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COMPARATIVE ATTENUATION OF AFRICAN HORSESICKNESS VIRUS IN MOSQUITO (AEDES ALBOPICTUS) AND IN HAMSTER KIDNEY (BHK-21) CELL LINES. (*)

H. Mirchamsy, A. Hazrati, S. Bahrami, A. Shafyi, and M. Mahinpour

In previous reports (6, 9) it was shown that African horsesickness (AHS) virus may be adapted to grow in primary hamster kidney cell with appearance of cytopathic effect (CPE) and in mosquito *Aëdes albopictus* cell line without showing CPE but with persistant infection. It was also demonstrated (7) that by a limited serial passage in primary hamster kidney cell culture, the virus was attenuated for the horse and induced immunity in this animal and that by increasing the number of passages the virus lost its immunogenic properties. The present investigation was undertaken partly to continue the observation of subcultures of chronically infected mosquito cells with AHS virus and to see how long this infection may persist; also partly to compare the antigenic stability of virulent virus, adapted to *A. albopictus* cell or to baby hamster kidney cell line, in order to assess the possibility of developing attenuated vaccine strains of AHS virus by limited cell culture passages instead of the time-consuming technique of attenuation of virus by at least 100 intracerebral passages in mice (1, 4).

Materials and Methods

Establishment of a Subline of AHS Virus Carrier of Mosquito Cells

This subline of *A. albopictus* was established according to the procedure already described (9). The cultures of three 4-oz medical flat bottles of *A. albopictus* cells were inoculated with neurotropic AHS virus (strain (S2) of type 9) adapted to MS cells at its 7 th passage. The multiplicity of infection was 1. After an adsorption period of 60 min at 30°C, the cell sheets

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were washed 3 times with warm PBS solution, 12 ml of LAH medium containing 2% type-specific AHS rabbit antiserum were added and the bottles were incubated at 26°C. After 5 days the supernatant fluid was harvested and cells were washed 3 times with PBS solution and were collected by a rubber policeman in 12 ml of LAH medium containing 2% calf serum. Three bottles were cultured with harvested cells of one bottle. The cells were grown in LAH medium with 5% calf serum at 26°C. At weekly intervals, the cell sheet became confluent and the fluid of each bottle was saved and stored frozen at – 70°C. The cultures of two bottles were washed 3 times with PBS solution and were subcultured as before. The cells of the third bottle were suspended in 12 ml of LAH medium, sonicated and centrifuged as mentioned before (10) and the suspension of virus was stored at – 70°C for viral titration.

Adaptation of Virulent AHS Virus to Mosquito and BHK-21 Cell Lines Virus

A 10-percent suspension of mouse brain of virus strain 10/60 type 9 in PBS pH 7.2 containing 2% calf serum was used. This strain was isolated from a sick horse in South Iran in 1960 (6) and was passed twice intracerebrally in baby mice. A pooled suspension of infected mouse brain was prepared in PBS plus 2% calf serum, distributed in ampoules of 1 ml and stored frozen at –70°C. The original virus had a titer of $10^{6 \cdot 5}$ mouse LD 50/ml. Before use, the content of each ampoule was thawed and centrifuged at 3,000 rpm for 15 min.

Cell lines. The original *A. albopictus* mosquito cell line, kindly supplied by Dr. K.R.P. Singh, of the Virus Research Institute, Poona (India), as a 54th passage, underwent 120 passages in this laboratory. Details about growth and maintenance of this cell line have been given before (9).

Roux bottles of confluent monolayer of baby hamster kidney cell line (BHK-21) were kindly supplied by Dr. M. Amighi, of the Department of Foot-and-Mouth Vaccine Production of this institute. The cells were grown in Hanks solution containing 0.5% lactalbumin, 0.1% yeast extract and 5% calf serum.

Infection of cells. The growth medium of mosquito or BHK-21 monolayers were discarded, the cell sheets were washed once with the growth medium and 1 ml of stock virus was added to each Roux bottle. Three bottles of each cell line were infected. Adsorption was allowed to proceed at 30°C for 4 h. Non adsorbed virus particles were removed by 3-time rinses of cell sheets with a large volume of growth medium before 90 ml of corrsponding growth media containing 2% heated calf serum were added in each bottle. The infected mosquito cells were incubated at 26°C and BHK cells were kept at 36°C in an incubator.

Passage of virus. After 96 h the bottles of infected mosquito cells were

removed from the incubator and kept overnight at –70°C. Next day the contents of bottles were quickly thawed, the pooled cell suspension was sonicated and centrifuged as previously described (9). 10 ml of the virus suspension of the first passage were used as inoculum for seeding bottles of the second passage. The bottles containing infected BHK-21 were treated in the same way when CPE was observed in most parts of the cell sheet. On the first passage CPE was complete in 4 days, but in the following passages alteration of cells was completed in 48–60 h. Virus was harvested and passed in new cultures of BHK/21 as mentioned for mosquito cells.

Horses. Nonvaccinated healthy horses, 2–3 years old, were obtained from North Iran where AHS has not been reported before. Horses were kept under close observation for 2 weeks and rectal temperature was taken twice daily for 1 week before the experiment started. The animals were shown to be free of AHS antibodies before being used in the experiment.

Neutralization test. Neutralization antibody (NA) titer in the serum of inoculated horses was determined before and 4 weeks after infection. Sera were inactivated at 56°C for 30 min and the antibody titers were determined against 100 TCID50 of homologous virus strain in MS cell culture tubes, as described previously (8).

Results

Persistent Infection in Viral Carrier *A. albopictus* Cells

The first two subcultures of this subline were kept in LAH medium containing 2% type-specific antibody at 26°C. The supernatant fluids of these subcultures were free of AHS virus but intracellular virus was present at a low level (fig. 1). The following subcultures were made at 7- to 10-day intervals at 26°C, the cell sheet was confluent. The growth of cells was normal up to 17th passage, cells were apparently healthy and the cell-released or cell-associated viruses were present at a low level (fig. 1). During the 18th subculture the cells grew less satisfactorily and, even after 2 weeks, the monolayer of cells was not confluent. From the 18th to 27th passages, the cells of each bottle were cultivated in two new bottles instead of 3 bottles, in this way the cell sheet was half confluent in about 2 weeks but after 27 subcultures the cells started to alter by forming irregular rounded cells giving rise to the formation of clamps scattered on glass surface. The subculture of this subline was then discontinued. It is worth mentioning that the carrier cells, subcultured 27 times in a period of over 200 days, synthetized and released mature viruses at a low rate, the cell-associated virus titer being always higher than the released virus titer (fig. 1).

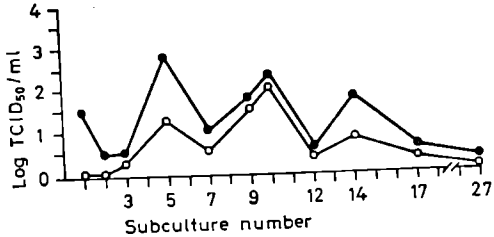


Fig. 1. The titer of virus in chronically infected cells in subculture series (0) free virus in supernatant fluid/ml (*) intracellular virus/ml.

Reactions of Horse to Virus Adapted to Mosquito Cells

After First Passage

Hosrses No. 7109, 7110, 7111 and 7112 were inoculated intravenously with 20 ml of virus passed only once in mosquito cells. These horses showed high fever, inappetance and viremia lasting for several days (fig. 2) but all had recovered 3 weeks later. After 1 month these horses were shown to have a high (NA) titer in their sera.

After Three Passages

Horses No. 7105–7108 were inoculated intravenously with 20 ml of AHS virus passed 3 times in mosquito cells. The virus had a titer of $10^{4.5}$ mouse LD50/ml. Control horses No. 7129 and 7132 were inoculated in the same way with 1 ml of original virus stock 10/60. Rectal temperature was recorded twice daily for 3 weeks. Horses inoculated with mosquito cell adapted virus had a febrile response 7–9 days postinoculation (fig. 3). Viremia was shown in these horses during the febrile period. At the end of the second week the rectal temperature became normal. These horses had a high NA titer 30 days after virus inoculation.

Horses No. 7129 and 7132 were inoculated as controls, with original virulent virus, and manifested a marked febrile response 6 days after virus inoculation characteristic clinical symptoms of AHS developed, which terminated in the death of the animals in the second week postinoculation (fig. 4).

Reversibility of Virus to Virulence after 3 *Passages in Mosquito Cells*

In order to investigate the reversibility of AHS virus, by passaging in susceptible horses, samples of blood collected from horses No. 7105–7108 during the high fever and when viremia was present were pooled. 75 ml of the pooled blood was inoculated intravenously and 75 ml intramuscularly to horses No. 7101–7104. These horses showed a high fever for 3–6 days, starting from 7 to 8 days postinoculation (fig. 5) but, in spite of a very severe clinical reaction, all recovered. Viremia was detected in these horses during the febrile period. All horses showed a high titer of NA in their sera 30 days postinoculation.

Blood samples of horses No. 7101–7104 collected during the febrile period with viremia were pooled. Horses No. 7126 and 7135 were inoculated with 150 ml of the pooled blood as mentioned above. These horses reacted by a marked rise of temperature 8–11 days after blood inoculation, together

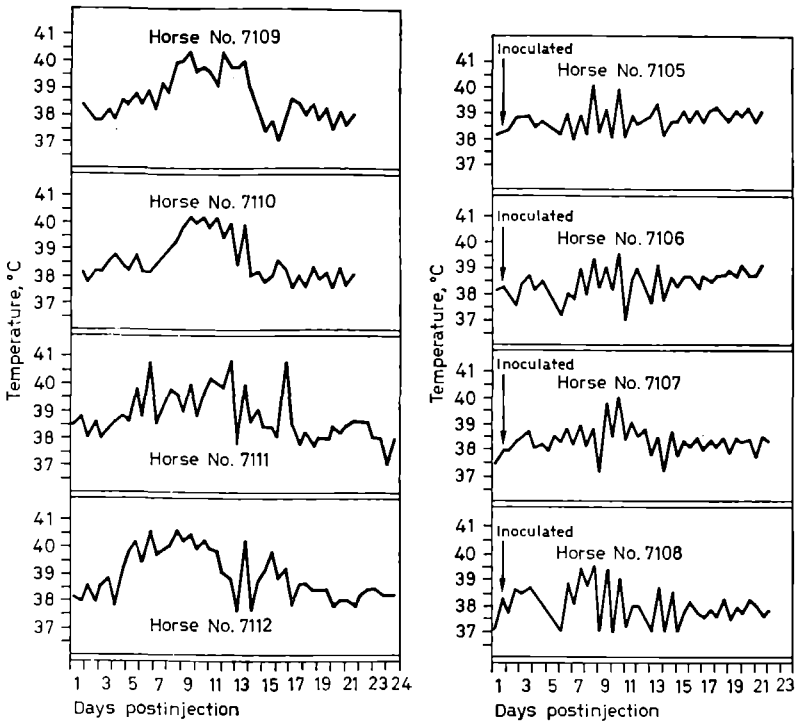


Fig. 2. Horses inoculated with 20 ml of virulent AHS virus passed once in mosquito cells.

Fig. 3. Daily temperature of horses inoculated with AHS virus, strain 10/60, passed 3 times in mosquito cell culture.

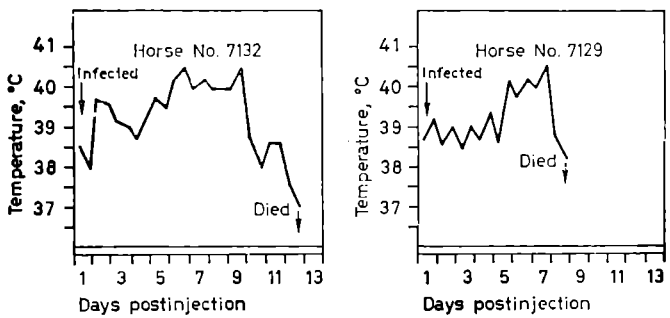


Fig. 4. Daily temperature charts of 2 horses infected with AHS virus, strain 10/60, 10% suspension of mouse brain, for control of virulence.

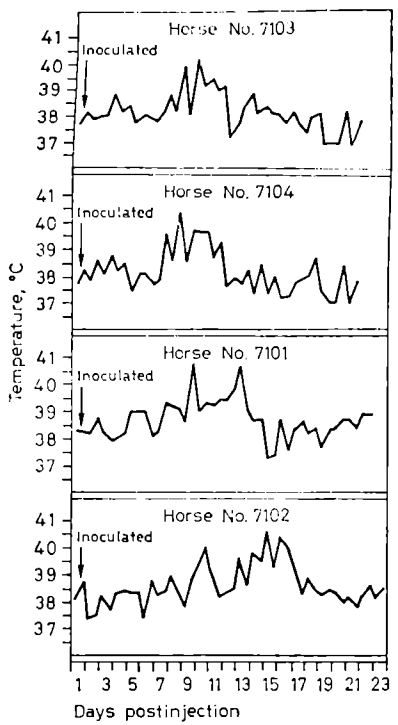
with lack of appetite and difficulty of respiration. They both died in the second week postinoculation with all symptoms of AHS (fig. 6).

After 7 Passages

Horses No. 7207 and 7208 were inoculated intravenously with 20 ml of AHS virus type 9, strain 10/60, passed 7 times in mosquito cells. After each passage virus was titrated in adult mice. It was found that after 5 and 7 passages the titer of virus in mice was $10^{4\cdot6}$ and $10^{4\cdot1}$ ID_{50}/ml, respectively.

The reaction of horses to this inoculation was mild, since only horse No. 7207 showed a slight hyperthermia 11 days after inoculation (fig. 7); at this stage viremia was present. Horse No. 7208 showed no reaction and viremia was not recorded. Both horses had a significant amount of NA in their blood 30 days after inoculation.

Stability of virus after 7 passages in mosquito celles. 150 ml of the blood of horse No. 7207 taken when viremia was present was inoculated into horses



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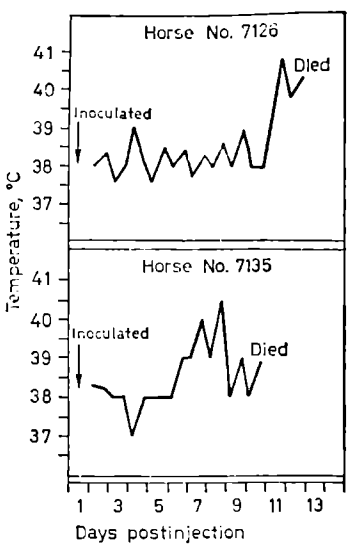

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Fig. 5. Horses inoculated with pooled blood of horses No. 7105–7108 (second passage in horse of AHS virus passed 3 times in mosquito cell).

Fig. 6. Horses inoculated with pooled blood of horses No. 7101–7104 (third horse passage of AHS virus passed 3 times in mosquito cell).

No. 7209 and 7210. These horses showed no thermal reaction (fig. 7) or symptoms of viral infection, the virus was not recovered in the following days from their blood but both had NA against type 9 one month after inoculation.

Reaction of Horses to Virus Adapted to BHK Cells

After 1 *Passage*

Horses No. 7211–7214 were inoculated by intravenous route with 20 ml of virus passed only once in BHK cells. The titer of virus was $10^{5\cdot5}$ mouse ID_{50}/ml. These horses reacted by a rise of temperature to over 40°C lasting

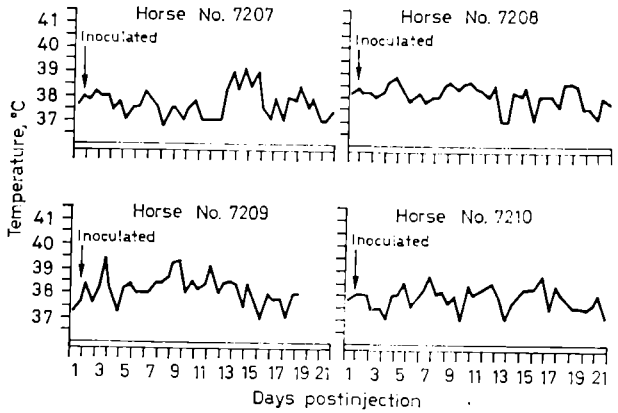

Fig. 7. Horses No. 7207 and 7208 inoculated with 20 ml of AHS virus passed 7 times in mosquito cell. Horses No. 7209 and 7210 inoculated with 150 ml of blood of horse No. 7207.

for several days (fig. 8), together with inappetance and viremia, but all of them had recovered 3 weeks later, showing a good serological response.

After 3 *Passages*

Horses No. 7215 and 7216 were inoculated intravenously with 20 ml of virus passed 3 consecutive times in a BHK cell line. The thermal reaction lasting 2 days was noticed in horse No. 7216 (fig. 9) together with viremia without any other clinical manifestation. Horse No. 7215 revealed no fever or any other sign of illness but showed viremia 9 days after inoculation. Both horses had large amounts of NA in their blood tested 1 month after virus inoculation.

Stability of attenuated virus. Horses No. 7217 and 7218 were both inoculated with 150 ml of pooled blood of horses No. 7215 and 7216 when the latter showed viremia. These horses showed no rise of temperature (fig. 9) or any other reaction in the following days but had a high NA titer 1 month after inoculation.

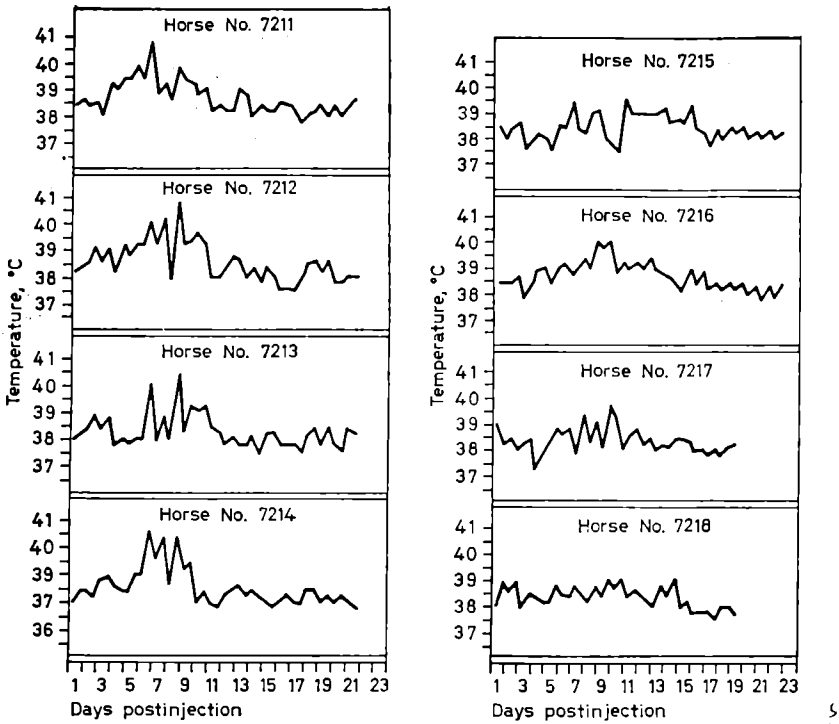

Fig. 8. Horses inoculated with 20 ml of AHS virus passed once in BHK-21 cells.

Fig. 9. Horses No. 7215 and 7216 inoculated with 20 ml of virus passed 3 times in BHK-21 cells. Horses No. 7217 and 7218 inoculated with 150 ml of pooled blood of horses No. 7215 and 7216.

After 7 Passages

Horses No. 7219 and 7220 were inoculated by intravenous route with 20 ml of the same virulent AHS virus passed 7 times in BHK cell line. The virus had a titer of $10^{4\cdot6}$ LD_{50}/ml mouse and $10^{4\cdot5}$ $TCID_{50}$/ml MS cells. No thermal or general reactions were recorded in the following days (fig. 10); however, viremia was observed in blood collected 8 and 10 days postinoculation.

Stability of virulence of attenuated virus. Blood samples of horses No. 7219 and 7220 were pooled and horses No. 7221 and 7222 were both inoculated with 150 ml of the pooled blood. Only horse No. 7221 showed a temporary thermal reaction not exceeding 39.8°C, 15 days after blood inoculation (fig. 10). AHS virus was isolated from blood samples of both horses.

A second transmission of the virus was done by inoculation of 150 ml of pooled blood of horses No. 7221 and 7222 to horses No. 7223 and 7224. Each horse received 75 ml of blood intravenously and 75 ml intramuscularly. Both horses remained healthy in the following days and showed an slight thermal rise of 39.6°C for a short duration (fig. 10). Viremia was observed in these

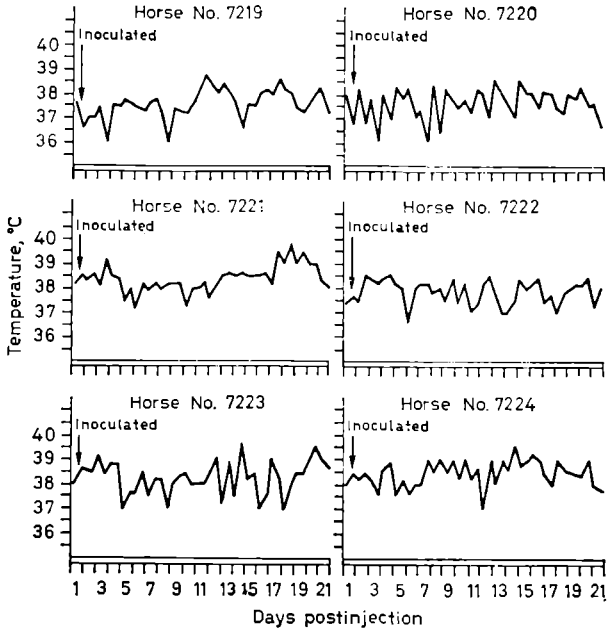

Fig. 10. Horses No. 7219 and 7220 inoculated with 20 ml of AHS virus passed 7 times in BHK-21 cell lines. Horses No. 7221 and 7222 inoculated with 150 ml of blood of horses No. 7219 and 7220. Horses No. 7223 and 7224 inoculated with 150 ml of blood of horses No. 7223 and 7224.

horses 9 days after blood inoculation. These horses had a high titer of NA 1 month after blood inoculation.

Discussion

In a previous report (9) it was indicated that AHS virus may persistently infect the mosquito *A. albopictus* cell line. The maturation of complete virus in cell generation and the release of virus were observed; however, cytopathic changes were not found in infected cells. In the present study a subline of *A. albopictus* cells infected with AHS virus was serially subcultured. No CPE was observed in the serially subcultured cells, but a decrease in multiplicity of carrier cells after 17 passages with a concomitant drop in virus titer indicated that the cells were damaged. These changes were similar to those observed by Zalkind *et al.* (11) in HEP-2 cell line persistently infected with tick-borne encephalitis virus (61 passages in 24 months). According to these authors, besides changes in metabolic activities of cells and absence of CPE in subcultures, a

marked heterogeneity of the cell population characterized mainly by the prevalence of small rounded cells forming peculiar conglomerates was observed in the 12th passage. The titration of AHS virus produced in and released from mosquito cells was done in MS cells. The CPE in the latter cell was the same as in the case of the original attenuated neurotropic virus strain S2, type 9, but the possible change of neurovirulence of this strain for mice following serial passage in mosquito cells was not studied.

The development of attenuated strains of AHS virus from virulent strains of virus in cell cultures for immunization of equine was first introduced by this laboratory (7) and was confirmed by Erasmus (3). The main purpose of the present attempts to immunize horses with virulent virus adapted to mosquito or to BKH-21 cell lines was to study the virus and mosquito cell interaction and the possible reversibility of cell culture attenuated virus to virulence.

The data presented here, and those not yet published, indicate that by only one passage of virulent virus in mosquito or in BHK cell lines, the virulence was decreased in a way that a large dose of virus could not kill a nonimmunized horse. The virus passed 3 times in mosquito cell line was quite attenuated for horses. The immunized animals showed hyperthermia and viremia without revealing any clinical symptoms of the disease. The pooled infectious blood of the horses, inoculated to new horses, caused thermal and general reactions specific to AHS, but horses had recovered when the infectious pooled blood of the second group of horses was inoculated to a third group; the animals showed a clear picture of AHS within 2 weeks after inoculation and died of the disease.

From these findings we may conclude that an apparently attenuated AHS virus may beecome virulent by 3 successive transmissions to susceptible horses.

It is reasonable to believe that this phenomenon may explain how outbreaks of the disease among the imported horses in enzootic areas where mosquitos or other vectors are in abundance is carried out. A virus of low virulence may be transmitted by some arthropod to horses who may not react in the first cycle of infection by a visible reaction, but in the second or third transmission cycle the virus may cause a new outbreak.

It is worth mentioning that in Iran, where an outbreak of AHS type 9 was observed in 1959, the new equine generations were fully susceptible to this type of virus and reservoirs of virus were not established. But in central, east and south Africa where epizootics are commonly recorded in hot and rainy season when biting insects and culicoides are in abundance the horse may also show neutralizing antibodies against AHS virus without apparent infection

(Perreau, personal communication). Wild animals such as zebra and elephant, from these areas, on the other hand, have shown neutralizing antibodies against different types of AHS virus in their sera (Hazrati, unpublished data). It is normal to believe that equines or wild animals of the enzootics regions have been inapparently infected in nature by insects or culicoides harboring slightly attenuated virus in their organism. The virus may gain full virulence by one or several passages in unprotected horses through arthropod bites, and outbreak of the disease will then be observed.

Although AHS virus can be adapted easily to mosquito *A. albopictus* cells and a subline of carrier virus was established, this mosquito has not been suspected to play a role in transmission of AHS virus. *A. albopictus* is the most common daytime-biting mosquito of man and, together with *A. aegypti,* are efficient vectors of Dengue virus (2) in southeast and central Asia, in Madagascar and in adjacent islands. The synthesis and release of complete virion from chronically infected mosquito cell may, however, support the idea of biological transmission of AHS by other species of mosquitoes.

It is also interesting to note that the virulent virus passed 7 times in mosquito cells loses its virulence for the horse and may be used as an immunogenic strain, since the response of horses to this strain was mild, the serological response was satisfactory and its virulence was not increased by one transfer of virus from horse to horse.

The lack of observation of viremia in horse No. 7208 does not mean the absence of viremia, since the duration of viremia for the attenuated strains may be so short that it may remain unnoticed.

The virulent AHS virus passed once in BHK cell line was still virulent for the horse, causing severe symptoms in inoculated horse who were finally recovered.

After 3 or 7 passages in BHK cell line the virus was quite attenuated. Horses immunized with virus passed 7 times in the latter cell line showed no clinical symptoms but viremia was noticed together with a high serological response. The stability of the attenuated virus was shown by two consecutive transmissions of this virus from horse to horse. The animals inoculated with pooled blood did not show any clinical symptoms and developed large amounts of type-specific neutralizing antibody.

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