Arch. Inst. Razi, 1969, 21, 25 - 34

# QUANTITATIVE STUDIES ON THE NEUTRALIZATION REACTION BETWEEN AFRICAN HORSE-SICKNESS VIRUS AND ANTISERUM (*)

By

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## Introduction

It has been reported (*Alexander*, 1935) that the African horse-sickness (AHS) neutralization test in mice is a reliable indicator of immunity. Since then, serum neutralization techniques have been accepted and used as the most reliable means for typing AHS virus and titrating antibodies in sera.

Recently, all types of AHS virus have been successfully adapted to monkey kidney stable (MS) cells ( *Ozawa* and *Hazrati*, 1964) and the usefulness of the new host cell system in serologic studies has been explored by *Hazrati* and *Ozawa* (1965). Based on this technique, neutralization indices have been exclusively used in previous papers (*Ozawa* and *Hazrati* 1965; *Hazrati*, and *Ozawa*, 1965) because of difficulties in maintaining constant titers of numerous AHS virus strains cultivated in tissue culture. Most of the difficulties were overcome in recent studies (*Ozawa* and *Bahrami*, 1968).

*McIntosh* (1958), titrating an AHS antiserum against graded doses of homologous virus, showed that a relatively small difference in virus concentration resulted in significant difference in serum titer. Therefore, more precise quantitiative studies of AHS virus neutralization tests are needed to compare antibody titers of sera and to determine serologic types of AHS viruses closely related, such as types 6 and 9.

For this reason and others which will be considered in this paper, several quantitative and kinetic neutralization tests of AHS virus with specific homologous antisera were made by using tissue-culture and plaque assay techniques.

## Materials and Methods

*Tissue cultures.* Monkey kidney stable cells were used for titrating virus and for

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*1* This work was undertaken at the Near East Animal Health Institute, a project established by the United Nations Development Program Special Fund through the Food and Agriculture Organization in cooperation with the Ministry of Agriculture of Iran.

(*) Reprinted from Archiv fur die gesamte Virusforschung 25,83-92 (1968)

neutralization tests in tubes. The source of the cell line, the method of cultivation, and its susceptibility to AHS virus have been described in previous papers (*Ozawa* and *Hazrati*, 1964; *Ozawa* et al., 1965). Growth medium consisted of Earle's buffered salt solution containing 0.5% lactalbumin hydrolysate, 0.005% yeast, 5% calf serum heated at 56° C for 30 minutes. 100 units of penicillin and 100 μg of streptomycin per ml and 0.0015% phenol red.

For plaque assay, African green monkey kidney (VERO) cell line (*Ozawa*, 1967) was used. The method of cultivation and media used were the same as for MS cells (*Hopkins* et al., 1966; *Hopkins* et al., 1967).

*Virus strains.* Types 1 through 7, neurotropic AHS vaccine strains A 501, OD, L, Vryheid, VH, 114, and Karen, respectively, were obtained from the Onderstepoort Veterinary Laboratory, South Africa. Strain S2 (*Hazrati* and *Taslimi*, 1963) was originally isolated in Iran and was attenuated through more than 100 successive intracerebral passages in adult Swiss albino mice. Strain FA 1/66 was isolated from a horse in Morocco and submitted to the Razi Institute for typing through the Institut d'Elevage et de Médicine Vétérinaire, Alfort. Strain T6/66 was recovered from horse blood which had been received from Tunisia.

A few additional passages of these strains were made in mice for the preparation of fresh seed virus to be used in the experiments or for their adaptation to MS cell cultures. The techniques for isolation and passage of virus in mouse brain and adaptation to MS cell cultures have been described (*Ozawa* and *Hazrati*, 1964).

Infectious mouse brains were stored at —20° C. Most of the infectious tissue culture fluids were stored at 4°C, and some were stored at —70°C.

*Antiserum.* Specific antisera were obtained from the blood of hyperimmune sheep and rabbits taken at 2-week intervals. They were immunized by inoculation with mouse or MS tissue-culture-adapted virus. Antigen preparation and hyperimmunization methods were similar to those described in a previous paper (*Hazrati* and *Ozawa*, 1965b) except for type 8 antiserum which was prepared by 3 consecutive injections of strain 18/60-adapted MS cell cultures at 2-week intervals. Equine antisera were obtained from recovered or immunized horses during the course of the experiment.

The sera without any preservative were stored at —20°C. All sera were inactivated by heating for 30 minutes at 56°C immediately before use.

*Diluent.* In making various dilutions of virus and serum, maintenance medium containing 2% calf serum heated for 30 minutes at 56°C was used throughout this study.

*Plaque-reduction test.* VERO cell line was employed in the tests. The nutrient medium and method of cultivation used are the same as those described in previous reports (*Hopkins* et al., 1966; *Hopkins* et al., 1967). A suspension of approximately $10^7$ PFU/ml of type 7 virus was mixed with an equal volume of antiserum diluted with maintenance medium. Both constituents of the neutralization mixture were equilibrated at 37°C prior to mixing. At intervals, samples were taken and diluted immediately 1:100 in maintenance medium chiled in an ice-bath, at pH 7.5, to arrest neutralization; from this, further 10-fold dilutions and intermediate dilutions were made. The diluted mixtures were inoculated into two 2-oz prescription bottles in 0.2 ml volumes immediately after dilution. Infected bottle cultures were incubated for 3 hours at 37°C. spreading the inoculum by rocking the bottles every 30 minutes. Then they were overlaid with the nutrient medium and incubated again at 37°C. Plaques were counted on the 7th and

10th days after inoculation. Most of the plaques were 2 to 3 mm in diameter at the time of counting, and the cultures which had less than 35 plaques per bottle were used for reading the endpoint.

*Dose-response pattern.* In order to determine the sensitivity of MS cell culture for the titration of AHS virus, the dose-response range from 0 to 100% infection with the virus in MS cells was studied according to the method of *Robson* et al. (1961). Two-fold dilutions of S2—6 (6th passage of strain S 2 in MS cells) were prepared, using a separate pipette for each dilution transfer. One-tenth ml of each virus dilution was then inoculated into each of 25 tube cultures prepared by using MS cells of two different passage levels. The tubes were incubated for 30 minutes at 37°C before 1.5 ml of maintenance medium was added. The tubes were then incubated 7 days at 37°C and observed for cytopathic effect (CPE). The proportion of tubes showing CPE in each dilution was determined from the results. The sensitivity range was calculated by subtracting the log dilution of virus which produced 100% CPE in cell cultures from the log dilution of virus which produced no CPE.

In a similar manner, the dose-response range for the neutralization tests was studied. Serial 2-fold dilutions of rabbit MS S 2 antiserum were prepared. Each dilution was mixed with an equal volume of homologous virus suspension containing approximately 100 tissue culture infectious dose (TCID) per 0.1 ml. The serum-virus mixtures were incubated for 1 hour at 37°C and 0.2 ml of each mixture was inoculated into a tube culture using 25 tubes per dilution. The final results were read on the 7th day after infection and the proportion of tubes showing CPE and dose-response range were calculated in the same manner as above. From this dose-response range an appropriate dilution rate of serum and the number of tubes to be inoculated per dilution to obtain a desired tolerance rate were calculated.

*Variations within and between neutralization tests. The* deviation within a test was determined by diluting hyperimmune rabbit serum in 4-fold increments. Titer of antibody in each serum dilution was measured, as if it were a separate serum, by using a 2-fold dilution rate. An equal volume of virus suspension containing approximately 100 TCID /0.1 ml was added to each serum dilution. The mixtures were incubated for 60 minutes at 37°C after which 0.2 ml of the mixture was inoculated into each of 4 MS tube cultures per serum dilution. After 30 minutes' adsorption, fresh maintenance medium was added. Titers were adjusted to account for the initial serum dilution. This test was repeated using a different batch of MS cell cultures.

*Neutralization tests for the study of neutralization slopes. Serial* 4-fold dilutions of virus and serum were prepared separately in maintenance medium. Each virus dilution and appropriate serum dilution was mixed in equal parts. After incubation for 60 minutes at 37°C, 0.2 ml of each serum-virus mixture was inoculated into each of 4 culture tubes per dilution. After 30 minutes' adsorption, fresh maintenance medium was added to each tube. Netralization tests in mice were carried out in the same manner, using 6 adult mice per serum dilution — each receiving 0.03 ml of the mixture by intracerebral inoculation. In one test, the virus-serum mixtures, incubated 60 minutes at 37°C, were inoculated into mice after overnight storage at 4°C.

*Calculation of endpoints.* Throughout this study, infected tissue culture tubes were incubated at 37°C for 7 days, at which time the final reading was made. Any evidence of characteristic CPE was considered as a criterion of positive response. 50% endpoints were calculated by the *Spearman-Karber* method *(Finney,* 1952).

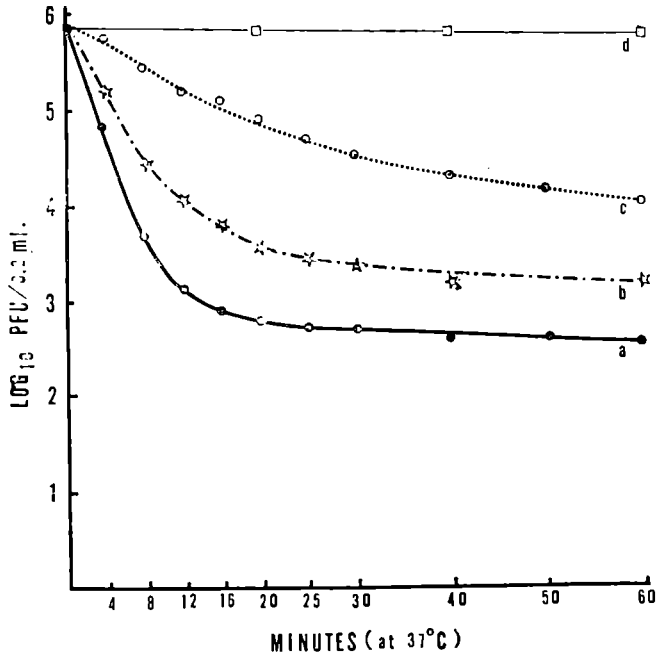


Fig. 1. Plaque reduction curves of AHS type 7 (Karen) virus with various concetrations of the hyperimmune rabbit serum; (a) 1:100 dilution; (b) 1:200 dilution; (c) 1:500 dilution; (d) 1:100 dilution of normal rabbit serum

## Results

Neutralization curves of AHS virus with homologus antiserum were studied by incubating type 7 (Karen strain) virus with equal volumes of either 1:100, 1:200, or 1:500 dilutions of rabbit hyperimmune serum. The control mixture contained an equivalent concentration of normal rabbit serum. Titre of PFU from samples taken at intervals are shown in Fig. 1. Neither significant inactivation of virus by incubating at 37°C nor dissociation of the virus-antibody union under stated conditions was observed. Kinetic neutralization curves of AHS virus with higher concentrations of antiserum were characterized by initial first-order phases of short duration followed by gradual, stationary phases. The higher the concentration of antiserum, the clearer and shorter was the first-order phase. From the results, it appears that 1-hour incubation of virus-serum mixtures at 37°C is appropriate for the neutralization test.

The dose-response patterns of the virus titrations are presented in Fig. 2. The range from 0 to 100% infection was 1.31 log units with type 7 (Karen) virus and 1.76 log units with type 9 ($S_2$) virus. The ranges of the response for the neutralization tests of types 7 and 9 viruses were 1.36 and 1.43 log units, respectively (Fig. 3). Therefore, the changes from no infection to essentially complete infectivity took place over an approximately 20- to 58-fold dilution range for virus titration, and

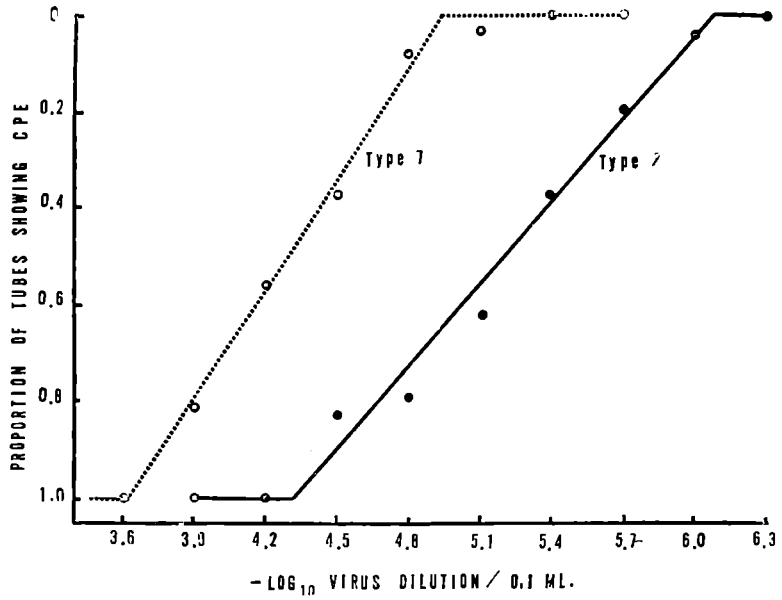


Fig. 2. Dose response patterns of AHS type 7 (Karen) and type 9 (S 2) virus in MS cell cultures.

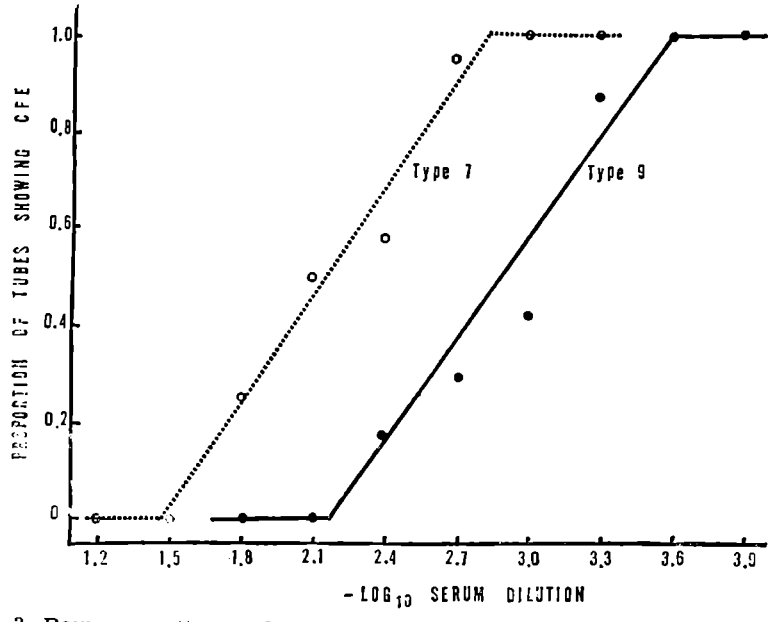


Fig. 3. Response patterns of African horse-sickness serum neutralization tests.

the change from complete neutralization to complete infection took place over an approximately 23-to 27-fold dilution range for the serum neutralization test.

When the same hyperimmune rabbit sera were first diluted and then titrated separately to calculate the deviation in a single neutralization test, differences in titers were found to range from 0.04 to 0.24 log units, indicating that the results did not vary more than in a 3-fold dilution (Table 1).

**Table 1. Accuracy of African Horse-sickness Neutralization Test with Types 7 and 9 Virus**

| Serum dilution | Type 7 | | | |
|---|---|---|---|---|
| | Test 1 | | Test 2 | |
| | −log 10 titer | −log 10 calculated titer | −log 10 titer | −log 10 calculated titer |
| 0 | 2.81 | 2.81 | 2.75 | 2.75 |
| 1:4 | 2.20 | 2.80 | 1.90 | 2.51 |
| 1:16 | 1.60 | 2.80 | 1.45 | 2.65 |
| 1:64 | 0.96 | 2.77 | 0.75 | 2.56 |
| Range | log $10^{0.04}$ | | log $10^{0.24}$ | |
| | Type 9 | | | |
| 0 | 3.05 | 3.05 | 2.90 | 2.90 |
| 1:4 | 2.35 | 2.95 | 2.35 | 2.95 |
| 1:16 | 1.81 | 3.01 | 1.60 | 2.80 |
| 1:64 | 1.29 | 3.10 | 1.05 | 2.85 |
| Range | log $10^{0.15}$ | | log $10^{0.15}$ | |

The relationship between the quantity of antibody and the quantity of virus neutralized in AHS virus neutralization tests is shown in Fig. 4 and Table 2. Straight lines were fitted to the data of all AHS virus types. The range of the neutralization slope was between 1.33 and 1.59 when tests were made in MS cell tube cultures. There was no remarkable difference in slopes of enutralization tests with immune sera prepared from the blood of the three different animals (Table 2). There were, however, significant differences between the slopes of neutralization tests made in tissue cultures and mice (Fig. 4).

When virus-serum mixtures were incubated 60 minutes at 37°C and titrated immediately in mice, the neutralization slope was approximately 1.0. The curve of the same virus-serum mixture was 0.7 when titrated in mice after storing the mixtures overnight at 4°C. Similar results were obtained in the repeated tests.

## Discussion

Kinetic neutralization curves were studied by plaque assay techniques developed by *Hopkins* et al.(1967). Type 7 (Karen) virus was used in the study because the virus produced uniform ,large plaques in VERO cell cultures. The homologous antiserum was diluted 1:100, 1:200, or 1:500 before mixing with the virus. At these

Table 2. Neutralization Slopes and Intercepts of Straight Lines Showing Relationship between Quantity of Specific Antibody and Quantity of Virus Neutralized. Virus-serum Mixtures were Incubated at 37°C for 60 Minutes and Titrated in MS Tube Cultures

| Virus types (strain) | Antiserum | Neutralization slope | Intercept (virus neutralized) |
|---|---|---|---|
| | | Cultures | |
| 1 (A501) | Rabbit (MS)[1] | $-1.33 \pm 0.20$ | $5.39 \pm 0.56$ |
| 1 (A501) | Sheep (MO)[2] | $-1.48 \pm 0.19$ | $5.63 \pm 0.32$ |
| 2 (OD) | Rabbit (MO) | $-1.43 \pm 0.22$ | $4.96 \pm 0.31$ |
| 3 (L) | Rabbit (MS) | $-1.52 \pm 0.30$ | $4.53 \pm 0.35$ |
| 4 (Vry) | Rabbit (MS) | $-1.33 \pm 0.14$ | $5.31 \pm 0.28$ |
| 5 (VH) | Rabbit (MS) | $-1.38 \pm 0.08$ | $5.45 \pm 0.15$ |
| 6 (114) | Rabbit (MO) | $-1.45 \pm 0.14$ | $5.35 \pm 0.27$ |
| 6 (114) | Sheep (MO) | $-1.47 \pm 0.05$ | $6.39 \pm 0.10$ |
| 7 (Karen) | Rabbit (MO) | $-1.59 \pm 0.11$ | $6.13 \pm 0.28$ |
| 9 (S2) | Rabbit (MO) | $-1.48 \pm 0.19$ | $6.15 \pm 0.46$ |
| 9 (S2) | Rabbit (MS) | $-1.42 \pm 0.07$ | $6.15 \pm 0.18$ |
| 9 (FA1/66) | Rabbit (MS) | $-1.34 \pm 0.32$ | $5.82 \pm 0.75$ |
| 9 (T6/66) | Rabbit (MS) | $-1.36 \pm 0.21$ | $5.13 \pm 0.33$ |
| 9 (S2) | Mule (recovered) | $-1.58 \pm 0.13$ | $5.95 \pm 0.31$ |

1 Rabbit antiserum against the homologous virus grown in MS cell cultures.
2 Shee antiserum against the homologous virus grown in mouse brains.

concentrations of serum, neutralization of virus during a 3-hour adsorption period was almost completely arrested by diluting the virus-serum mixtures 1:100 or more in maintenance medium chilled in an ice bath before inoculating them into bottle cultures. Under the conditions employed in this study, there was no evidence of the recovery of neutralized virus as was the case with Japanese encephalitis virus and its antiserum (*Hashimoto* and *Prince*, 1963).

The dose-response range of AHS virus in MS cell cultures was measured in order to determine a suitable dilution rate and the number of culture tubes to be inoculated per dilution. Only types 7 and 9 viruses were tested in this study, but the results indicate that the ranges are wider than those of other animal viruses such as bovine virus diarrhea virus (*Coggins*, 1964) and distemper virus (*Robson* et al., 1961). According to the proposed procedure by *Robson* et al. (1961), the virus dilution rate should be approximately equal to the log range and no more than log range plus 0.5 ($10^{R+0.5}$). After the response range and the dilution rate have been determined, the number of cultures per dilution necessary for the resulting titer, not to deviate by more than a 3-fold dilution, can be calculated by the formula: number of tubes $= \frac{4D^2}{(2E-D+R)^2}$. Where D is the log dilution rate, E is the log allowable deviation (3-fold = 0.5), and R is the log range as determined above. The ranges for types 7 and 9 virus titrations were 1.31 and 1.76 log units, and the neutralization ranges 1.36 and 1.43, respectively. This indicates that using 10-fold dilutions, the resulting titer will not deviate by more than 3-fold. However, a 4-fold dilution rate was applied in this study to obtain more accurate

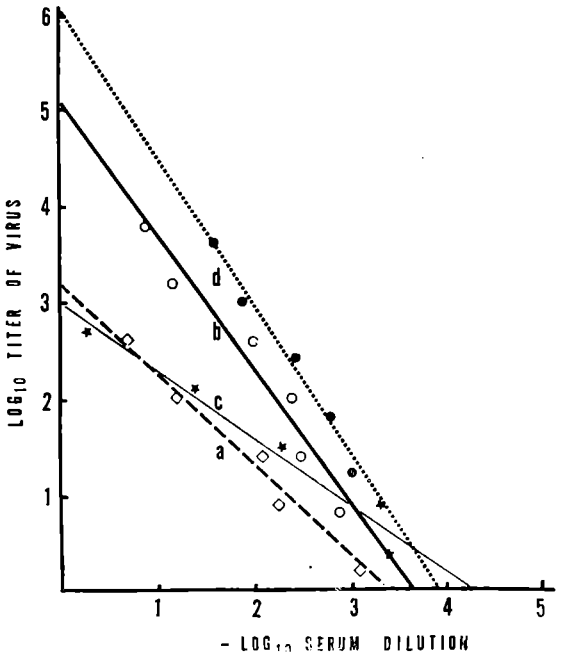


Fig. 4. Relation between amount of serum antibody and amount of virus neutralized. AHS type 9 (S 2) pirus grown in MS cell culture was mixed with parious dilutions of hyperimmune rabbit serum prepared by inoculating S 2 virus grown in mouse brains. The mixtures were incubated 1 hour at 37°C and titrated in mice (a), or in MS cell cultures (b). The same virus-serum mixtures were stored overnight at 4°C after 1 hour incubation at 37°C and titrated in mice (c). Type 7 (Karen) virus grown in MS cell cultures was incubated at 37°C for 1 hour with rabbit hyperimmune serum prepared by inoculating Karen virus grown in mice, and titrated in MS cell cultures (d). Neutralization slopes of these regression lines were (a) $-0.99 \pm 0.11$, (b) $-1.39 \pm$ (c) $-0.70 \pm 0.09$, and (d) $-1.59 \pm 0.11$.

results. Calculations show that, theoretically, only 1 tube per dilution is sufficient if a 4-fold dilution is used. Taking possible unforeseen human errors into consideration, it might be safer to use more than one tube—preferably 2 to 4 tubes—per dilution. In practice, a 2-stage procedure, first using 10-fold dilutions and then 4- or 5-fold dilutions could be followed for testing unknown specimens.

The results of the repeated neutralization tests were satisfactory when 4 tubes were used for each dilution. The maximum difference was 0.24 log unit which was within a 2-fold dilution deviation.

When neutralization tests were carried out in MS tube cultures, there was no significant difference in the neutralization slopes, regardless of virus types and sources of immune sera. However, the slopes were different when the same samples were tested in mice, especially when the virus-serum mixtures were stored overnight at 4°C. This may be partly because it takes longer to complete neutralization

reaction with the higher dilution of serum as shown in Fig. 1. and, also, because the antibodies remain in the maintenance medium of tube cultures, but not in the mouse brain.


## Summary

Kinetic neutralization curves formed with African horse-sickness virus were studied by the plaque reduction technique. There was no recovery phase in surviving virus titer during a 60-minute incubation period at 37°C when antiserum was diluted 1:100 or more.

Factors affecting the accuracy of the serum neutralization test in tube cultures of monkey kidney stable (MS) cells were studied to ascertain optimal conditions. Dose-response patterns, variations within and between tests, and the relationship between the amount of virus and serum titers were examined to develop a test procedure showing accuracy of less than a 3-fold dilution deviation under circumstances.

With antisera against 8 different types of AHS virus ,it was demonstrated that there is linear relationship between the amount of serum antibody and amount of virus neutralized. When tested in MS cell tube cultures, neutralization slopes of all AHS virus types ranged between 1.33 and 1.59. A decrease in the slope was noticed when tested in mice.



## Acknowledgements

The authors thank Dr. *M. Kaveh* of Razi Institue, and Dr. *W. M. Moulton* of the Near East Animal Health Institute for their helpful advice and support. Authors are indebted to the staff of the Plum Island Animal Disease Laboratory for their kind support in preparing this manuscript.


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