (4,9)

Antisera: Specific antisera against 9 types of the virus were obtained from hyperimmunized rabbits. The animals were hyper-immunized by using mouse or tissue culture adapted virus as antigens. The methods of antigen preparation and hyperimmunization were similar to those described in a previous paper. (5)

Antiserum against one of the newly isolated strains, i.e. FA 1/66, was prepared in rabbits in a similar manner using mouse adapted virus as antigen.

The sera, without any preservative, were stored at —20 C. until use.

Complement fixation tests: Ten per cent suspension of infected mouse brains harvested from an early passage of the virus in suckling mice were prepared in sterile saline. This preparation was allowed to stand at 4 C. overnight and then centrifuged at 10,000 r.p.m. for one hour and the supernatant was used as antigen in a complement fixation test. Normal mouse brains and horse-sickness infected mouse brains prepared in a similar manner were used as negative and positive control antigens. Known positive and negative horse sera were used as positive and negative sera in each test.

In each test dilutions of antigens were tested against different dilutions of complement. Controls for all the reagents were included in each test.

The set was stored at 4 C. overnight. The hemolytic system consisting of optimal hemolysin dilution and 2.8 per cent washed sheep red blood cells were added to the test after being incubated at 37 C. for 30 minutes. The tubes were centrifuged at 800 r.p.m. for 5 minutes and the degree of hemolysis was determined by comparison with a colour standard prepared by the same red blood cells on the same day.

Neutralization tests: For screening tests 1/5, 1/50 and 1/500 dilutions of each inactivated hyperimmune serum against nine different types of horse-sickness virus, were mixed with an equal volume of a suspension of mouse brains infected with the virus to be tested containing 100 to 300 mouse LD 50 per 0.03 ml. The virus- serum mixtures were throughly mixed and were then incubated for 60 minutes at 37 C. The serum - virus mixtures were then tested for unneutralized virus by inoculating 8 mice per mixture, inoculum being 0.03 ml. The inoculated mice were observed for 2 weeks and the titer of sera was calculated by the Reed and Muench method. (11)

More precise neutralization in order to find the exact relation of the strain to the type of virus, was carried out in MS cell culture tubes. The technique of the tests in MS cells was described previously. (5) The neutralization index and the titer of serum against a given dose of virus was calculated by the Reed and Muench method. (11)

Ether sensitivity tests: To the virus suspension diethyl ether was added at the rate of 20 per cent. (2) The mixture was held in a screw-capped bottle firmly closed with adhesive tape around the cap to prevent evaporation of the ether. The bottle was kept at 4 C. for 18 hours and then the ether was removed by pouring the treated virus suspension into an uncovered sterile petri dish and leaving at room temperature for 15 minutes. Some of the virus suspension without ether was

similarly treated and served as control. The infectivity of both, ether treated virus suspension and the control, were titrated in MS cell culture tubes.

Chloroform sensitivity tests: It was found that a mixture of 0.05 ml. of chloroform and 1 ml. of virus suspension represented a suitable proportion for chloroform sensitivity test. (3) The virus containing tissue culture fluid was mixed with the chloroform. The resulting emulsion was gently mixed for 10 to 15 minutes at room temperature and was then centrifuged at 400 r.p.m for 5 minutes. The clear part of the supernatant was removed and titrated as before. A part of virus suspension, without chloroform was similarly treated and used as control.

Results

Pathogenicity of the strains for Laboratory animals: Each strain was isolated in suckling mice and then its pathogenicity for adult mice, guinea pigs and rabbits was studied. Mice and guinea pigs were found fully susceptible to the strains by intracerebral inoculation. The inoculated animals reacted and produced symptoms identical to those caused by horse-sickness virus in these animals. (1) Within limited passages of the virus in these animals the incubation period gradually decreased.

Rabbits were found to be resistant to the virus strains when inoculated by intracerebral, intraperitoneal, and intravenous route of injection.

Susceptibility of cell cultures to the strains: All virus strains were easily adapted to MS cell cultures. BHK and green monkey cells were also found to be susceptible to the isolates by mouse adapted or MS adapted virus.

Pathogenicity of the isolates for horses: An Iranian mixed breed yearling female horse (Horse No. 77) obtained from a village by the Caspian sea and kept under observation for more than one month was inoculated intravenously by 6 ml. O.C.G. blood of an infected horse received from Tunisia (Gabes). Starin designated T. 6/66 was isolated from this sample.

The horse was kept in a mosquito proof stable and closely observed. The animal showed a significant rise in body temperature from 7 days after inoculation, (Fig. 1). Swelling of the supra-orbital fossae became evident on the same day and increased gradually during the rest of the period. On the 11th day after inoculation the animal depressed hanging its head down and showing anorexia. During the rest of the observation period the animal was depressed and was often found lying down. Slight temporal swelling, slight petechial haemorrhages of the ventral surface of the tongue and slight nasal discharge were also observed.

On the 15th day after inoculation the horse was found lying down with abnormal breathing of 20/min. and a pulse of 72/min. The condition of the animal was very bad and it was killed for an immediate necroscopy.

The autopsy revealed changes similar to those found in the cardiac form of horse-sickness, the major pathological finding was on the heart. (6) The lungs were not particularly involved. There was a lot of yellowish gelatinous exudate in the subcutaneous tissues along the head and neck

The virus was isolated from the blood taken during the last 9 days of the observation period. The same virus was also isolated from the post mortem tissue materials.

In another experiment horse No. 80 was inoculated intravenously with 5 ml. of defibrinated blood of horse No. 77 (blood was collected on the 9th day after infection). The animal showed a significant rise in body temperature from the 5th day after infection showing similar symptoms as horse No. 77 (Fig. 2). The horse was found dead on the 8th day having a lot of foam in the nose.

In autopsy, much serous fluid was observed in the pericardial cavity. There were haemorrhages in epicard, myocard and endocard. The subendocardial haemorrhages were rather diffuse. The lungs were oedematous with a wet appearance. The trachea and bronchi to the oedematous lungs were filled with frothy fluid and there was an excess amount of pleural fluid. The intralobular septa stood out prominently. The other organs appeared normal except slight congestion in some parts of the liver.

The virus was isolated from blood taken during the last 4 days of the observation period and from the post mortem tissues.

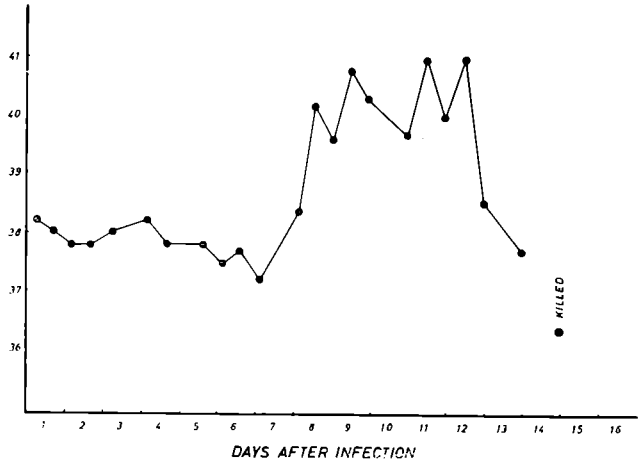

Fig. 1. Postexposure temperature (C.) of horse No. 77

Complement fixation tests: Mouse brain antigen for each strain was prepared as mentioned before. Different dilutions of this antigen were tested against different amounts of complement. The control for all the reagents were included in the test. The complete test carried out with strain FA1/66 virus is showen in table 1, and the results of the similar tests performed with the other strains are summarized in table 2.

The results showed clearly that the virus strains fixed the complement in the presence of specific horse-sickness antiserum.

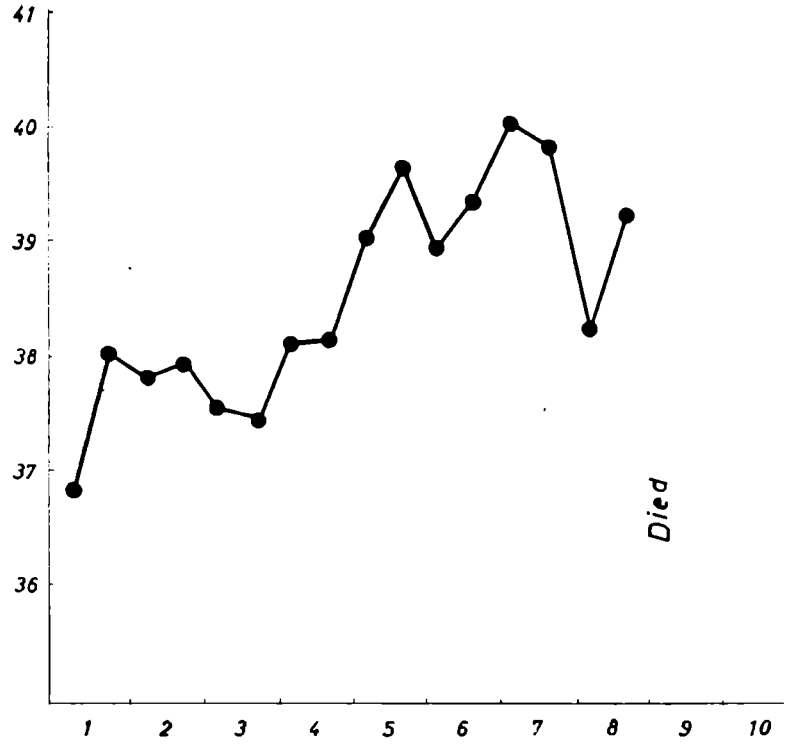

Fig. 2. Postexposure temperature (C.) of horse No. 80

Table 1 : Complement fixation test; testing different dilutions of strain FA1/66, as antigen, against dilutions of complement.

Antigen	Serum	Complement dilutions 1/80	1/50	1/30	1/20	1/15
Veronal buffer (VB)	VB	3	0	0	0	0
Antigen und.	"	3	TR	0	0	0
Antigen 1/2	"	3	TR	0	0	0
Antigen 1/4	"	2	0	0	0	0
Normal mouse brain und.	"	3	0	0	0	0
Normal mouse brain 1/2	"	3	0	0	0	0
VB	+ve serum	3	0	0	0	0
Control antigen	"	4	4	4	4	4
Antigen und.	"	4	4	4	4	4
Antigen 1/2	"	4	4	4	4	1
Antigen 1/4	"	4	4	4	1	0
Antigen 1/8	"	4	3	0	0	0
Normal mouse brain und.	"	4	2	0	0	0
Normal mouse brain 1/2	"	4	0	0	0	0
VB	—ve serum	3	0	0	0	0
Control antigen	"	3	0	0	0	0
Antigen 1/2	"	3	0	0	0	0
Antigen 1/4	"	3	0	0	0	0
Normal mouse brain und.	"	4	3	0	0	0
Normal mouse brain 1/2	"	4	2	0	0	0

Und. undiluted.
0 Complete hemolysis.
4 No hemolysis.

Table 2 : Complement fixation tests with newly isolated strains of horse-sickness virus.

Antigen	Serum	Complement dilutions				
		1/80	1/50	1/30	1/20	1/15
Alg 2/66+	+serum	4	4	4	3	0
M 3/66	"	4	4	3	2	1
Alg 5/66	"	4	4	4	3	3
Alg 12/66	"	4	4	4	1	0
Alg 13/66	"	4	4	4	0	0
Alg 14/66	"	4	4	3	1	0
Alg 15/66	"	4	4	4	2	2
Alg 16/66	"	4	4	4	4	1
Alg 17/66	"	4	4	4	3	0
VB	"	3	0	0	0	0
Control antigen	"	4	4	4	4	4
Normal mouse brain	"	4	2	0	0	0
Alg 2/66	—ve serum	4	TR	0	0	0
M 3/66	"	4	0	0	0	0
Alg 5/66	"	4	0	0	0	0
Alg 12/66	"	4	0	0	0	0
Alg 13/66	"	4	0	0	0	0
Alg 14/66	"	4	0	0	0	0
Alg 15/66	"	4	1	0	0	0
Alg 16/66	"	4	1	0	0	0
Alg 17/66	"	4	0	0	0	0
Control antigen	"	4	0	0	0	0
Normal mouse brain	"	4	1	0	0	0

+ The results of the test with 1/2 dilution of antigens were only recorded here.

0 = Complete hemolysis; 4 = No hemolysis.

Ether and Chloroform Sensitivity: Passages 6 or 7 of the virus strains in MS cell cultures were used in this experiment. The virus was harvested whenever the cynthopathic changes were widespread, the suspension was then centrifuged at 2500 r.p.m. for 15 minutes and the supernatant was used in the test. The procedure was exactly as mentioned above and the titration of treated and untreated virus suspensions were performed in MS cell culture tubes.

The titers of control and viruses exposed to ether and chloroform are presented in tables 3 and 4, which indicate that, under the conditions of the tests, the virus strains were only slightly inactivated by chloroform and ether. However, the inactivation appeared to be in the same range as that of S2 strain, a known type 9 horse-sickness virus, exposed simoultaneously to the chemicals. On the basis of these results it was concluded that the newly isolated strains are ether and chloroform resistant.

Table 3: Action of Ether on newly isolated strains of horse-sickness virus.

Strain	Log. TCID50 titer		
	Treated virus	Control	Difference
FA 1/66	5.5	6.0	—0.5
Alg 2/66	6.0	6.8	—0.8
M3/66	5.0	5.5	—0.5
Alg 5/66	5.2	5.5	—0.3
T 6/66	6.4	7.0	—0.6
Alg 12/66	6.5	7.0	—0.5
Alg 14/66	4.0	4.5	—0.5
Alg 15/66	6.0	6.3	—0.3
Alg 16/66	5.2	6.0	—0.8
Alg 17/66	6.5	7.0	—0.5
S2	5.7	6.5	—0.8

Table 4: Action of Chloroform on newly isolated strains of horse-sickness virus.

Strain	Log. TCID50 titer		
	Treated virus	Control	Difference
FA 1/66	5.5	6.5	—0.5
Alg 2/66	4.5	5.2	—0.7
M 3/66	5.2	6.0	—0.8
Alg 5/66	5.0	5.5	—0.5
T 5/66	6.2	7.0	—0.8
Alg 12/66	6.0	6.5	—0.5
Alg 14/66	4.5	5.0	—0.5
Alg 15/66	5.5	6.3	—0.8
Alg 16/66	5.5	6.5	—1.0
Alg 17/66	6.4	7.0	—0.6
S2	5.5	6.2	—0.7

Serologic relationship between newly isolated virus strains and their similarity with known types of horse-sickness virus: For the purpose of rapid estimation of serologic similarity of each isolate to known types of HS virus, a neutraliza-

tion test was performed as soon as the virus was adapted to mouse brain. The virus was tested against 9 different type specific antisera in mice as outlined before. The results of these tests are showen in table 5.

Based on the results of these experiments indicating the similarity of newly isolates to type 9, and to some extent, to type 6 horse-sickness virus, it was decided to prepare antiserum against one of these strains and investigate its exact relation to known type of HS virus.

Strain FA1/66 was chosen and a complete cross neutralization test was performed with each of 9 serologically different types of HS virus vaccine strains. The tests were done in MS cell culture tubes as previously described. The neutralization index of each serum against homologous and heterologous virus, as well as, the titer of each serum against approximately 100 TCID 50 of homologous and heterologous virus were calculated. The results summarized in table 6 indicate that there is a complete cross neutralization reactions between strain FA1/66 and type 9 horse-sickness virus. There was no reaction between this strain and type 1,2,3,4,5,7, and 8. The strain was neutralized slightly by type 6 antiserum and vice versa.

In a similar manner the degree of serologic relationship of all the newly isolates to strain S2 (type 9) and FA1/66 was studied by testing each strain against S2 and FA1/66 antisera. The results showed that all isolates are identical being strongly neutralized by FA1/66 and S2 antisera, Table 7.

Table 5 : Neutralization of newly isolated strains by horse-sickness type specific antisera in mice.

Strain	LD50	Type antisera								
		T.1	T.2	T.3	T.4	T.5	T.6	T.7	T.8	T.9
FA1/66	2.3[+]	0	0	0	0	0	0.7	0	0	1.7[++]
M 3/66	2.4	0	0	0	0	0	0.7	0	0	>1.7
Alg 5/66	2.0	0	0	0	0	0	1.7	0	0	>2.7
T.6/66	2.5	0	0	0	0	0	0.5	0	0	1.7
Alg 12/66	1.8	0	0	0	0	0	1.7	0	0	>2.7
Alg 14/66	2.0	0	0	0	0	0	1.2	0	0	>2.7
Alg 15/66	2.3	0	0	0	0	0	0.7	0	0	>2.7
Alg 16/66	2.5	0	0	0	0	0	0.5	0	0	1.7
Alg 17/66	2.0	0	0	0	0	0	0.7	0	0	>2.7

\+ Log virus used in the test.

++ —Log serum titer.

0 = Serum at 1/5 dilution did not give 50 per cent protection.

Table 6 : Cross neutralization tests of strain FA1/66 and nine types of horse-sickness virus by specific antisera.

Virus	Specific antisera									
	T.1	T.2	T.3	T.4	T.5	T.6	T.7	T.8	T.9	FA1/66
T.1	5.6+ 2.4++									0 0
T.2		5.0 2.1								0 0
T.3			5.0 2.0							0 0
T.4				5.4 2.4						0 0
T.5					5.5 2.4					0 0
T.6						5.6 2.3				1.8 0.8
T.7							6.0 2.5			0 0
T.8								5.6 2.4		0 0
T.9									6.2 2.9	4.9 2.0
FA1/66	0 0	0 0	0.6 0	0 0	0 0	2.2 1.0	0 0	0 0	6.2 2.8	6.0 2.5

\+ = Logarithm of neutralization index of the serum.
++ = —Log serum titer against approximately 100 TCID50 of the virus.
0 = 0 or negligible.

Table 7 : Neutralization of newly isolated strains by S2 and FA1/66 antisera.

Virus Strain	Antisera FA 1/66	S2(T.9)
Alg 2/66	>4.5 + 2.1 ++	5.5 2.3
M 3/66	>4.5 2.0	5.0 2.1
Alg 5/66	>4.2 2.1	NT
T. 6/66	4.8 1.8	5.6 2.2
Alg 12/66	5.8 2.8	6.0 2.4
Alg 13/66	>4.0 1.8	NT
Alg14/66	4.8 2.0	NT
Alg 15/66	5.0 2.8	NT
Alg16/66	5.5 2.0	>5.0 2.2
Alg 17/66	5.5 2.2	6.0 2.4

\+ = Log Neutralization index of the serum.
++ = - Log titer of serum against approximate 100 TCID 50 of the virus.
NT = not tested.

Discussion

It has been shown that for the control and eradication of African horse-sickness and to prevent the introduction of the disease from infected to clean areas, in addition to sanitary control measures, a mass vaccination scheme of solipeds by a suitable vaccine is of absolute necessity.

There exists 9 known immunologic types of horse-sickness virus, (7,8) and due to this plurality of virus, in the enzootic regions where more than one type of virus has been identified, a polyvalent vaccine consisting of the existing types is necessary, whereas in areas where only one type of virus exists, monovalent vaccine giving a better immunity is preferable. This is especially true when horse-sickness appears in a hitherto horse-sickness-free area. In such a case as the 1959 epizootic of the disease in the Middle East, (4) outbreaks usually originate from a single focus of infection and only one type of virus is present. In order to control the disease and to prevent its spreading, which due to the susceptibility of soliped population, is very quick, a rapid typing of virus is of much importance.

McIntosh (8) showed that it was possible to classify new horse-sickness virus strains by neutralization test without the necessity of preparing antisera against these strains. This technique was utilized in the rapid classification of virus strains isolated in the recent outbreak of horse-sickness in North Africa. Each strain was tested against 9 type specific antisera and the results showed that the isolates were more strongly neutralized by type 9 and to some extent by type 6 antisera. These findings correspond to those of Pilo-Moron et al, (10) who typed several virus strains isolated during the recent outbreak of the disease in Algeria, and that of Taslimi working with Virus strains isolated in Morocco (Personal Communication).

More precise neutralization tests were done after adaptation of the isolates to monkey kidney stable (MS) cells and preparation of antiserum against one of the isolates. Based on the results of these tests (Tables 6 and 7). The isolates were classified as type 9 horse-sickness virus with some antigenic similarity to type 6.

Several convalescent sera collected from recovered horses and mules during the epizootic of horse-sickness in Algeria, Morocco and Tunisia, were tested for horse-sickness antibodies. The results of these tests were not presented in this communication, but, however, it would be of interest to note that the sera showed a high titer in complement fixation tests against horse-sickness antigen. Whereas in neutralization tests only type 9 horse-sickness virus was strongly neutralized by the sera.

Complement fixation tests, Pathogenicity tests, ether and chloroform sensitivity tests were done for confirmation of virus identification. The isolates were found to be pathogenic for mice and guinea pigs, readily adapted to MS, BHK and GM cells, ether and chloroform resistant and had the ability to fix complement in the presence of horse-sickness antiserum in complement fixation tests.

An opportunity to investigate the pathogenicity of one of the isolates, namely strain T6/66, for horses, was offered during "The transmission study of horse-sickness by mosquitoes". Horse No. 77 inoculated by the blood sample T.6/66 received from Tunisia, and horse No. 80 inoculated with the blood of horse No. 77, developed a classical form of horse-sickness. (6) Horse-sickness virus serologically identical to type 9 was isolated from the blood of both animals during the febrile reactions and from the tissue materials after death.

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