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## **AN OUTBREAK OF EQUINE VIRUS ABORTION IN TURKEY I. ISOLATION AND IDENTIFICATION OF RHINOPNEUMONITIS VIRUS IN CELL CULTURES**

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During the last two decades it has been established that the disease formerly described as equine influenza was not a single entity. There are, however, three distinct virus diseases which may have similar symptoms and similar complications, and each of which may be fitted into the complex of respiratory disorders of horses. These diseases are equine rhinopneumonitis, viral arteritis of horses and a true equine influenza caused by a virus typical of the myxovirus group (5, 7).

Dimock and Edwards (1, 2) was the first to describe the equine virus abortion and to determine its filterable etiology. The results of investigation conducted by Manninger (II), Manninger and Cson-tos (12) led them to decide that virus abortion of pregnant mares was caused by equine influenza virus. Other studies (7) revealed that equine influenza virus was identical with the equine abortion virus. Subsequent studies by Doll et al. (5) established that the disease was primarily a respiratory infection with abortion occurring as a result of infection in pregnant mares. They named the disease equine rhino pneumonitis and the agent equine rhinopneumonitis virus.

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The initial research on the rhinopneumonitis virus abortion problem was directed toward finding a suitable technique that the virus could be introduced directly into the fetus carried by pregnant mares, and that 100 per cent of fetuses so inoculated would be aborted (4). This procedure enabled laboratory workers to maintain and to identify strains of virus. The first success in the basic laboratory studies was development of a complement fixation test for the virus (8). Through this test, it was possible to study the prevalence and spread of the disease, and to learn that on nearly all farms almost every horse was infected prior to occurrence of the first abortion.

Doll et al. (9) was the first to propagate the virus in suckling Syrian hamsters. The hamster-virus system has provided a sensitive laboratory tool for virus neutralization tests which serve for specifically identifying the virus (6). Soon there after, hamster-propagated abortion viruses were cultivated on the chorioallantoic membrane of the chicken embryo (10).

Since 1953, various cell cultures were investigated for their capacity to support propagation of the rhinopneumonitis virus (15, 16, 17, 18).

Shimizu et al. (20, 21) reported on the use of horse kidney culture system in the isolation of virus from natural cases of equine abortion. McCollum et al. (13) described the isolation and propagation of rhinopneumonitis virus in primary monolayer cell cultures of equine, ovine and porcine kidney.

The etiological agent of equine abortion in Turkey has been, for a long time, considered to be *Salmonella abortus equi*. But in those of all bacteriologically negative cases the cause was unknown and remained to be sought. However recent study (3) suggested that viral abortion which occurs in most of the horse-raising regions of the world was also prevalent in Turkey. The subsequent histopathological studies by Pamukçu et al. (14) definitely revealed the presence, in Turkey, of equine abortion due to rhinopneumonitis virus by demonstrating specific intranuclear inclusion bodies in various specimens of tissues obtained from aborted fetuses.

So far, there have been no isolations of etiologic agents of equine abortion virus in Turkey. This report describes the isolation and identification of the virus in primary monolayer culture of kidney cells derived from various ages of sheep, and compares its immunological relationship to the equine rhinopneumonitis virus as well as the properties of the virus in this cell culture.

## Materials and Methods

Specimens of liver, lung and spleen were harvested with sterile precautions from five naturally aborted fetuses. Portions of each organ dispensed into vials, and stored at  $-20^{\circ}\text{C}$ . Vials of tissue were discarded after being thawed once. To prepare tissue suspensions for the biologic test, frozen tissues were thawed slowly in a refrigerator at  $+4^{\circ}\text{C}$ ., minced with sterile scissors and grinded in a ultra-mixer. Earle's balanced salt solution was used as a diluent to give a 10 per cent crude suspension of the tissue by weight. This suspension was centrifuged at 3000 rpm for 5 minutes. The supernate was the inoculum. 2000 units of penicillin and 2 mgr. of streptomycin were added to each ml. of the inoculum.

### *Cell Culture.*

Primary monolayer culture of kidney cells prepared from various ages of sheep were employed in the present study. Dispersed cells were obtained by digesting tissue fragments of the kidney cortex with trypsin as described by Youngner (22). The growth medium employed consists of Hanks' balanced salt solution, containing 0.5 per cent lactoalbumine hydrolysate, 10 per cent calf serum inactivated by heating at  $56^{\circ}\text{C}$ . for 30 minutes after filtration through a Seitz EK disc. 100 units of penicillin and 0.1 mgr. of streptomycin per ml. The maintenance medium used was the same. One milliliter of 0.5 per cent cell suspension prepared was used for standard culture tube, and 15 ml. for a milk dilution bottle. Incubation was carried out without motion at  $37^{\circ}\text{C}$ . Bottles were laid flat in trays while tubes were inclined at a slight angle. Cultures were left undisturbed for 48 hours, after which time they were usually stuck to the glass and beginning to multiply. The old growth medium was always replaced after 48 hours. Subsequent changes of medium were at intervals of 3 to 4 days. Excellent cell sheets were produced after 5 days of incubation and maintained in good condition for at least 8-10. days.

For virus isolation, the growth medium was removed from milk dilution bottles, and 1.5 ml. of the specimen was inoculated. After incubation at room temperature for 2 minutes, 13.5 ml. of growth medium was added into the bottles and left at  $37^{\circ}\text{C}$ . for 2 hours. At the end of this period, fluids were removed from bottles and after adding 15 ml. of fresh medium the cell cultures were incubated at  $37^{\circ}\text{C}$ ., and examined daily under a microscope for cytopathic effects

37°C., and examined daily under a microscope for cytopathic effects. At the termination of trial period, the culture fluids and cells were harvested and either inoculated immediately or frozen.

#### *Equine Rhinopneumonitis Virus.*

RAC-H strain was obtained through the courtesy of Prof. Dr. V. A. Mayr, Germany. This cell culture adapted strain was passed in monolayer culture of sheep kidney cells at our laboratory, and the virus at the IIth passage was employed as seed virus in the present study.

#### *Antisera.*

Antisera against the RAC-H, and one of the our isolate was prepared in rabbits. Cell culture of sheep kidney in milk dilution bottles were infected with RAC-H and our isolate. When the cytopathic effect was complete, the whole culture was homogenized and centrifuged at 3000 rpm for 10 minutes. The supernatant fluid was employed as viral antigen for immunization of rabbits. Animals were given 5 intravenous injections with RAC-H ( $10^7$  TCID<sub>50</sub>), and our isolate ( $10^5$ TCID<sub>50</sub>) at intervals of 3 days. The amounts administered were 0.3 ml., 0.5 ml., 1.0 ml. 1.5 ml., and 2.0 ml. The rabbits were bled to death on the sixth day after the last injection. Sera were separated and stored frozen at - 20°C.

#### *Neutralization Test.*

A stock of the isolated virus was prepared in cell cultures cultivated in milk dilution bottles. After 3 days of incubation virus growth was complete as indicated by cytopathogenic effects, and the suspending medium and cells was removed from several bottles and pooled. From this homogenized whole culture the cellular debris was removed by centrifugation at 3000 rpm for 20 minutes, and the clear supernatant distributed in ampoules and lyophilized.

For virus titration, the virus material in an ampoule was diluted with Hanks' solution to make serial tenfold dilutions, and 0.1 ml. of each of the dilutions was inoculated into groups of 4 tube cultures per dilution. The inoculated tubes were incubated at 37°C. for one week. TCID<sub>50</sub> titre was calculated by the Reed and Muench method (19) based on the developing cytopathic effects.

Serum was inactivated by heating at 56°C. for 30 minutes, and it was then diluted in twofold steps with Hanks' solution starting with a dilution of 1:2. The serum-virus neutralization tests were set

up by mixing one ml. of each serum dilution with one ml. of virus suspension containing 100 TCID<sub>50</sub> per ml. The mixtures left at room temperature for one hour, and each of the serum-virus mixture was then seeded into 4 tubes of cell culture with 0.1 ml. amounts. 0.9 ml. of maintenance medium was added to each tube and incubated at 37°C. The results of neutralization trials was recorded on the bases of cytopathic effects. Complete neutralization of virus in 4 tubes was accepted as the titration end point.

## Results

### *Virus Isolation.*

Attempts were made to isolate the etiological agent of equine abortion in primary monolayer cultures of ovine kidney cells. Specimens of liver, lung and spleen were collected from 5 aborted fetuses. The first isolation of virus was obtained from a fetal liver. Bacteriological tests failed to reveal any pathogens in these specimens of organs. Six milk dilution bottles of monolayer cell sheets were inoculated with a 1:10 suspension of liver tissue in Earle's solution. Among these cell cultures only three of them showed obvious foci cytopathic change which began 36 hours after inoculation, and affected the whole cell sheet at 3 - 4 days. In the remaining cell cultures the cytopathic changes were detected 72 hours following inoculation. Infected cultures were harvested at 5th day and transferred in fresh monolayer culture of kidney cells. In the second and later passages the cytopathogenic effects were observed in all inoculated of cell cultures, 36 hours after inoculation. The infected cell cultures compared with uninfected ones, and the control cultures were found to be unaffected during the trial period. The first cytopathic effects appeared in scattered areas as rounded cells and the number of such cells increased rapidly affecting adjoining cells. By the 5th to 6th day the degenerated cells aggregated and were falling off the glass.

### *Neutralization Test.*

The 4 isolates which were recovered from cases of the same outbreak found to be strains of a single virus. Because cytopathogenic changes developed in ovine kidney cell cultures were indistinguishable from each other, and all of the 4 isolated viruses were well neutralized by the anti-serum produced against one of them to a similar extent. The antigenic property of the isolated viruses was investigated by neutralization test employing rabbit antiserum produced against

the RAC-H strain and one of the new isolate. The anti-RAC.H serum neutralized all of the 4 agents to a lesser extent, but it neutralized well the homologous virus.

In view of the serum-neutralization test result, along with their cytopathogenicity in cell culture, it was concluded that the agents were strains of rhinopneumonitis virus. The presence of antigenic relation between isolated viruses and the RAC-H strain was also determined.

Lyophilized culture fluid of one our isolate was sent to Germany for type determination. This virus strain was identified as "equine rhinopneumonitis subtype 2" by Prof. V. A. Mayr, Faculty of Veterinary Medicine, Munich.

### Discussion

Various cell culture systems have been investigated for the isolation and propagation of rhinopneumonitis virus by many workers (13, 15, 16, 17, 18, 20, 21). Recent work on an outbreak of abortion in mares, in Turkey, has revealed the presence of specific eosinophilic intranuclear inclusion bodies in specimens of tissues of aborted fetuses. These findings were pathognomic for virus abortion (14).

The present study has shown that the virus recovered from an outbreak of abortion occurred in a government stud in Turkey can be easily grown in primary monolayer cultures of ovine kidney cells. This confirms the results of other investigators (13, 20). Serial passages were readily accomplished in this cell culture. Definite cytopathic effects was evident 36 hours following inoculation. The 4 isolated agents considered to be strains of a single virus. Because they were isolated from cases of a single outbreak, their cytopathogenic effect in culture of ovine kidney cells was indistinguishable from each other, and in the cross-neutralization test no difference was demonstrated among them. On the other hand, the cross-neutralization test revealed a distinction between the German RAC-H strain and the newly isolated virus strain.

One of the lyophilized strain was sent to Germany, and typed as "rhinopneumonitis subtype 2".

This is the first to report the isolation of the rhinopneumonitis virus from an outbreak of abortion in Turkey.

### S u m m a r y

An outbreak of abortion occurred among mares in Karacabey stud in Turkey during the 1966 foaling season. 4 cytopathogenic viral agents were isolated from specimens of 5 aborted fetuses in primary monolayer culture of ovine kidney cells. The isolated strains were found to multiply and produce cytopathic changes in this cell culture. Serial passages were readily accomplished. The 4 isolated agents considered to be strains of a single virus because they were recovered from cases of a single outbreak, their cytopathogenicity in cell cultures was indistinguishable from each other, and in the cross neutralization test no difference was demonstrated among them. However, the cross neutralization test revealed a difference between the German RAC-H strain and the newly isolated strains.

The isolated agents were identified as equine rhinopneumonitis virus, and one of the lyophilized strain was sent to Germany and typed as "rhinopneumonitis subtype 2".

### T ü r k ç e Ö z e t

1966 yılı doğum mevsiminde Karacabey Harası kısıraklarında görülen yavru atma olaylarından 4 adet sitopatojenik virus izole edilmiştir. İzalasyonda koyun böbreği hücre kültürleri kullanıldı. İzole edilmiş suşların seri halindeki pasajlarına bu hücre kültürlerinde muvaffak olunmuştur. Bu etkenlerin, koyun böbreği hücre kültürlerinde husule getirdikleri sitopatolojik değişikliklerin birbirine benzemesi, izole suşlardan birine karşı hazırlanmış immun serumla hepsinin nötraliye olmaları ve aynı salgın olaylarından izole edilmeleri sebebiyle, aynı virusun suşları oldukları kanaatine varıldı. Çapraz nötralizasyon testi Almanya orijinli RAC-H suşu ile yeni izole edilmiş yerli suşlar arasında antijenik bir fark göstermiştir. Yerli suşlardan biri si liyofilize edilerek Almanya'ya gönderilmiş ve Hannover Yüksek Veteriner Okulunda ve Münih Veteriner Fakültesinde "rhinopneumonitis subtype 2" olduğu tesbit edilmiştir.

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