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STUDY ON HORSE SICKNESS VIRUS STRAINS ISOLATED IN IRAN

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Summary:

1. Some 53 strains of Horsesickness virus have been isolated from a total of 167 samples received from different parts of Iran, during the recent outbreaks of the disease in the Middle Eastern countries.
   The method of isolating the virus from the infected materials using 4—5 day-old suckling mice is described, and it is shown that the incubation period in this animal when injected intracerebrally with the infected material, varied from 5 to 22 days.
2. Strain S 2, a locally isolated strain, was successfully attenuated by successive intracerebral passages in adult mice and it was found that the 65th generation of this strain was sufficiently attenuated while possessing a relatively good immunizing potency for equine animals.
3. It is shown, by cross-neutralization tests in mice using 5 local Horsesickness virus strains, that these strains are closely related to each other and all have an antigenic similarity to type six of Horsesickness virus.
4. The technique used for neutralization tests is also described in detail.

Introduction

In the spring of 1960, Horsesickness, which had first appeared in Middle Eastern countries in the summer of 1959, was reported from all areas of the Persian Gulf. The spreading of the disease was so intensive and rapid that its presence had also been recognized in Pakistan, Afghanistan, Turkey, and India by the time Horsesickness was officially confirmed in Iran in May 1960.

The disease spread rapidly and continuously into the neighbouring countries so that, by October 1960, all countries stretching from Turkey to

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India, having a horse population of about 13,000,000 were overrun with the disease.

At the begining, the mortality of infected soliped animals was very high due to lack or shortage of means to cope with the onslaught. The disease however, was eventually controlled by a mass scale vaccination of all equines throughout the affected areas.

Although Horsesickness is now under control, the danger of its reappearance in infected areas or of appearance in hitherto unaffected countries still remains. Therefore, it is of the utmost importance to increase knowledge of the disease in various aspects, such as possibility of preparing an economically and immunologically more satisfactory vaccine, determination of the vectors and reservoires of the virus, serological identification of the virus strains isolated in this part of the world, and so on.

In the present paper, a summary of the primary investigations on Horsesickness strains isolated in various parts of Iran is described. Although the experiment is not complete, it could prove helpful in the future studies of this disease.

1. Horsesickness virus strains isolated in Iran

Some 53 strains of Horsesickness (H. S.) virus have been isolated from a total of 167 samples received from different parts of Iran, since May 1960, (table 1).

The samples were submitted as a mixture of equal volumes of O.C.G. solution and blood of the suspected animals, having been sent mostly in iced thermos flasks by the fastest available means.

Table 1

Animal	No. of specimens	Positive	Negative
Horse	132	43	89
Mule	15	6	9
Donkey	20	4	16
Total	167	53	114

(Technique) : Although guinea-pig and adult mice are susceptible to the viscerotropic virus of Horsesickness, (Alexander, 1935), to isolate the virus

from the infected materials preference was given to the method using 4—5 day-old suckling mice.

Each sample was treated as follows :

The O.C.G. blood was diluted in equal volume with sterile distilled water. Penicillin 1,000 units, streptomycin 2 mgm. and mycostatin 80 units per ml. of the O.C.G. blood were added. The final product was left at room temperature for 30 minutes, and then 6 suckling mice were injected intracerebrally receiving 0.025 ml. each.

The incubation period in suckling mice varied from 5 to 22 days at the first passage, (table 2). Thus all the negative results were read after 3 weeks' observation.

Table 2

No. of isolated strains	Incubation period
9	5 days
7	6 days
9	7 days
10	8 days
3	10 days
3	11 days
2	13 days
3	15 days
2	17 days
1	18 days
2	20 days
2	22 days
Total 53	

The second and third passages were carried out either through suckling or adult mice, but for further passages only adult mice were used.

In each passage the brains of sick mice were harvested in extremis, and the supernatant of a 10 per cent suspension of these infected brain materials in serum saline containing 500 units penicillin and 0.5 mgm. streptomysin Per ml., after centrifugation at 3,000 r.p.m. for 30 minutes, being bacteriologically sterile, was used for the next passage. The doses employed were 0.025 and 0.05 ml. in suckling and adult mice, respectively.

11 - Neurotropic fixation and attenuation of strain S 2.

This strain has been isolated from the O.C.G. blood sample received

from Shiraz. The blood has been collected from a horse, showing symptoms of acute cardiac form of Horsesickness, while having a body temperature of 40.5° C.

The incubation period in suckling mice was 6 days at the first passage. The technique used for neurotropic fixation, i.e. neurotropic fixation of the virus with serial intracerebral passages in mice, was that described, for the first time, by Dr. Alexander in 1935. The first three passages were carried out through suckling mice and the others, up to 100 passages, into adult mice brain. From the first passage the mortality rate was almost 100 per cent in adult mice. The incubation period varied between 5 to 7 days at the early passages but gradually decreased, and after 20 passages it reached a constant period minimum of about 4 days.

To determine the degree of attenuation, as the result of these successive passages, the 65th generation of this strain was selected and its pathogenicity was examined as follows :

Seven susceptible horses and donkeys, whose sera had been shown, by serum neutralization test, to be free from H.S. antibody, were injected intravenously with different doses (1—5 million mouse m.l.d.) of this strain.

The animals were closely observed for a period of 2 months and their body temperature was recorded every morning and afternoon. No sign of illness or remarkable variation in the body temperature was noticed, (table 3).

Table 3

Animal No.	Species	H.S. antibody in the blood	Date of inoc.	Rout	Inoculum m. m. l. d.	React.
1	Horse	0	22. 4. 61	I/V	1×10^6	Nil
2	Horse	0	22. 4. 61	I/V	1×10^6	Nil
3	Horse	0	22. 4. 61	I/V	2×10^6	Nil
4	Horse	0	22. 4. 61	I/V	2×10^6	Nil
5	Horse	0	22. 4. 61	I/V	5×10^6	Nil
6	Donkey	0	22. 4. 61	I/V	1×10^6	Nil
7	Donkey	0	22. 4. 61	I/V	2×10^6	Nil

3. Antigenic potentiality of neurotropic strain S 2

The brains of sick mice, infected by 65th generation of strain S 2, while in extremis were harvested. A 5 per cent suspension was prepared from these infected brain materials in lactose-peptone buffer. This suspension, which had been proved bacteriologically sterile, was titrated in mice.

Seven susceptible horses were injected with the above mentioned virus

suspension, doses varying from 50 to 500 thousands mouse m.l.d.

The inoculated horses showed only a slight rise in body temperature on the 5th, 7th and 8th day after inoculation.

Thirty five days later each inoculated horse received 6×10^6 mouse m.l.d. of viscerotropic strain, (strain S 2, generation 4), intravenously. Two other susceptible horses were also injected by 3×10^6 mouse m.l.d. of the same virus in the same manner.

A very close observation of these 9 horses during a period of 4—5 weeks showed that :

— The body temperature of all vaccinated horses remained normal and no sign of illness which could be attributed to Horsesickness was observed.

— Both of the control horses showed a rise in temperature on the 7th day after injection. The temperature reached 41.5°C on the 11th day. One of these horses died on the 15th day, showing a typical cardiac form of Horsesickness. The virus was isolated from the blood and internal organs of the carcass.

Although the other horse showed characteristic symptoms of the cardiac form of the disease, the animal survived. The body temperature of this horse remained at 41.5° C for about 5 days but gradually returned to normal (table 4).

It must be mentioned here that all horses were given complete rest during the investigation.

From these two experiments it will be seen that the neurotropic fixation

Table 4

Horse No.	antibody in the serum	Date of inoc.	Inoculum mouse m.l.d.	Route	Reac.	Immunity test 35 D. A. I. ,N I, of serum	Inoculum mouse m. I. d.	Route	Reaction
8	0	5. 8. 61	50 000	S/C	S. R. T.	31	6×10^6	I/V	Nil
9	0	5. 8. 61	50 000	S/C	S. R. T.	50	6×10^6	I/V	Nil
10	0	5. 8. 61	100 000	S/C	S. R. T.	50	6×10^6	I/V	Nil
11	0	5. 8. 61	100 000	S/C	S. R. T.	79	6×10^6	I/V	Nil
12	0	5. 8. 61	150 000	S/C	S. R. T.	63	6×10^6	I/V	Nil
13	0	5. 8. 61	150 000	S/C	S. R. T.	100	6×10^6	I/V	Nil
14	0	5. 8. 61	500 000	S/C	S. R. T.	250	6×10^6	I/V	Nil
15*)	0	—	—	—	—	0	3×10^6	I/V	Died of H. S.
16*)	0	—	—	—	—	0	3×10^6	I/V	H. S.

NB:
S. R. T. = Slight rise in temperature on 5th, 7th and 8th day after inoculation.
D. A. I. = Days after inoculation.
,NI, = Neutralization Index.
*) = Control.

and attenuation of strain S 2 by the successive intracerebral passages in adult mice was successfully carried out and that the 65th generation of this strain was sufficiently attenuated and maintained its antigenicity for equine animals.

4. Serological types of H.S. virus strains isolated in Iran.

It has been known for a long time that there exist an antigenic plurality of H.S. virus. Theiler (1908, 1915, 1921) showed, by cross immunization, that while the horses and mules vaccinated with a certain strain of H.S. virus developed a durable and solid immunity to that particular strain, resistance was inadequate when challenged with a heterologuous one.

Alexander (1935) confirmed the existence of this antigenic multiplicity and a valuable technique for the serological identification of the different strains of the virus was demonstrated by employing the intracerebral neutralization test.

McIntosh (1958), in the same manner using 42 mouse adapted strains, showed that these strains could be grouped into 7 immunological types.

From these findings, it is obvious that the most satisfactory vaccine to be used in a certain affected country must contain those virus strains that have been proved to be present in that particular area. Moreover, the vaccine used in an infected area, where the strains of virus have not been identified, should contain as many known antigenically different attenuated virus strains to afford adequate immunity against the unknown numbers of different constitution.

However, the serological identification of H.S. virus strains in affected areas is of the utmost importance.

During the recent outbreaks of the disease in the Middle East in 1960, in addition to sanitary measures, thousands of doses of polyvalent neurotropic vaccine consisting of 6 strains were administered. It was found that this vaccine afforded adequate immunity against the Horsesickness in this part of the world, and indicates that there is, at least, a similarity between the strain presents in the Middle East and the vaccine strains.

The virus isolated at Onderstepoort Laboratory South Africa from a blood sample submitted by the Pakistan government was found to be of an antigenic similarity with the type six of Horsesickness virus.

This has been also demonstrated by cross immunization tests at the Razi Institute of Iran, using a locally isolated virus, (Rafyi 1961).

In order to identify the serological types of strains isolated in Iran by means of intracerebral neutralization in mice, five mouse adapted local strains were chosen and an attempt was made to demonstrate their antigenic similarity, if any, with the vaccine strains, as follows :

A. Cross neutralization tests of the vaccine strains and strain S 2.

Material:

1. Hyperimmune sera. — Antisera against each of the seven vaccine strains, as well as strain S 2, were prepared by hyperimmunization of rabbits. Five rabbits were used for each strain and the collected sera were pooled.

To hyperimmunize, healthy rabbits were intravenously injected with 1 ml. of freshly prepared infected mouse brains suspension in normal saline. The infected mouse brains suspension was centrifuged at 3,000 r.p.m. for 30 minutes and the supernatant was used for the injection. The rabbits received 10 weekly interval injection, (McIntosh 1958).

The anti-S 2 serum was also prepared by hyperimmunization of sheep. The animal was given five intravenous injections with 3—4 days' interval, followed by another five injections after 10 days rest. The dose was 2 ml. of a suspension of suckling mouse brains, infected with strain S 2, prepared in the same manner as the above.

The sera, from the immunized rabbits and sheep, were collected 10 days after the last injection. The bacteriologically sterile unheated sera were distributed in penicillin vials. After sealing, the vials were labelled and stored at—20° C without any preservatives.

2. Viral antigen. — The day before performing the neutralization test, a 10 per cent suspension of the newly harvested mouse brains of each strains was prepared in serum saline. The supernatant of this suspension after being centrifuged at 3,000 r.p.m. for 30 minutes, having been proved bacteriologically sterile, was used as the viral antigen.

Method:

1. A serial ten fold dilutin, 10^{-1} to 10^{-5} , of the strain S 2 in serum saline was prepared in sterile test tubes.

2. Four rows of five sterile C.F. tubes were set up.

3. Using a sterile 1 ml. serological pipette, 0.3 ml. of the 10^{-5} virus dilution was transferred to the fifth tube of each of (2) above. Then, 0.3 ml. of 10^{-4} , 10^{-3} , 10^{-2} and 10^{-1} virus dilutions were transferred to the forth, third, second and the first tube of each row, respectively, using the same pipette throughout. Thus four rows of virus dilutions from 10^{-1} to 10^{-5} in 0.3 ml. volume were prepared.

4. To each tube of the first row of the above, 0.3 ml. of rabbit anti-S 2 serum; to the second row, 0.3 ml. of sheep anti-S 2 serum; to the third row, 0.3 ml. of antiserum of the vaccine strain to be tested; and to the fourth row, 0.3 ml. of normal rabbit serum were added.

a) In the same manner four rows of 10^{1-} to 10^{5-} dilutions of the vaccine strain to be tested were prepared in sterile C. F. tubes in 0.3 ml. volumes.

b) To each tube of the first row of (a) above, 0.3 ml. of rabbit anti-S 2 serum; to the second row, 0.3 ml. of sheep anti-S 2 serum; to the third row, 0.3 ml. of normal rabbit serum were distributed.

The tubes were thoroughly shaken and incubated at 37° C for 2 hours, and then mice were injected intracerebrally, using 0.05 ml. inoculum and 6 mice for each dilution.

The inoculated mice were kept separately in suitable cages, under close observation for 15 days. Deaths occuring sooner than 3 days were not considered as the result of virus multiplication.

LD 50 and the neutralization indexes were calculated by the method of Reed and Muench (1937), and the results thus obtained are summarized in table (5).

Table 5

Virus ...	T. 1	T. 2	T. 3	T. 4	T. 5	T. 6	T. 7	S. 2
Rabbit anti-T. 1 serum	250*)	—	—	—	—	—	—	10
Rabbit-anti-T. 2 serum	—	1000	—	—	—	—	—	7.9
Rabbit anti-T. 3 serum	—	—	630	—	—	—	—	10
Rabbit anti-T. 4 serum	—	—	—	310	—	—	—	10
Rabbit anti-T. 5 serum	—	—	—	—	1023	—	—	30
Rabbit anti-T. 6 serum	—	—	—	—	—	450	—	310
Rabbit anti-T. 7 serum	—	—	—	—	—	—	1000	30
Rabbit anti-S 2 serum	1.9	1.2	2.5	1	3.1	790	2.5	1200
Sheep anti-S 2 serum	1	1	1.9	1	1	150	1.5	790

NB : number indicates the neutralization index of serum T. 1 against virus T. 1, and so on.

B. Neutralization tests of 4 local H.S. strains with anti-S 2, anti-T. 6, and anti-T. 5, serum.

Materials:

1. Virus strains.

The 26th passage of strain S 1 isolated from a sample received from Boushehr, Iran.

The 28th passage of strain S 3 isolated at the Razi Institute from a horse

blood sample received from Tcheshmah Fathi, Iran.

The 25th passage of strain S 4 isolated at the Razi Institute from a horse blood sample received from Tcheshmah Fathi, Iran.

The 100th passage of strain S 5, Peshawar, isolated at the Onderstepoort Laboratory from a blood sample submitted from Pakistan (the 18th passage of this strain was received at the Razi Institute and additional passages through mice were carried out at the Razi Institute).

2. Hyperimmune sera.

Rabbit anti-S 2 serum, sheep anti-S 2 serum, rabbit anti-T. 5 serum, and rabbit anti-T. 6 serum prepared as for the previous experiment.
Rabbit anti-T. 6 serum prepared at Onderstepoort Laboratory.

Method:

1. A serial ten fold dilutions, 10^{-1} to 10^{-5} , of the strain to be tested was prepared, in serum saline, in sterile test tubes.

2. Six rows of 10^{-1} to 10^{-5} virus dilutions were prepared from the above in C.F. tubes in 0.3 ml. quantities.

3. To each tube of the first row of the above, 0.3 ml. of rabbit anti-S 2 serum; to the second row, 0.3 ml. of sheep anti-S 2 serum; to the third row, 0.3 ml. of rabbit anti-T. 5 serum; to the fourth row, 0.3 ml. of rabbit anti-T. 6 serum prepared at the Razi Institute; to the fifth row, 0.3 ml. of anti-T. 6 serum prepared at Onderstepoort; and to the sixth row, 0.3 ml. of normal rabbit serum were added.

The tubes were treated as described in the first experiment and each serum-virus mixture was injected intracerebrally into a group of 6 mice using 0.05 ml. of inoculum per mouse.

The results obtained are summarized in table (6).

Table 6

Virus ...	S 1	S 3	S 4	S 5*)
Rabbit anti-S 2 serum	1000**)	630	630	1000
Sheep anti-S 2 serum	1000	250	630	790
Rabbit anti-T. 5 serum	79	3.1	2.5	79
Anti-T. 6 serum***)	280	50	25	316
Rabbit anti-T. 6 serum****)	500	250	100	500

NB:
*) Peshawar strain.
**) The number indicates the neutralization index of serum S 2 against strain S 1, and so on
***) Prepared at Onderstepoort.
****) Prepared at the Razi Institute.

The results of the neutralization tests shown in table 5 and 6 indicate clearly that :

1 Anti-S 2 serum has a high neutralization index against type 6, but not against other types of vaccine strains.

2. Among the seven different type antisera only anti-T. 6 serum gives a good protection against strain S 2, and the neutralization index of the others are of no significance.

3. Anti-S 2 serum, as well as anti T. 6 serum, has a good neutralization index against strains S 1, S 3, S 4, and S 5.

4. Finally, strain S 2 and four other strains, namely S 1, S 3, S 4, isolated in Iran, and S 5, isolated at Onderstepoort Laboratory of South Africa from a sample received from Pakistan, are closely related to each other and all have an antigenic similarity to type six of Horsesickness virus.

References

1. Alexander, R.A. (1935) : Studies on the neutropic virus of Horsesickness, 1. Neurotropic fixation. Onderstepoort J. Vol. 4, No. 2, PP. 291—322. 2. Alexander, R.A. (1935) : Studies on the neurotropic virus of Horsesisckness, III. The intracerebral protection test and its aplication to the study of immunity. Onderstepoort J. Vol. 4, No. 2, pp. 349—377. 3. McIntosh, B.M. (1958) : Immunological type of Horsesickness virus and their significance in immunization. Onderstepoort J. Vol. 27, No. 4, pp. 465—538. 4. Rafyi, A. (1961) : La peste équine. Arch. Inst. Razi, 13, pp. 60—106. 5. Reed, I. J., and Muench, H. (1937) : A simple method of estimating 50 per cent endpoints. Am. J. Hyg., Vol. 27, pp. 493—497. 6. Theiler, A. (1908) : Further notes on immunity in Horsesickness. Rept. Gov. Vet. Bact., 1906—07, pp. 85—161. 7. Theiler, A. (1915) : The problem of Horsesickness. Rept. 13th. Ann. Meeting S.A. Assoc. Adv. Sc. pp. 65—83. 8. Theiler, A. (1921) : African Horsessickness. Sc. Bull. No. 19, Dep. Agric. S.A. Pretoria.