Isolation an Characterization of the Virus of Epizootic Hemorrhagic Disease (EHD) of Deer

Hsing Kuei Liu

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ISOLATION AND CHARACTERIZATION OF THE VIRUS OF

EPIZOOTIC HEMORRHAGIC DISEASE

(EHD) OF DEER

BY

HSING KUEI LIU

A thesis submitted
in partial fulfillment of the requirements for the
degree Master of Science, Major in
Bacteriology, South Dakota
State University
1970
This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable as meeting the thesis requirements for this degree, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.
ACKNOWLEDGMENTS

I wish to extend my sincere appreciation to my major professor, Dr. George C. Parikh, for his advice, counsel, and patience during the course of this study and the preparation of this thesis.

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The assistance of Dr. Robert M. Pengra and Dr. Paul R. Middaugh with proofreading is gratefully acknowledged.

The friendship and understanding of Miss Elisabeth Y. J. Wu is also appreciated.
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INTRODUCTION

The name "epizootic hemorrhagic disease" (EHD) of deer was first given by Shope et al. in 1955 because of the occurrence of the hemorrhagic disease in large epizootic proportions and because of its pathological features. It was also determined by them that the causative agent for the disease is a virus.

Difficulties in studying the causative agent have been encountered because no host system other than the deer itself was available to support replication or detect the virus. However, isolation of the New Jersey strain of EHD virus was accomplished by Mettler et al. in 1962 using newborn mice and HeLa cells. Limited success of the isolation of South Dakota strains of EHD virus was achieved by Pirtle and Layton in 1961. Yet there is no one ideal tissue culture system that has been applied to the study of EHD virus, especially the South Dakota strain. Therefore, there is a need for a cell culture system in which the cytopathogenic effect is obvious, rapid in onset, regularly reproducible, and the yield of virus is high.

This study was undertaken to develop a tissue culture system which would allow the detection, isolation and characterization of the virus. In order to develop a rapid laboratory diagnostic method for the
disease, the study was extended to determine the relationship be-
 tween the EHD disease and the total serum cholesterol level.
LITERATURE REVIEW

The complex nature of the pathogenesis of EHD coupled with the difficulties in conducting experiments on large wild animals have contributed to inconsistent reports on properties of various EHD strains. In order to obtain a thorough understanding of the previous research work on EHD of deer, an extensive literature review was undertaken.

Occurrence of the Disease

EHD of deer was first reported by Shope et al. in 1955. This highly fatal disease of white-tailed deer occurred in New Jersey, in August of 1955. Fatalities from this epizootic outbreak approached 1,000 deer. A year later Fey et al. (1956) reported an epizootic of hemorrhagic disease of white-tailed deer in Michigan. This outbreak occurred in 1955 and was similar to the New Jersey outbreak in etiology, pathology and epizootiology. During the late summer and early fall of 1956, a laboratory-confirmed outbreak of EHD occurred in white-tailed deer in South Dakota (Shope et al., 1960).

Although these reports were the first actual laboratory-confirmed cases of this malady, a search of the literature by Trainer (1964) revealed that deer deaths resulting from a disease similar to EHD have occurred in various sections of North America since 1890. Extensive mortality from a fatal hemorrhagic disease similar to EHD
occurred at irregular intervals for many years among white-tailed
deer in several Southeastern states (Ruff, 1949). Other similar
deer losses occurred in British Columbia (Cowan, 1962), Missouri
(Murphy, 1957), Nebraska (Daily, 1957), North Dakota (Richard et
al., 1956), Washington (Shope et al., 1960), Alberta (Vance, personal
communication), California (Herman, personal communication), and
Iowa (Haugen, personal communication), Wyoming (Parikh, personal
communication), Texas (Trainer, personal communication). Figure 1
shows the distribution of EHD in North America which suggests that
the disease may be more widespread than had been realized. Although
the causative agent in many of these outbreaks was not isolated,
mortality patterns with postmortem symptoms always were observed.
Parikh et al. (1968) stated that 75% of the epizootics occurred on

Cultivation of the Virus

Pirtle and Layton (1961) cultivated the South Dakota strain of EHD
virus in the cells of fetal deer spleen but failed in their attempts to
grow the virus in deer kidney cells. These authors reported that the
cytopathogenic effect, involving gradual degeneration of cells, began
with the second passage. The deer inoculated with the infected tissue
culture material died after 21 days. These authors suspected that this
may have been because their tissue culture material contained a
Fig. 1. Distribution of Epizootic Hemorrhagic Disease in North America.
minimal infective quantity of virus or that the virus lost some
virulence during passages through tissue culture.

Mettler et al. (1962) propagated the New Jersey strain of EHD
virus in newborn Swiss mice by intracerebral inoculation. It was
usually lethal beyond the first serial passage in the mouse, however,
the mouse-passaged virus induced an inapparent infection in an ex-
perimental deer. Mettler et al. (1962) were successful in growing the
New Jersey strain of EHD virus serially in HeLa cells which showed
a characteristic cytopathic effect, but these workers failed to
grow the South Dakota strain in deer kidney and HeLa cell culture
systems.

Properties of the Virus

Ditchfield and Debbie (1964) reported that the Alberta strain of
EHD virus is relatively heat stable and has a particle size of about
25-30 mμ. These authors also stated that Alberta strain EHD virus
is an RNA virus and has a simple cubic symmetry. The New Jersey strain
of the EHD virus was considered to have a limited sensitivity to sodium
desoxycholate by Mettler et al. (1962) and the Alberta strain of EHD
virus was considered chloroform resistant by Ditchfield and Debbie
(1964). The New Jersey strain of EHD virus is quite stable when
stored at low temperatures with dry ice (Shope et al., 1960).
Immunological Properties of the Virus

In vivo, serum neutralization tests using experimental deer were successfully carried out by Mettler et al. in 1962. In vitro, neutralization tests were carried out in HeLa cells using EHD-recovered deer sera. EHD specific complement fixation antibody in deer was first demonstrated by Mettler et al. (1962) using an antigen prepared from mouse brain and infected tissue culture fluid. In their experiments normal deer serum did not react with uninfected tissue culture fluid and uninfected mouse brain. Debbie et al. (1965) reported that complement fixation antibody was found in 2 out of 7 deer which were experimentally exposed to EHD virus. Parikh et al. (1967) developed a standardized complement fixation test to detect EHD antibody using a sucrose acetone extract of mouse brain and Genetron (trifluorotrichloroethane) extract of EHD infected deer liver or spleen as an antigen source. These workers confirmed the work of Mettler et al. (1962) on the applicability of a complement fixation test by testing for the virus specific antibody which was produced in the sera of 23 experimentally infected deer, 40 experimentally infected rabbits and 6 horses. Also, Parikh et al. (1967) were first able to demonstrate the EHD specific precipitation reaction in agar.
Symptoms of the Disease

Shope et al. (1960) described the disease of naturally infected deer. The deer had rough coats and they lay listlessly on the ground with their necks extended before them and their ears drooping. They walked with an unsteady, wobbly gait and some even had a bloody diarrhea. Observations were also made on experimentally infected deer. Incubation periods varied from 4 to 12 days, with most animals showing manifest signs of illness on either the 6th or 7th day after inoculation. Shope et al. (1960) also stated that about half of the inoculated deer reached the acute disease course seen in nature and died in apparent shock within 24 to 48 hours after the first visible sign of illness. The most prominent signs in these animals were loss of appetite, roughening of the coats, marked congestion of the conjuntiva and the mucosa of the lips and sometimes rather pronounced salivation.

Clinical Findings of the Disease

Karstad et al. (1961) observed the following in artificially infected deer. Fever usually appeared in diseased deer on the fifth or sixth day postinoculation. The body temperature rose sharply to about 105 F, then decreased more or less gradually until death within the next 2 to 5 days. The animals ceased to eat and rarely drank after the onset of fever.
The bleeding time was greatly prolonged during the later stages of the disease. Clotting time was also prolonged. The increase in blood coagulation time was somewhat parallel to the elevation in body temperature. Sometimes just before the death of the deer the clotting time changed from the normal clotting time of 3 to 5 minutes to a clotting time of 23 minutes.

Total red blood cell counts changed a little during the course of the infection. The counts were slightly elevated near the end of the illness. The percentage of lymphocytes decreased and the percentage of neutrophils increased while total white blood cell count remained about the same.

Pathology of the Disease

The same workers (Karstad, et al., 1961) described the pathology as found in their experimentally infected deer. They found that hemorrhages, both gross and microscopic, were present in many organs and tissues of the body such as heart, intestine, mesentric vessels, mucosal surfaces, etc. Hemorrhages occurred in the later stage of the disease. Sometimes large size infarct-like lesions of hemorrhages were found in the heart. Microscopic vascular changes, consisting of over-dilatation of vessels with degeneration and necrosis of smooth muscle and adventititia, were seen throughout the body, which appeared to be associated with the hemorrhages.
Arterioles and venules were most likely to be affected. Signs of inflammation were usually absent.

Lymphatic organs throughout the body were hemorrhagic and partially depleted of lymphatic tissue. Lymph nodes were grossly enlarged, edematous and hemorrhagic. Similar changes were seen in the spleen. The brain lesions consisted of perivascular hemorrhages and edema.

Gross changes in the lungs consisting of congestion, generalized edema and emphysema were seen. The pathologic changes in the kidney consisted of extreme congestion and necrosis of the convoluted tubules. Liver lesions were few and consisted chiefly of congestion and atrophy of cells bordering the distended sinusoids. The diseased organ also had mild changes such as fatty metamorphosis of hepatic cells. The adrenal glands were congested, the pancreas had lesions of congestion, hemorrhages, vascular degeneration and necrosis.

They also found that the myocardia had hemorrhagic lesions, areas of degeneration and necrosis of the muscle and connective elements. The aorta had degeneration, necrosis of the media, and degeneration of the adventitia associated with perivascular hemorrhages. Sometimes there were dissecting hemorrhages of the aorta. The thymus had extreme congestion, edema, vascular necrosis, and hemorrhages. Edema and degeneration were prominent in the fibrous supporting tissues of the gland. The gastrointestinal tract pathology
consisted mainly of hemorrhages in all tissue layers and associated walls. The connective tissue elements, submucosal, intermuscular, and subserosal were considerably swollen and edematous. Skin on varied parts of the body had gross and microscopic hemorrhages.

Pathogenesis of the Disease

Three factors may be involved in the hemorrhagic pathogenesis: damage to the blood vessel, a decreased number of the blood platelets and prothrombin deficiency. The extensive hemorrhages in the infected deer led Karstad et al. (1961) to study the derangement of the normal blood clotting mechanism. They found that deer infected with EHD virus had prolonged blood coagulation and prothrombin time and in hematologic studies they observed that this prolonged blood time was related to prothrombin deficiency. The derangement of the blood clotting mechanism plus vessel wall damage resulted in multiple microscopic to massive hemorrhages.

In a study by Debbie et al. (1965) a decrease in blood platelets in the peripheral blood of deer exposed to the virus of EHD was observed. The means by which EHD-induced degeneration and necrosis of the muscle and connective tissue of blood vessel walls occurs is unknown. Karstad et al. (1961) assumed that the primary cause may have been over-dilatation as the result of a toxin liberated from damaged tissue, or, the observed degenerative changes may have been the direct
result of viral action upon cells. Another explanation for this change may be that these tissues were damaged as the direct result of interrupted blood supply, which in turn was caused by the hemorrhages observed.

Mode of Transmission of the Disease

The wealth of knowledge on human hemorrhagic disease suggests that the EHD virus could be transmitted in nature by (1) insect bites and/or (2) by oral-fecal routes. Shope et al. (1960) made an investigation on both of the modes of EHD transmission and they found that whatever the natural mode of the virus transmission may be, it is not by contact. Their insect transmission experiments also yielded negative results, yet they suspected that there might be technical problems involved. Experimental transmission of EHD was first successfully performed by Shope et al. (1955) by injecting the deer intramuscularly or subcutaneously with suspensions of infected deer organs which produced EHD pathological features. Experimental transmission of EHD was also tried by Debbie et al. (1965) by stomach tube implantation of deer which did not produce apparent EHD but complement fixing antibodies were present in the deer. Ticks were utilized by Trainer (1961) in an unsuccessful attempt to transmit EHD.
MATERIALS AND METHODS

Solution: and Media for Tissue Culture Cell Lines

Deer kidney cell cultivation was first attempted in lactoalbumin yeast-extract medium (IA medium, Fough and Lund, 1957). After two unsuccessful trials with IA medium, a modification of Eagle's minimum essential medium (MEM, Eagle, 1965) was tried. Growth of deer kidney cells was satisfactory in this medium. In addition to MEM, LA medium was used to cultivate baby hamster kidney cells while Mitsuhashi-Maramorosch medium (MM medium, Singh, 1967) was used to cultivate *Aedes albopictus* insect cells.

Various base solutions, growth and maintenance media and other chemical solutions utilized in EHD virus and tissue culture research are described below.

**Hanks’ Balanced Salt Solution** (Hanks’ BSS)

Stock Solution (10X)

Solution A

<table>
<thead>
<tr>
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<th>Amount</th>
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<tbody>
<tr>
<td>NaCl</td>
<td>80.0 g</td>
</tr>
<tr>
<td>KCl</td>
<td>4.0 g</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Na₂HPO₄·H₂O</td>
<td>0.6 g</td>
</tr>
</tbody>
</table>
Solution B

\[
\begin{align*}
\text{CaCl}_2 & \quad 1.4 \text{ g} \\
\text{Distilled water} & \quad 200.0 \text{ ml}
\end{align*}
\]

Each salt was dissolved in distilled water in the order above. Solution B was added to solution A and 20 ml of 1% phenol red (Difco, catalog number 5358-60) was added as a pH indicator. The 10X stock solution was held at 4°C. For use in cell cultures the stock solution was diluted 1:10 with distilled water and sterilized by autoclaving for 15 minutes at 121°C.

**Earle's Balanced Salt Solution**

**Stock Solution (10X)**

**Solution A**

\[
\begin{align*}
\text{NaCl} & \quad 68 \text{ g} \\
\text{KCl} & \quad 4 \text{ g} \\
\text{MgSO}_4 \cdot 7\text{H}_2\text{O} & \quad 2 \text{ g} \\
\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O} & \quad 1.25 \text{ g} \\
\text{Glucose} & \quad 10 \text{ g} \\
\text{Distilled water} & \quad 800 \text{ ml}
\end{align*}
\]

**Solution B**

\[
\begin{align*}
\text{CaCl}_2 & \quad 2 \text{ g} \\
\text{Distilled water} & \quad 200 \text{ ml}
\end{align*}
\]
Each salt was dissolved in distilled water in the order above. Solution B was added to solution A and 20 ml of 1% phenol red was added as a pH indicator. This solution was also held at 4 C. For use in cell cultivation the stock solution was diluted 1:10 in distilled water and sterilized by autoclaving 15 minutes at 121 C.

Hanks' BSS free of Ca$^{++}$ and Mg$^{++}$

For washing cells and for trypsinization procedures the 10X stock solution of Hanks' BSS was prepared by omitting CaCl$_2$ and MgSO$_4$·7H$_2$O. This solution will be referred to as "GKNP". The working solution was made by diluting 1:10 with distilled water and sterilized by autoclaving for 15 minutes at 121 C. Again 1% phenol red was added as a pH indicator.

Trypsin Solution

The trypsin stock solution was prepared by dissolving 1 g of trypsin (Difco 1:250) in 100 ml of 1X GKNP. The pH was adjusted to 7.2 to 7.4 with 0.2 N NaOH. Most of the powder was dissolved upon the pH adjustment. The solution was then sterilized by filtration and stored in the freezer at -20 C.

For use, the stock solution was diluted 1:5 in GKNP. Sufficient 2.8% NaHCO$_3$ was added to hold the pH above 7.2 to 7.4 during the trypsinization procedure.
Glutamine Solution, 200 mM

Glutamine (powdered) was purchased from Hyland Laboratories, Los Angeles, Calif. To obtain a 200 mM glutamine solution, 5.8 g of glutamine was dissolved in 200 ml of distilled water and sterilized by passing through a Millipore filter, 0.45 µ, type HAWG.

Antibiotic Solutions

The usual concentrations of penicillin and streptomycin in the media were 100 g and 100 units per ml respectively. Ordinarily only penicillin and streptomycin were included in the media. Mycostatin was seldom used for control of yeasts and fungi. A concentration of 25 units of Mycostatin per ml of medium was used. Since the Mycostatin is inhibitory to cell growth, it is better if it is not employed in the growth medium.

Amino Acid Solution

The amino acid solution was purchased from Grand Island Company Berkeley, California. The amino acid solution was the "MEM Amino Acids" (catalog number 113) and contained arginine, cystine, glutamine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine and valine.
Vitamin Solution

The vitamin solution was Grand Island Company's "MEM Vitamins" (catalog number 112) and contained choline, folic acid, pantothenic acid, pyridoxal, riboflavin, thiamin, nicotinamide and myo-inositol.

Eagle's Minimum Essential Medium (MEM)

<table>
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<th>Growth medium</th>
<th>Maintenance medium</th>
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<tbody>
<tr>
<td>BSS</td>
<td>80.0 ml</td>
<td>80.0 ml</td>
</tr>
<tr>
<td>Amino acid (50X)</td>
<td>2.0 ml</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>NaOH, 0.2 N</td>
<td>2.0 ml</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>Vitamins (100X)</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Glutamine (200 mM)</td>
<td>1.0 ml</td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Fetal calf serum</td>
<td>10.0 ml</td>
<td>2.0 ml</td>
</tr>
<tr>
<td>NaHCO₃, 2.8%</td>
<td>1.5 ml</td>
<td>2.0 ml</td>
</tr>
</tbody>
</table>

Amino acids solution was added directly to BSS before addition of other components so that pH was adjusted without denaturing other substances in the complete medium. The bicarbonate concentration may be raised or lowered depending on number of cells in the culture. Calf serum may be used in place of fetal calf serum.

The pH of the growth and the maintenance media was 7.0 to 7.3. After the media components were combined, they were stored at 4°C for not more than 2 to 3 weeks.
**Process of Preparing Deer Kidney Cells**

Kidneys were removed aseptically from freshly killed deer. Kidneys were decapsulated and cut in half and the medulla was removed with sterile scissors and forceps. The remainder of the cortex was placed in a wide mouth centrifuge tube and cut into small pieces.

The tissue was washed with cold Hanks' BSS to remove blood. The cut tissue was transferred to a trypsinization flask and approximately 50 ml of prewarmed trypsin solution (0.2%) was added to the flask. This was gently stirred with a magnetic stirrer for 5 minutes. It was important to prevent bubbling during stirring. The supernatant liquid, after momentary settling, contained damaged cells, blood cells and debris. This was decanted off and 50 ml of fresh prewarmed trypsin solution was added to the remaining tissue which was again stirred as above for 10 minutes.

The cell suspension was collected in a 1000 ml flask and kept at 4°C. Treatment with trypsin was repeated until the tissues were trypsinated for 2 to 3 hours. The pooled cell suspension was filtered through six layers of sterile gauze and collected in a wide mouth bottle.

The cell suspension was then centrifuged at 600 rpm with the 822a head for 10 minutes in the International Refrigerated centrifuge model PR-2. The supernatant trypsin solution was removed, the packed cells were resuspended in cold Hanks' BSS, mixed well and centrifuged
as above for 10 minutes. This washing procedure was repeated three times. The supernatant fluid was removed and the cells were diluted 1:300 in the growth medium. This cell suspension gave approximately 300,000 to 500,000 cells per ml. The cell suspension was distributed in 0.8 ml amounts into 16 mm diameter test tubes.

It was noticed that when too concentrated a cell suspension was seeded, a large portion of the cells originally planted was destroyed. This may have been due to a cytotoxic material liberated from the deer kidney cells, as has been demonstrated with freshly trypsinized monkey kidney cells (Melnick, 1955). Thus, in order to get optimal growth of the deer kidney cells, a concentration of 3 to 5 x 10^5 cells per ml was used.

The cells were incubated at 37 C for 72 hours. The 1 ml of the outgrowth medium was replaced. They were used after 5 to 7 days incubation when confluent monolayers of cells were present. Prior to inoculation with virus, the growth medium was removed and 1 ml of the maintenance medium was added. After the inoculation of the virus, the cells were fed twice weekly with maintenance medium to prevent the cell degeneration caused by starvation.

**Virus Source of South Dakota Strain #10**

Splenic material from an EHD infected white-tailed deer, which died in an epizootic that occurred near Philip, South Dakota in 1964,
was used to infect a healthy deer. The South Dakota Strain #10 of EHD virus was isolated from this experimental infection by workers from the Bacteriology Department at South Dakota State University. The virus from the experimentally infected deer spleen suspension was adapted to grow in African green monkey kidney cells by Dr. William Hann of Arbovirus Section, Laboratory of Tropical Virology at National Institute of Allergy and Infectious Disease. Tissue culture infected fluid from African green monkey kidney cells was used in this study and designated as South Dakota Strain #10 of EHD virus.

Applications of Cell Cultures

I. Detection of Viral Multiplication in Cell Cultures

A. Cellular Degeneration

After inoculation with the virus, both the infected and the non-infected cells were observed at 100X every other day to detect cellular degeneration produced by the virus. When the degeneration of the cells started to show, the cells were observed more frequently (every 24 hours). The infected cells were harvested when about 75% of cell destruction had occurred. The cells were harvested using the following method. They were put into the -60 C Revco freezer to be frozen. The culture vessels were then put into a 37 C water bath to thaw the cells. This step was repeated twice in order to loosen the cell sheets
from the culture vessels. The control cells were also harvested the same way. Fluids harvested from the infected and non-infected cells were kept in the -60 C Revco freezer for the subsequent passages.

B. Virus Titration

To determine the TCID\textsubscript{50} (tissue culture infecting dose for 50% of the cultures inoculated) of the viral suspension logarithmic dilutions of tissue culture grown virus were prepared in Hank's BSS. In preparing these viral dilutions it is very important to discard pipettes between each dilution to avoid carrying virus particles on the pipette to the next dilution. Each of four tube tissue cultures which had just been fed with 0.9 ml of fresh maintenance medium was inoculated with 0.1 ml of viral suspension at each dilution.

The inoculated culture tubes were incubated at 37 C and observed microscopically every other day over a 14 day period. Cultures were considered positive (or infected) if one-half or more of the cells in the culture showed cytopathogenic effects (CPE). The TCID\textsubscript{50} was calculated by the method of Reed and Muench (1938).

C. Neutralization Test

Ten fold dilutions of virus were prepared. Equal volumes of diluent, normal serum and antiserum were added to each virus
dilution and shaken well. (All sera had been inactivated at 56 C for 30 minutes). The virus-sera and the virus-diluent (virus control) mixtures were then allowed to remain at room temperature for 1 hour. After 1 hour 0.2 ml of each mixture was inoculated into each of four culture tubes.

CPE in all tubes were checked at 3, 5 and 7 days post-inoculation. Titers of each inocula (virus plus diluent, virus plus normal serum and virus plus EHD antiserum) were calculated by the method of Reed and Muench (1938). Inhibition of CPE demonstrated by decreasing titers was considered a positive neutralization test.

II. Sensitivity to Diethyl Ether

The test was carried out following the method of Andrewes and Horstmann (1949). Viral suspensions were centrifuged in the Beckman model L ultracentrifuge with the type 30 rotor for 60 minutes. Half of the viral suspension supernate was saved as a control. No diethyl ether was added to it. Diethyl ether was added to half of the viral suspension. Four volumes of viral suspension supernate were added to 1 volume of C.P. diethyl ether and thoroughly mixed. The mixture and the control both were stored in a tightly stoppered container at 4 C for 24 hours. On the following day, the container of ether-exposed suspension was unstoppered and the ether removed under vacuum at room temperature, the control suspension being held at a comparable
Tenfold dilutions of each viral suspension were titrated in the deer kidney cell culture system. The results of titration were calculated by the method of Reed and Muench (1938). When there was one-half or more than half a log drop in titer of the ether-exposed suspension, it was considered to have limited ether sensitivity or complete ether sensitivity.

III. Chromosome Spreading and Staining Technique

1. 0.8 ml of fresh growth medium was added to each Leighton tube culture just before adding colchicine.

2. 0.2 ml colchicine solution was added 6 to 8 hours before harvesting to accumulate metaphases. (Colchicine solution was prepared by dissolving 25 mg colchicine in 100 ml distilled water and sterilized by filtration. Colchicine was purchased from K & K Laboratories Inc., catalog number 8170.

3. The coverslips were transferred to warm hypotonic solution (1 part of Hanks' BSS plus 9 parts of distilled H₂O) for 1 hour.

4. The coverslips were fixed by transferring to acetic-alcohol (1 part glacial acetic acid:3 parts ethanol) for 10 minutes.

5. The coverslips were placed horizontally and left to dry at room temperature.

6. The chromosomes were stained by the aceto-orcein stain. The stain was prepared by dissolving orcein in 100 ml 50% glacial
acetic acid and filtered through filter paper. To stain the cell preparation, a drop of stain was added to the coverslip and it was mounted on another coverslip immediately. Then the edges of the coverslips were mounted with Permount medium to prevent the evaporation of the aceto-orcein stain. To prevent the evaporation of the dye it is very important to stain the chromosomes just before observation.

7. Chromosomes were examined under the oil immersion lens (1000X)

8. Chromosome pictures were taken by using a Polaroid film type 107, ASA speed 3,000. When the transformer setting was at 6.5 the shutter speed was at 1/10 second. The neutral density filter and the condenser filter both were not used. The time of development for each Polaroid picture was 15 to 20 seconds.

Determination of Total Serum Cholesterol in Deer

A new micro method for determination of the serum cholesterol as described by Clark et al., (1968) was slightly modified and used to measure the deer serum cholesterol.

A. Deer Inoculation and Serum Collection

EHD infected African green monkey cell culture fluid provided by Dr. William Hann, Arbovirus Section, Laboratory of Tropical Virology, National Institute of Allergy and Infectious Disease was used for the inoculation of deer #23, #2-145, #12 and #14.
Sera were collected on different days post-inoculation. Post-mortems were done when the deer died of the infection and organs were collected. Deer #2-129, #2-210, #4-291, #4-290, #198 and #1983 were all inoculated with South Dakota Strain #10 Virus and sera of them were collected when the animal died of the infections.

B. Reagents

Absolute ethanol

KOH-ethanol reagent, 2 ml of 33% aqueous KOH in 100 ml absolute ethanol (KOH from Mallinckrodt Chemical Works, analytical reagent, KOH 85% meets A.C.S. specifications)

Methylene chloride, Fisher certified A.C.S., code number D-37

Concentrated sulfuric acid, (Mallinckrodt Chemical Works, Analytical Reagent)

Iron-ethanol reagent (FeAlc), 5.0 gm Fisher certified reagent grade FeCl₃·6H₂O dissolved in 1000 ml absolute ethanol.

Cholesterol working standard:

Fifty mg of cholesterol was dissolved in 45 ml absolute alcohol in a 50 ml volumetric flask, made to 50 ml volume with additional ethanol. Two ml aliquots were transferred to vials, capped, and stored at -20 C. A fresh vial of standard (1 mg/ml) was used each week.
C. Equipment

Beckman model DU spectrophotometer

Beckman Pyrex cuvette cells with 10 mm light path

Beckman model L ultracentrifuge, type 50 rotor, tube size:

5/8" x 2 1/2"

Gyratory water bath shaker, model G 76

25 ml wide mouth, Bellco (catalog number 1800) screw cap

culture tubes with rubber lined caps.

Reciprocating shaker, model Sp/630-AK3-5110

Hamilton syringe, serial number 705

D. Procedures

1. With a 100 µl size Hamilton syringe, 50 µl of well mixed serum sample were transferred to the bottom of a 25 ml screw cap tube. The syringe was rinsed with distilled water between serum samples. A standard and a blank were likewise prepared with 50 µl of cholesterol standard and 50 µl of water respectively.

2. Two ml of iron-ethanol reagent was added to each tube along the edge of each tube with a volumetric pipet.

3. One ml of KOH-ethanol reagent was added to each tube with a 1 ml volumetric pipet.

4. The tubes were capped and vigorously shaken in the gyratory water bath shaker at the maximum speed setting
for 5 minutes at 65 C to aid the protein-Fe(OH)₃ precipitation.

5. Each sample in the test tubes was centrifuged for 5 minutes at 20,000 rpm to separate the precipitate, the type 50 rotor head was used, the centrifuge tubes were balanced and capped tightly to avoid any evaporation.

6. The supernatent in each centrifuge tube was carefully pipetted into a clean test tube and shaken well.

7. One ml of each supernatent was transferred to each 25 ml culture tube with a volumetric pipet.

8. Two ml of concentrated sulfuric acid was added to each tube without mixing by pipetting the acid slowly down the side of the tube. Any tube in which accidental mixing occurred was discarded.

9. The tubes were carefully capped and vigorously shaken by hand for one and one-half minutes. A red-orange color developed.

10. An additional 2 ml of concentrated sulfuric acid was gently layered beneath the color solution.

11. Without mixing, 5.0 ml of methylene chloride was added with a 5 ml volumetric pipet.
12. The tubes were capped tightly and laid in the reciprocal shaker and shaken vigorously at a speed of around 175 strokes per minute for 10 minutes.

13. The tubes were allowed to stand for 10 minutes to separate the phases.

14. A portion of the upper layer (methylene chloride phase) was transferred to a spectrophotometer cell. The spectrophotometer was set to 100% transmission at 525 μm with the reagent blank against distilled water in the reference cell. The light transmission of the methylene chloride phase was then read against the distilled water reference.

15. Each tube was left capped until it was ready to read to prevent adsorption of atmospheric water and evaporation of methylene chloride.
RESULTS AND DISCUSSION

The South Dakota strain of virus was cultivated in BHK-21 cells, deer kidney cells and insect cells (Aedes albopictus). Partial characterization of EHD virus after the isolation of the virus included its diethyl ether sensitivity and its effect on the deer kidney chromosomes. A measure of changes in blood cholesterol of EHD infected deer was carried out to help in characterization of the disease.

I. Cultivation of EHD Virus in Various Tissue Culture Cell Lines

A. Deer Kidney Monolayer Cell Culture

Deer kidney cells were cultured two ways, in the bottles of a roller culture apparatus and in the tissue culture test tubes (Fig. 2 and Fig. 3).

Fig. 2. Roller culture apparatus utilized to grow deer kidney cells on the inner surface of the bottles (Bellco Biological Glassware and Equipment, Vineland, N. J.)
Fig. 3. Rolling device for incubation of test tubes cultures. It was utilized to grow deer kidney cells. Dilution bottles on the top shelf were utilized to grow the BHK-21 cells.

Normally, it took 5-7 days for the cell suspension to form a sheet of cells ready for virus inoculation. Multiplication of EHD virus was detected microscopically when cellular degeneration had occurred. Virus induced cytopathological effect (CPE) was observed in animal cells cultured either by roller or stationary means. While the control or uninfected cells looked healthy and did not have any cytological abnormality (Fig. 4), the cells when initially infected showed granular type CPE which finally led to a total destruction of the cells and contraction of the cells leaving empty spaces on glass surface (Fig. 5).
Fig. 4. Uninfected deer kidney monolayer cell culture. X100.

Fig. 5. Deer kidney monolayer cell culture 9 days post-inoculation with South Dakota strain #10 EHD virus. X100.
To determine the potential of deer kidney cells in primary isolation of EHD virus, attempts were made to isolate the virus from experimentally infected deer organs and urine. Results are shown in Table 1.

<table>
<thead>
<tr>
<th>Animals</th>
<th>EHD virus isolation specimens</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Lung</td>
</tr>
<tr>
<td>Deer #689</td>
<td>Positive</td>
</tr>
<tr>
<td>Deer #368</td>
<td>Not tested</td>
</tr>
<tr>
<td>Deer #369</td>
<td>Not tested</td>
</tr>
</tbody>
</table>

Inoculation of tissue cultures with spleen of an infected deer gave rapid CPE in 3 to 4 days after inoculation showing that spleen is a good source for primary EHD virus isolation. Mettler et al. (1962) had success in primary isolation with infected organs of New Jersey strain EHD virus in HeLa cell cultures.

The maintenance of South Dakota strain #10 virus through 3 passages with minimal inocula, and the regular development of specific cellular degeneration following inoculation with the virus clearly indicates that EHD virus multiplies in deer kidney cell cultures.
Adaption of the virus for growth in this deer culture system is shown in Figure 6. The time required for the virus to exhibit CPE varied with the number of passages through which it had gone. With increasing number of passages of the virus, not only the onset of the initial CPE occurred sooner but also the time for the total CPE decreased.

A very interesting phenomenon noticed during the EHD virus infection experiment was that the deer kidney cells had a "foamy type" degeneration. This has been observed in certain simian virus infected monkey kidney cells (Hsing and Gaylord, 1961). Deer kidney cells formed vacuoles in the cytoplasm in both the normal (Fig. 7) and the EHD infected cells (Fig. 8). The EHD infected deer cells had a larger proportion of cells showing foamy type degeneration. This foamy agent is considered an endogenous virus of deer. Further work needs to be done for its isolation and identification.

Since EHD agent afflicts deer as its host, success in culturing deer kidney cells in vitro should afford one of the best systems for cultivation of the EHD virus and probably other viruses afflicting deer. Using this tissue culture system, an oral vaccine may be developed in the laboratory by comparing the virulence of various strains of EHD virus from the deer kidney tissue culture system using serum neutralization tests. Other fastidious hemorrhagic disease viruses may also grow in this deer tissue culture system.
Fig. 6. Effect of Tissue Culture Passage on Time for Degeneration of Deer Kidney Cells by South Dakota Strain #10 of EHD Virus in Deer Kidney Cells
Fig. 7. Uninfected deer kidney monolayer cell culture showing autogeneous foamy cell formation, 11 day old cells. X100.

Fig. 8. Deer kidney monolayer cell culture 9 days after inoculation with South Dakota strain #10 EHD virus also showing "Foamy Type" cells. X100.
B. Baby Hamster Kidney (BHK-21) Monolayer Cell Culture

The susceptibility of hamster kidney cell cultures to the cytopathogenic effect of certain arthropod-borne viruses (arboviruses) were first described by Kissling (1957). Other investigators have also found this cell type to be a useful host for the propagation and isolation of a number of arboviruses. (Diercks and Hammon, 1958, Rosenberger and Shaw, 1961, Lennette et al., 1961).

Because EHD virus has been suspected to be an arbovirus, attempts were made to grow the South Dakota strain #10 EHD virus in hamster kidney cells. Evidence of replication of this strain of EHD virus in this cell line was obtained; the infected hamster kidney cells showed CPE between 5 to 9 days. This varied with the passage of the virus through the cells (Fig. 9). Infected BHK-21 cells observed were granulated and scattered from each other. Figure 10 shows the appearance of normal, uninfected BHK-21 cells while Figure 11 shows early infection of BHK-21 cells and Figure 12 shows a later period during infection of BHK-21 cells. Another kind of morphological change that was observed in South Dakota EHD virus infected BHK-21 infected cells was the formation of giant cells. Giant cells arose by fusion of adjacent cells (Fig. 13). Roizman, who has made a study of viral-induced giant cell formation has reviewed the subject (1962). Although the mechanism for giant cell formation is not known,
Fig. 9. Effect of Tissue Culture Passage on Time for Degeneration of BHK-21 Cells by South Dakota Strain #10 of EHD Virus
Fig. 10. Uninfected BHK-21 monolayer cell culture. X100.
Fig. 11. BHK-21 Monolayer cell culture 5 days after inoculation with EHD virus South Dakota strain #10 showing 2+ C.P.E. X100.

Fig. 12. BHK-21 monolayer cell culture 9 days after inoculation with EHD virus South Dakota strain #10 showing 4+ C.P.E. X100.
presumably it is related somehow to alterations in the cell membrane caused by viral replication.

Fig. 13. BHK-21 cells infected with South Dakota strain #10 EHD virus 9 days post-inoculation showing giant cell formation. X200. Two cells are fusing. Nuclei of the two cells are much enlarged.

C. Monolayer Culture of Aedes albopictus Insect Cells

That arthropod-borne animal viruses might be propagated in insect tissue in vitro was first shown nearly 25 years ago by Trager (1938), who cultured western equine encephalitis virus in surviving mosquito tissues. Since then, no further experiments of this kind, at least with animal viruses, have been done until recently. Progress in the cultivation of Aedes aegypti embryonic cells and of ovarian tissue from two species of mosquito (Kitamura, 1966), and establishment of
a cell line derived from tissue of *A. aegypti* larvae (Grace, 1966) have opened the possibility for studying mosquito-borne viruses in *vitro* in mosquito tissue culture.

Attempts to propagate the South Dakota strain #10 EHD virus were made in this study. A cytopathogenic effect appeared 6 days after inoculation. The cytopathic effect first became evident as granulation in cells. These cells then rounded-up, contracted, and finally formed an empty area in the cell sheet. Enlarged and disintegrated cells also were present in 6 day post-inoculated insect cells (Fig. 14 and Fig. 15).

![Fig 14. Uninfected *Aedes albopictus* monolayer cell culture. X100.](image)

The ability of South Dakota EHD virus growth in a mosquito cell line supports the idea that the virus is an arbovirus. Also, this
finding suggests the role of mosquitoes as a vector in the transmission of EHD.

Fig. 15. *Aedes albopictus* monolayer cell culture 6 days after inoculation with South Dakota strain #10 EHD virus. X100.

By chance an observation was made in maintaining or subculturing the *Aedes aegypti* and *Aedes albopictus* cells. Both of these cell lines failed to recover (i.e. to grow and multiply as well) after they were fed the media containing Mycostatin at 10 units per ml. In 1967 Millam and Vaughn made a study on the effect of one of the polyenes, amphotericin B on *Aedes aegypti*. He found that the insect cells failed to recover after they had been treated with the medium containing amphotericin B. Table 2 shows a comparison of the properties of Mycostatin and amphotericin B.
As it shows in Table 2, Mycostatin binds membrane sterols, thus damaging the membrane. The susceptibility of cell lines of A. aegypti and A. albopictus to Mycostatin not only supports the hypothesis that insects do not synthesize sterols (Clark and Block, 1959), but also explains the limited usefulness of anti-fungal agents in insect tissue cultures.

D. Determination of Titer of South Dakota Strain #10 EHD Virus

Tissue culture infected fluid containing EHD virus from third passage of deer kidney cells was used to inoculate deer kidney cell cultures after ten-fold serial dilutions. The results obtained are shown in Table 3. The virus titer, as calculated by the Reed and Muench (1938) method, representing TCID\textsubscript{50} (tissue culture infecting dose for 50\% of the cultures inoculated) is equal to $10^{-3}$/0.1 ml.
Table 3. Determination of titer of South Dakota strain #10 EHD virus in tissue culture fluid

<table>
<thead>
<tr>
<th>Dilution of Virus</th>
<th>No. of Cultures Showing CPE/No. Inoculated</th>
<th>Cumulative No. Not Infected</th>
<th>Calculated Infectivity Ratio %</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^{-1}$</td>
<td>4/4</td>
<td>0</td>
<td>10/10 100</td>
</tr>
<tr>
<td>$10^{-2}$</td>
<td>3/4</td>
<td>1</td>
<td>6/7 86</td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td>2/4</td>
<td>3</td>
<td>3/6 50</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>1/4</td>
<td>6</td>
<td>1/7 14</td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>0/4</td>
<td>10</td>
<td>0/10 0</td>
</tr>
</tbody>
</table>

E. Virus Neutralization Test in Deer Kidney Cells - Serological Evidence that the Agent Propagated in Deer Kidney Cell Culture Was the South Dakota Strain EHD Virus

In order to confirm that the agent propagated in deer kidney cell culture was South Dakota Strain EHD Virus, attempts to neutralize the agent in deer kidney cell culture with EHD antisera were made. Pre-inoculation and post-inoculation sera from deer no. 1982 were used. The results obtained are outlined in Table 4.

As shown in Table 4, the serum of the deer that had recovered from infection with the South Dakota strain of EHD neutralized the agent propagated in deer kidney cells. The serological data indicated
that the agent propagated in deer kidney cell cultures was indeed the South Dakota strain of EHD virus.

Table 4. Neutralization by the serum of EHD-recovered deer of the agent propagated in deer kidney cell culture

<table>
<thead>
<tr>
<th>Virus neutralized with</th>
<th>Virus (Vero-1, BHK-21-1, Deer Kidney-3</th>
<th>Log Titer of virus</th>
<th>Neutralization index</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1982 South Dakota strain</td>
<td>EHD virus immune serum</td>
<td>10^{-1.3}</td>
<td>1.5</td>
</tr>
<tr>
<td>#1982 pre-inoculation serum</td>
<td>10^{-2.3}</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Diluent (Hanks' BSS)</td>
<td>10^{-2.8}</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

II. Sensitivity to Diethyl Ether

Among the simplest and most important methods for screening a virus isolate for clues as to whether it is or is not an arbovirus are the tests for chemical sensitivity to inactivation by diethyl ether or sodium desoxycholate (SDC). As it was pointed out before, EHD has been considered either a Picorna or an arbo type virus. In order to characterize this agent, the ether sensitivity of EHD was tested following the method of Andrews and Horstmann (1949). Results are recorded in Table 5.
Table 5. Sensitivity of South Dakota strain EHD virus to diethyl ether

<table>
<thead>
<tr>
<th>Virus source</th>
<th>Titer of Virus</th>
<th>Inactivation in Logs</th>
</tr>
</thead>
<tbody>
<tr>
<td>South Dakota Strain EHD Virus (control)</td>
<td>10^{-1.5}</td>
<td></td>
</tr>
<tr>
<td>South Dakota Strain EHD Virus Treated with Diethyl ether</td>
<td>10^{-1.0}</td>
<td>0.5</td>
</tr>
</tbody>
</table>

As is evident from Table 5, the South Dakota strain #10 of EHD virus exhibited, although to a limited extent, a sensitivity to diethyl ether, thus adding one more piece of evidence that this virus is an arbovirus.

This finding agrees with the data of Mettler et al. (1962), although he used the New Jersey strain of EHD virus and Theiler's (1957) technique. If the virus has an essential lipid in its coat, treatment with a fat solvent should remove its structural lipid and decrease its infectivity.

III. Analysis of the Effect of EHD Virus on Deer Kidney Chromosomes

One of the consequences of infection of cells by viruses (RNA and DNA) is derangement of the karyotype. Most of the changes observed are random in nature. Frequently, breakage, fragmentation and
rearrangement of the chromosomes occurs; abnormal chromosomes and changes in chromosome number have also been observed. Herpes Zoster virus induces a colchicine-like effect in human cells; (Benyesh-Melnick et al., 1964). The mitotic cycle is interrupted at metaphase, the chromosomes over-contract and micronuclei form. Some of the chromosomes undergo fragmentation. Chromosome breaks have also been observed after adding Rous sarcoma virus in vitro to leukocytes (Nichols et al., 1964) and in leukocytes from patients with chicken pox and from patients with measles (Aula, 1963, Nichols et al., 1962). Cells infected with or transformed to malignancy by papovavirus SV 40 and cells exposed to adeno-virus type 12, also exhibit random chromosomal abnormalities (Koprowski et al., 1962, MacKinnon, et al., 1966, zur Hausen, 1967). Many of these findings are preliminary, and analysis of the effect of various viruses on chromosomes is continuing. Studies were undertaken to determine the effect of EHD virus on chromosomes of infected deer kidney cells.

It was found that the normal, uninfected deer kidney cells of Virginia white-tailed deer had a diploid chromosome number of 70 (Fig. 16). Similar results were obtained by Wurster et al., in 1967. Of the autosomes, there were 33 pairs of acrocentrics and one pair of medium-sized submetacentrics. The sex chromosomes were of the usual XY variety, the X being a large submetacentric and the Y a small metacentric element.
Fig. 16. Chromosomes of Female White-tailed Deer (*Odocoileus hemionus*). X1900. 2n=70.
The same techniques were used to study the chromosomes of the EHD infected deer kidney cells. Table 6 shows the metaphase chromosomal aberrations of the deer cells 36 hours post-EHD virus inoculation. The data indicate that after infection with EHD virus there are fragmentations and changes in chromosome numbers. Lower and higher chromosomal numbers in infected deer cells are shown in Figure 17 and Figure 18. Further investigation needs to be done in this area.

<table>
<thead>
<tr>
<th>Number of chromosomes</th>
<th>61</th>
<th>61 + fragment(s)</th>
<th>62</th>
<th>65</th>
<th>70</th>
<th>70 + fragment(s)</th>
<th>71 + fragment(s)</th>
<th>80 + fragment(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell counts</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

IV. Determination of Total Cholesterol in Deer Serum

Hypocholesterolemia has been noticed in rodent malaria disease (Desowitz and Langer, 1968). Relationship between the lipids of Venezuelan encephalomyelitis virus and the host has been investigated and established (Heydrick et al., 1968). Since it is well known that severe sudden hemorrhages may cause total serum cholesterol increase
Fig. 17. Chromosome aberrations showing fragments and lower chromosomal number (61 + fragments) in metaphase. Plate of South Dakota strain #10 EHD virus infected white-tailed deer kidney cell after 36 hours infection of the cell. (X1900)

Fig. 18. Chromosome aberrations showing higher chromosomal number (80 + fragments) in metaphase plate of South Dakota strain #10 EHD virus infected white-tailed deer kidney cell after 36 hours infection of the cell. (X1900).
(Goodale, 1955), the experiments described here were performed in order to establish the relationship between the EHD and the total serum cholesterol level.

Cholesterol from Mann Research Laboratories was used as working standards through the experiments. Figure 19 shows a linear graph of optical density vs. iron chromogen concentration, expressed as mg cholesterol/ml ethanol solvent, using the color reaction of Clark et al. (1968) with Mann's cholesterol. Cholesterol measurements of the deer sera were carried out the same way as the standards.

Total serum cholesterol changes of deer that survived the EHD infection are shown in Table 7 and Figure 20. Table 8 shows total serum cholesterol changes of deer that died of the EHD infection. Total cholesterol of ten penned normal wild deer before virus inoculation ranged from 100.3 mg to 26.4 mg (average 66.6 mg), Table 7, row 1 and Table 8, column 2, in 100 ml sera. Total deer serum in cholesterol in the infected deer ranged from 138.4 to 0.0 mg during the course of the EHD disease. Preliminary examination of Table 7 and Table 8 indicates that during the disease the animal developed both hypocholesterolemia (decrease of cholesterol) and hypercholesterolemia (increase of cholesterol). Among the group of the deer that survived the infection, total serum cholesterol increases of each set (#2-145, #12, and #14) of deer were observed to
Fig. 19. Standard Curve for Total Serum Cholesterol Determination
Table 7. Total serum cholesterol changes before and after EHD virus inoculation in deer that survived the infection

<table>
<thead>
<tr>
<th>Deer no.</th>
<th>#2-145</th>
<th>#12</th>
<th>#14</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days of serum post-inoculation</td>
<td>cholest-</td>
<td>Percentage of</td>
<td>cholest-</td>
</tr>
<tr>
<td>post-inoculation</td>
<td>rol(mg/</td>
<td>Increase</td>
<td>Decrease</td>
</tr>
<tr>
<td>0</td>
<td>100.4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>No sample</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>43.9</td>
<td>56.2</td>
<td>46.1</td>
</tr>
<tr>
<td>24</td>
<td>138.4</td>
<td>37.8</td>
<td>4.4</td>
</tr>
<tr>
<td>31</td>
<td>125.2</td>
<td>24.7</td>
<td>116.9</td>
</tr>
<tr>
<td>38</td>
<td>21.6</td>
<td>78.4</td>
<td>60.5</td>
</tr>
<tr>
<td>45</td>
<td>24.6</td>
<td>75.5</td>
<td>90.4</td>
</tr>
<tr>
<td>54</td>
<td>13.8</td>
<td>86.3</td>
<td>91.6</td>
</tr>
<tr>
<td>76</td>
<td>43.9</td>
<td>56.2</td>
<td>92.5</td>
</tr>
</tbody>
</table>
Fig. 20. Percentages of Total Serum Cholesterol Decrease and Increase in Deer which Recovered after EHD Infection. ▲-▲ deer #2-145, ○-○ deer #12, ●-● deer #14
Table 8. Total serum cholesterol in deer that died of experimental EHD infection

<table>
<thead>
<tr>
<th>Deer no.</th>
<th>Total serum cholesterol (mg/100ml serum) pre-inoculation</th>
<th>Total serum cholesterol (mg/100ml) post-inoculation</th>
<th>Days of serum</th>
<th>Percentage of Increase</th>
<th>Decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>#2-129</td>
<td>43.9</td>
<td>48.1</td>
<td>4</td>
<td>9.6</td>
<td></td>
</tr>
<tr>
<td>#23</td>
<td>56.3</td>
<td>77.7</td>
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be on the 31st day post-inoculation; then they were followed by a sharp decrease of total serum cholesterol. Serum cholesterol of the deer that survived the infection was measured periodically from 0 to 76th day post-inoculation, maximum percentage of cholesterol decrease was found to be 100, while maximum percentage of increase was found only to be 124.2. Among the group of deer that died of the infection, hypocholesterolemia occurred early -- 4 to 7 days post-inoculation and hypocholesterolemia occurred on the 11th and 16th day post-inoculation. Maximum percentage of cholesterol decrease was found to be 92.6. Maximum percentage of cholesterol increase was found to be 65.8.

At this point it may be concluded from the experiment that EHD of deer will not cause hypercholesterolemia unless the deer develops hemorrhages. The occurrence of severe hypocholesterolemia may be due to a metabolic disorder; i.e. congestive heart failure and/or liver disease; i.e. hepatocellular damage (this causes a decrease particularly in cholesterol esters). It may be possible to use hypocholesterolemia as an additional indicator for the diagnosis of the EHD disease in the experimentally infected deer. Although information on both days after inoculation and symptoms of the deer is needed in interpreting the laboratory data.
CONCLUSIONS

1. Tissue culture methods for growing and maintaining deer kidney cells were developed.

2. Not only the deer kidney cells but also BHK-21 cells and Aedes albopictus cells supported the replication of South Dakota strain #10 of EHD virus. Cytopathogenic effects detected in deer kidney cells infected by South Dakota strain #10 EHD virus from the 4th to 14th days post-inoculation. CPE were detected in infected BHK-21 cells from the 5th to 9th day post-inoculation.

3. An in vitro serum neutralization test was performed in a deer kidney tissue culture cell system. Results confirmed the agent propagated was South Dakota strain EHD virus.

4. South Dakota strain #10 EHD virus may be considered as an arbovirus because of its ability to grow in the mosquito cell line (Aedes albopictus) and its susceptibility to diethyl ether.

5. Chromosomes of normal white-tailed deer had a diploid number of 70. Chromosomes of EHD virus infected white-tailed deer kidney cells had changed chromosomal numbers and fragmentation of the chromosomes.

6. Total serum cholesterol of deer generally decreased from the EHD infection unless the deer were in a severely hemorrhagic condition.
LITERATURE CITED


