THE HOST RANGE OF AN ENTERIC CYTOPATHOGENIC ORPHAN "ECBO" VIRUS ISOLATED FROM HEALTHY DAIRY CATTLE

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Abbas Mohamed Soliman

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This is to certify that the

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An Abstract

A virus isolated from the stools of "healthy" dairy cattle was tested for its host range.

The virus was found to multiply in the amnionic cavity of White Leghorn embryonated eggs, and after several passages, the agent was adapted to the allantoic sac.

When the virus was injected intracerebrally into adult mice, hamsters, white rats, cotton rats, guinea pigs, cats, dogs, chicks and chickens, no evidence of disease was found in these animals.

Suckling mice and hamsters were found to be susceptible to the virus. Both animals developed paralysis followed by death. The virus could be maintained serially in both suckling mice and hamsters.

Two calves were exposed to this virus. The first animal was given the virus per os. Although the animal died after 4 days, it was thought that death was not produced by the virus. The virus was recovered from the animals intestinal content. A second calf was injected intraveneously with the virus. This animal showed no symptoms of disease although the virus could be recovered from the feces for 13 days following inoculation.

Neutralization tests were done on paired sera from cats, dogs, chickens and one calf. Only the calf's post-inoculation serum sample showed evidence of antibodies to this virus.

Antiserum, for a different enteric virus isolated from cattle, did not neutralize the virus under study; indicating that there are probably several antigenic different enteric viruses in cattle.

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PART I

INTRODUCTION AND HISTORY

Interest in the enteric viruses was stimulated by a study of the human digestive system in which successive investigations indicated that an uncertain number of unknown viruses exist. Poliomyelitis and the Coxsackie group of viruses were known, for several years, to be harbored in the digestive tract. Recently, another group of viruses was found in the human digestive tract but these viruses were orphans, that is the diseases they produced, if any, were not known. This group of viruses was called enteric cytopathogenic human orphan (ECHO) viruses because they were enteric cytopathogenic agents from humans and were orphans. A search for viruses in the digestive tract of animals revealed that cattle also have their group of orphan viruses which were called Enteric Cytopathogenic Bovine Orphans, or ECBO viruses.

PART II

LITERATURE REVIEW

Poliomyelitis is characterized by gastrointestinal symptoms with possible involvement of the central nervous system. Wickman (1,13) published the first extensive review of its infectious nature.

Landsteiner and Popper (1909) and Flexner and Lewis (1909) transmitted the disease to monkeys following intraperitoneal inoculation with a saline emulsion of the spinal cord from a fatal case of poliomyelitis. Successive workers established the disease through other routes in primates.

Symptoms, identical to those occurring in man, developed in the monkey as a result of intranasal installation or oral administration of the infective material. A viremia, similar to that observed in man, was found during the incubation period (Horstmann, 1952; Bodian, 1952; et al, 1953 and 1954; Horstmann et al, 1954) with development of antibodies. Evans, et al, (1954), tried intratesticular inoculation in the living rhesus and cynomolgus monkeys, mice, hamsters and guinea pigs, but failed to produce typical poliomyelitis symptoms.

Chimpanzees were found to be more susceptible to the different types of the poliovirus, while monkeys of various genera were susceptible only to certain strains (Melnick and Ledinko, 1951). The Atales geoferyi monkey is highly susceptible to infection with three strains of type I poliovirus but is insusceptible to two strains of type II and III poliovirus. Asymptomatic infection with types II or III poliovirus conferred various degrees of protection against reinfection with one strain of type I poliovirus (Jungeblut, et al, 1954). However, at times both chimpanzees and monkeys failed to show evidence of disease, when the

attenuated "TN" strain, was used as the test agent. With this strain of virus, more resistance to the disease was evident in the monkey as compared with chimpanzees (Koprowski, et al, 1954). Burnet and Macnamara (1931) reported that monkeys, convalescent from paralytic attacks induced by a certain strain of poliomyelitis virus, were highly resistant to further infection by the same strain of virus. However, in chimpanzees inoculated orally, a second infection was initiated by the same strain when a period of a year separated the two exposures (Melnick and Horstmann, 1947; Howe, et al, 1950).

Armstrong (1939) adapted the type II Lansing strain of poliovirus to cotton rats and then to mice, thus enhancing experimental work with this virus. Schlesinger, et al, (1943) succeeded in adapting a Type II strain (Middle East Force - MEF-I) to mice. Casals, et al, (1951) found that newborn mice were more susceptible than adult mice to MEF-I strain. Suckling hamsters were found to be susceptible to the same strain and develop paralysis following intramuscular injection (Moyer, et al, 1952).

Through alternate passage in tissue culture and intracerebrally in mice, Krech (1954) was able to adapt the Type I, Mahoney strain, and the Type III, Leon strain, to the mouse intracerebrally. Similarly, Koprowski, et al (1954) propagated the Mahoney and the Sickle strains in mice and cotton rats. Stanley, et al (1954) adapted Types I and III intracerebrally in the "Prince Henry", but not in white Swiss mice.

Li, and Habel (1951) adapted the Type III Leon strain to mice intraspinally. Later Li and Schaeffer (1953) modified and adapted the method for use with the neutralization test.

Syrian hamsters inoculated intracerebrally with the Saukett strain (Type III) developed paralysis within 5 - 8 days (Keegan, Chang,

and Brueckner, 1954). Schwartzman (1950) reported that hamsters, treated with cortisone prior to intraperitoneal inoculation with MEF-1 strain, showed an increased percentage mortality rate and paralysis.

All known strains of the poliovirus belong to three types: Type I(Brunhilde), Type II (Lansing), and Type III (Leon) which are serologically distinct. The specific antiserum of any type fails to cross neutralize a heterologous type (The Committee on Typing of the National Foundation for Infantile Paralysis, 1951).

For classification and identification, the serum neutralization test was performed in vitro (Netter and Levaditi, 1910; Shaughnessey, Harmon and Gordon, 1930, Paul and Trask, 1935), in white mice (Haas and Armstrong 1940, Hammon and Izumi, 1942), and in tissue culture (Robbins, Enders, Weller and Florentino, 1951).

A specific complement fixation test for poliomyelitis has been described by Casals, Olitsky, and Anslow (1951). Casals and Olitsky (1951), used type II virus as antigen. A promising virus antigen has been prepared from tissue cultures (Svedmyr, Enders, and Holloway, 1953). However, the test is still in the experimental stage.

For many years, the in vitro cultivation of the poliovirus received a great deal of attention. Levaditi (1913), Flexner and Nogouchi (1913), and Long, Olitsky and Rhodes (1930), attempted to cultivate the virus in monkey spinal ganglia, rabbit kidney and human ascitic fluid, but results were irregular and controversial.

Sabin and Olitsky (1936), were probably the first to propagate the poliovirus in vitro on nerve tissue in Tyrode's solution. Enders,

Weller and Robbins (1949) introduced the use of extraneural tissue for the propagation of poliovirus.

Later, Weller, Robbins and Enders (1949) were able to grow the virus on tissue cultures of skin and subcutaneous tissues from children. Smith, Chambers, and Evans (1951) found that adult human testicular tissue would support growth of the poliovirus. Soon various adult tissues such as kidneys, lungs, and spleen were found capable of growing the virus when properly prepared in tissue cultures (Weller, Enders, Robbins and Stoddard, 1952). A strain of neoplastic cells, "HeLa" (Gey, Coffman and Kubicek, 1952), was found to propagate the virus with evident cytopathogenicity (Scherer, Syverton, and Gey, 1953). The agent was also grown in monkey testicular cells (Scherer, Butorac and Syverton, 1951) but was not observed to multiply on testicular tissues from mouse, guinea pig, hamster, cow or dog (Robbins, Weller, and Enders, 1952; Evans, Chambers, Smith and Byatt, 1954).

The virus growth in these tissues from humans or animals, normal or abnormal in origin (tumors), was accompanied by cytopathogenicity which is a visual criterion of virus multiplication. Kidneys from all primates were thought to share this property but Kaplan and Melnick (1955) found that the Cebus capucina kidney was an exception. Tissue cultures derived from the kidney of this monkey supported the growth of Type II (YSK) strain without evident cytopathogenicity.

After 119 serial passages in suckling hamsters, Roca-Garcia, Moyer and Cox (1952) maintained the MEF-I strain for 41 generations in the yolk sac of the embryonate chick, Cabasso, Stebbins, Dutcher,

Moyer, and Cox (1952) adapted the same strain to the allantoic sac. Without previous passage of the virus in suckling hamsters, Durham and Ewing (1953) reported apparent adaptation to the chick allantoic sac after cortisone treatment of the chick embryo.

Li and Schaeffer (1954) reported the multiplication of a number of substrains of modified poliomyelitis virus (Type I Mahoney strain) grown on monkey skin or kidney tissue culture grafted onto the chorio-allantoic membrane.

Dalldorf and Sickles (1948) injected the processed stool from a child ill with poliomyelitis into one day old white mice. A virus was isolated which they believed to be the poliovirus. A complete investigation of this virus, however, revealed that it was not the poliovirus but an unknown agent. The virus was called "Coxsackie" by the two workers because the child from whom the virus was isolated was from Coxsackie, New York. Soon after the isolation of the Coxsackie virus, Melnick, Shaw and Curnen (1949), Curnen and Melnick (1951), Hummeler, Kirb, Pa., and Ostapiak (1954), Tyrrell and Snell (1956), demonstrated that the virus could also be found in cases of aseptic meningitis.

From cases of pleurodynia, a syndrome described by Sylvest early in 1933; Curnen, Shaw and Melnick (1949), Curnen (1950), Kilbourne (1950), Shaw, Melnick and Curnen (1950) were able to isolate an agent which proved to be identical to the Coxsackie virus.

Coxsackie virus was also found to be responsible for herpangia by Melnick and Ledinko (1950), Shaw, Melnick and Curnen (1950, Huebner, Armstrong, Beeman and Cole (1950), and by Webb, Wolf and Howitt (1950), Since then, Coxsackie virus has been found associated with a variety of illnesses ranging from aseptic meningitis to

"summer flu" and also, in a large number of healthy carriers (Melnick and Agren, 1952). The Coxsackie viruses have been repeatedly isolated from the stools of paralytic poliomyelitis patients along with the poliovirus (Melnick and Ledinko, 1951; Rhodes, et al., 1950; Melnick, 1951).

On the basis of their antigenic relationship and pathogenicity for suckling mice and hamsters, the Coxsackie viruses have been classified into A and B groups (Dalldorf, 1950).

Most investigators agree that group A viruses are responsible for the herpangia syndrome (Cole et al., 1951; Huebner, 1951; Huebner, et al., 1952; Huebner, 1957) and that group B is the etiological agent of epidemic pleurodynia (Finn, Weller and Morgan, 1949) or the aseptic meningitis syndrome (Hummeler et al., 1954; Tyrrell and Snell, 1956).

Group A viruses produce typical lesions in the striated muscle tissue of baby mice and suckling hamsters. These resemble Zenker's hyaline degeneration and are observed with all Coxsackie viruses irrespective of their antigenic type (Dalldorf et al., 1949; Dalldorf, 1950; Melnick and Godman, 1951).

In suckling mice Group B viruses produce moderate muscular lesions, characteristic encephalopathy, inflammatory changes in dorsal fat and pads of the feet (Dolldorf, 1950; Rhodes, 1956). The suckling mouse may be infected by intracerebral, intraperitoneal or subcutaneous injection of the virus, but rarely by oral administration. As the animal becomes older, susceptibility declines. Mature mice are refractory to infection with the virus (Kaplan and Melnick, 1951). Kilbourne and Horsfall (1951) reported that treating adult mice with

cortisone increased their susceptibility to infection with Conn. 5 strain of Coxsackie virus. Monkeys inoculated with Coxsackie viruses sometimes develop subclinical infections. When administered orally to various types of monkeys, including the cynomolgus, rhesus and cercopethicus, the Ohio strain produced subclinical infection in the cynomolgus only. Following a viremia, these animals finally produced neutralizing and complement fixing antibodies (Melnick and Ledinko, 1950).

Co-existence of the Coxsackie and poliovirus creates a peculiar situation which some workers describe as a "sparing effect", others as an "interference" phenomenon. Dalldorf (1951) demonstrated the "sparing" effect of this dual infection with mice in which the "Nancy" strain of group B Coxsackie virus was inoculated and followed 5-8 days later with the "Lansing" strain of poliovirus. The survival rate was higher in those mice receiving both viruses. Stanley (1952) attempted to demonstrate "interference" between the Coxsackie and the poliovirus in monkeys and mice. He found, that if mice received group A Coxsackie virus first, followed by MEF-I strain of poliovirus, there was a decrease in the incubation period of the poliovirus. On the other hand, if group B Coxsackie virus was used, there was an increased incubation period. A similar effect was not demonstrated in monkeys.

Sixteen antigenic types of the Coxsackie viruses have been recognized to date. Identification and classification of these viruses were accomplished with the aid of cross neutralization test in mice (Sickles, Dalldorf, 1949; Sulkin, Manire and Farmer, 1950) and the

complement fixation test (Casals, Olitsky, and Murphy, 1949).

Using tissues of newborn mice, Slater and Syverton (1950) propagated the Coxsackie virus in tissue culture. The virus was also adapted to the chicken embryo (Huebner, Ransom, and Beeman, 1950; Godenne and Curnen, 1952) and was grown on chicken embryo culture in serum ultrafiltrate (Shaw, 1952). Stulberg, Schapira and Eidam (1952) found that a tissue culture of fibroblasts derived from the footpads of newborn mice supported the growth of some strains of group B. Sickles, Mutterer, Florino, and Plager (1955) reported that group A strains, designated 11-19-13-15-18, are cytopathogenic for human uterine tissue culture in plasma clot, for the HeLa cells, but not for trypsinized monkey kidney and testicular cells.

The ECHO Viruses (Enteric Cytopathogenic Human Orphans) are a group of human intestinal agents which were discovered as a result of refined tissue culture technic.

In Egypt, Melnick and Agren (1952) isolated two agents by means of tissue culture. One proved to be a Coxsackie virus and the other failed to produce lesions in the central nervous system of rhesus or cynomolgus monkeys. The latter virus was also non-pathogenic for adult and suckling mice. Antisera for Types I, II and III poliovirus failed to neutralize its cytopathogenicity in tissue culture. This agent was antigenically distinct from similar agents isolated in the United States. Steigman, Kokko and Silverberg (1953) isolated a similar "unidentified" agent from the stools of a hospitalized child in Cincinnati, Ohio. Following this, a large number of strains was isolated. They were at first believed to have no significant role as

disease agents (Committee on ECHO Viruses, 1955). However, since these viruses were found in the stools from patients with either poliomyelitis or Coxsackie infection, there was some apprehension regarding their pathogenicity. Occasionally, some of these agents could be isolated from patients with aseptic meningitis in which no poliovirus or Coxsackie virus was found (Enders, 1957; Winkelstein et al., 1957). They were also isolated from stool specimens from individuals not showing any evidence of disease (Alvarez and Sabin, 1956; Honig, et al., 1956; David and Melnick, 1956; Enders, 1957).

Laboratory animals, including adult and suckling mice, hamsters, guinea pigs and rabbits (Steigman, Kokko and Silverberg, 1953), monkeys (Alvarez and Sabin, 1956; Ormsbee and Melnick, 1957) were found to be non-susceptible to experimental infection.

In tissue culture, the ECHO viruses are highly pathogenic to cells of rhesus monkey (Macacca mulata) kidney (Steigman, Kokko and Silverberg, 1953; Committee on ECHO Viruses, 1955; Duncan et al., 1955; Alvarez and Sabin, 1956). Kidney cells of the South American capuchin monkey (Cebus capucina) support the growth of type 10 virus, while cells from the African red grass military monkey (Erytherocebus patas) propagate type 7. HeLa cells also support viral growth but are not as susceptible as rhesus monkey kidney cells (Committee on ECHO Viruses, 1955).

Some types of ECHO viruses grow in cells derived from human embryonic skin and muscle, postnatal uterine and human kidney tissues (Hammon et al., 1957) and human amnion cells (Zitcer, Fogle and Dunnebacke, 1955).

Plaque morphology of types 1, 3, 4, 5, 6, 7 and 9 was studied. All except type 7 could be differentiated from the poliovirus plaques by their diffuse irregular margins. Cells in some areas of these tissue cultures failed to show cytopathogenicity (Committee on ECHO Viruses, 1955).

At the present time 19 strains of the ECHO viruses are known. Serologically, this group of viruses is not neutralized by poliomyelitis antiserum or by antisera for the Coxsackie viruses (Committee on the ECHO Viruses, 1955). However, the members of this group are neutralized by human gamma globulin and individual human serum. Complement fixing antigens for this group have been detected in tissue culture fluids (Committee on ECHO Viruses, 1955).

For the sizes of the viral particles, the Committee on ECHO Viruses in 1955 approved the following measurements obtained by the gradocol membrane filtration: types 1, 2 and 3 are 11- 17 mu; type 10 is 60-90 mu. Alvarez and Sabin, (1956) found by the same method, that types 7, 8, 9 and 11 measured 300 mu and type 10 measured 60-90 mu.

In 1946 Olafson, McCallum and Fox described an explosive infection in cattle with a high morbidity and a low mortality rate. Affected animals showed anorexia, weakness, salivation and nasal discharge, ulceration of the buccal mucus membrane and diarrhea with sometimes a rise of temperature.

Post mortem examination findings were: inflammation and ulcerations along the digestive system, congestion of subcutaneous tissues, hemorrhages on the epicardium and vaginal mucus membrane.

Calves experimentally infected with the isolated virus did not always develop the symptoms described above. Mice, rabbits, guinea pigs and sheep were not susceptible to infection by any route of inoculation. Embryonated eggs inoculated by different routes did not support virus growth (Olafson, McCallum and Fox, 1946; Olafson and Rickard, 1947).

Lee and Gillespie (1957) propagated this virus in tissue culture of bovine embryonic skin and muscle in chick embryo extract, and in trypsinized bovine embryonic kidney cortex tissue culture. In bovine skin and muscle tissue culture, twenty serial passages were made with this virus. In cultures of bovine kidney, 15 passages were made. The titer obtained in the former system was 10^5 LD50 and in the latter, 10^7 LD50. No cytopathogenicity was observed in either system.

Moll and Finlayson (1958) described a febrile disease in calves characterized by cough, nasal discharge accompanied by considerable mucus in the stools. The virus isolated from the stools of these calves was demonstrated to be cytopathogenic in tissue culture but paralysis was not induced in suckling mice.

The agent reported in this thesis is probably an ECBO (Enteric Cytopathogenic Bovine Orphan) virus. It was obtained from Miss Elva Minuse of the School of Public Health, University of Michigan. The virus was found to propagate with evident cytopathogenicity on tissue cultures originating from: cattle, and rhesus monkey kidney cells, and chick embryo body tissue cultures.

In embryonate chicken eggs the agent multiplied when introduced onto the chorioallantoic membrane producing pock-like lesions. It also developed in the yolk and amnionic sacs of the fertile chicken egg.

The virus did not grow in HeLa cell tissue cultures, and when introduced into the allantoic sac of embryonate eggs, did not multiply. Suckling mice succumbed with paralysis upon intraperitoneal inoculation with viris propagated in eggs or tissue culture but not with the virus directly isolated from stools (Kunin and Minues, 1957).

Antisera prepared by intravenous inoculation of rabbits with this virus neutralized the virus from tissue cultures and embryonate eggs. Serum from the host cattle did not protect chicken embryos but did delay their death (Kunin and Minuse, 1957). Antisera from 14 of the ECHO viruses, Vesicular Stomatitis-types New Jersey and Indiana, calf pnemonitis enteric virus, the Sabin calf 25 enteric cytopathogenic virus, bovine mucosal disease and bovine rhinotracheitis failed to neutralize this virus.

Kunin and Minuse (1957) found that the viral particle has a sedimentation constant ranging between 150-200 Svd.

Storage of the virus at 4C for 5 days did not affect the titer while at -70C for few weeks, the titer was reduced. Infected amnionic fluid stored for several months at 4C retained the titer.

When this agent was inoculated into the amniotic sac of embryonate eggs from Barred Rock chickens, Kunin and Minuse (1957) found that the harvested fluid contained what appeared to be melanin particles.

Since the virus reported in this study was isolated as an "enteric agent" from cows, it was of interest to determine the infectivity spectrum of this agent. To also determine the effect of this virus on cattle and to describe the symptoms seen in experimentally

infected animals.

PART III

MATERIAL AND METHODS

Embryonate Eggs

Eggs used were from White Leghorn hens and were incubated for 9 consecutive days prior to inoculation. The incubated eggs were inoculated by the allantoic sac route, each was given 0.1 ml of the appropriate dilution of inoculum. Before harvesting the allantoic fluid, all eggs were chilled at 4 C. Control eggs were, in every case, one-fourth the number of inoculated eggs and were inoculated with the same amount of phosphate buffer solution (pH 7.6). All test and control eggs were candled daily for 10 days following inoculation. As death of the embryos was the criterion of infectivity, all survivals were discarded after this period. All eggs with dead embryos were stored at 4 C until the allantoic fluid was harvested aseptically. A sterility test was performed on the allantoic fluid removed from every egg. When proved sterile, the material was pooled and stored at -20 C in a screw cap vial.

Virus Titration

The virus contained in the pooled allantoic fluid was titrated in embryonate hens' eggs. The virus suspension, in allantoic fluid, was quickly thawed under tap water and centrifuged at 4000 rpm for 15 minutes to remove coarse particles. The supernatant fluid was removed from the sediment. Using the phosphate buffer solution as diluent, serial ten fold dilutions ranging between 10^{-1} and 10^{-10} were prepared

from the undiluted stored allantoic fluid. One tenth ml of each dilution was injected into the allantoic sac of six 9-day old embryonate eggs. Control eggs received 0.1 ml of the buffered solution only. From each dilution, two serum broth tubes were inoculated with 0.1 ml of the suspension and incubated for 5 days at 37 C to test for sterility.

The LD50 titer of the virus as determined by the method of Reed and Muench (1938) was $10^{7.0}$ per 0.1 ml of the allantoic fluid. This pool of allantoic fluid served as a virus source for all the eggs and animals tested in this work.

White Mice

All mice used were Swiss white mice of the Webster strain. Mice 3 weeks old or more were considered as adults. Inoculations were intracerebral, intraperitoneal or subcutaneous, with 0.03, 0.5, 0.2 ml of the virus respectively. Oral administration of the virus was in 0.1 ml amounts. Uninoculated control mice were also included and all animals were observed for a period of 21 days subsequent to inoculation for the development of symptoms.

Suckling Mice

Immature mice were one or two days of age when injected with the virus or material suspected of containing a virus. Those animals inoculated intracerebrally received 0.01 ml, and those inoculated intraperitoneally were given 0.05 ml of the material.

Control animals received no injections. All mice used as test animals were observed for 21 days subsequent to inoculation for symptoms of disease.

Guinea Pigs

Only adult animals were used. These animals were raised in the Department of Microbiology and Public Health, Michigan State University. Animals receiving intracerebral inoculations were injected with 0.1 ml, animals intraperitoneally inoculated were given 0.5 ml, and the subcutaneously injected hosts received 0.5 ml of the material. Some guinea pigs were given 0.5 ml of the virus *per os*.

White Rats

Adult white rats were obtained from a stock raised in the Department of Chemistry, Michigan State University. The animals were injected intracerebrally, intraperitoneally, subcutaneously or received the material orally with 0.1, 0.5, 0.5, and 0.5 ml of the virus, respectively. Uninoculated controls were also included. All animals were observed for 21 days for symptoms of disease.

Cotton Rats

Adult cotton rats (*Sigmodon hispidus hespidus*) were obtained from the Michigan State Department of Health Laboratories in Lansing.

Animals were inoculated intraperitoneally, subcutaneously or received the material orally with 0.1, 0.5, and 0.5 ml of virus respectively. These uninoculated controls and test animals were observed for 21 days for symptoms of disease.

Hamsters

Adult Syrian hamsters were obtained from the Leptospirosis Laboratory, Michigan State University, and received the same amounts of virus as described above for the white rats.

Suckling one to two day old hamsters were injected with 0.01 ml of the virus intracerebrally or 0.5 ml intraperitoneally. Again, uninoculated controls were included and observed for symptoms of disease for 21 days.

Cats

Six cats ranging in age between 2 and 4 months were used in this study. The animals were of mixed breeds and sex. History of previous exposure to the virus under study was unknown in these animals; therefore, four of the animals were bled for serum samples prior to exposure to the virus. Each cat, while under anaesthesia, was inoculated intracerebrally with 0.5 ml of the virus. Two cats were observed as controls.

On the second day following inoculation, one of the animals died presumably from trauma and was discarded. The remaining test animals were observed daily and the temperatures recorded. After the usual observation period of 21 days,. the test animals were again bled to obtain a post-inoculation serum sample.

Dogs

Two dogs of mixed breed, 3-4 months of age, were obtained from the Department of Physiology, Michigan State University. Thirty-five ml of blood were obtained from the external saphenous vein of each dog. The site of intracerebral inoculation was shaved, cleaned with soap and water and tincture of metaphen applied to the areas of inoculation. Using a sterile 4 cm x 2 mm needle as a trephine, 1.5 ml of the virus was introduced into the left cerebral hemisphere of each dog.

During the 21 days observation period, temperatures were recorded

twice daily and at the end of the observation period the two dogs were bled. The second blood sample represents the post-inoculation serum sample.

Chickens

A number of White Leghorn chickens, 6-8 weeks of age were kindly supplied by the Department of Poultry Husbandry, Michigan State University. Some were inoculated while others served as controls. Test chickens were first bled from the wing vein, then each was inoculated intracerebrally with 0.1 ml of the virus. Following the observation period, the inoculated chickens were bled for the second time. Serum was secured from pre- and post-inoculation blood for serum neutralization tests.

Baby Chicks

A group of one day old White Leghorn chicks were kindly offered by the Department of Poultry Husbandry, Michigan State University. Test birds were inoculated intracerebrally with 0.03 ml of the virus. The control and test birds were observed for 21 days subsequent to inoculation for any symptoms of disease.

Calves

Two calves were exposed experimentally to the virus in an attempt to produce disease.

The first was a five day old Jersey calf obtained from the Dairy Department, Michigan State University. It was not in good physical condition when obtained but had a good appetite and normal temperature. The calf was observed for 7 days prior to being used as a test animal.

On the eighth day, a fecal sample was obtained and a blood sample was collected aseptically from the jugular vein prior to feeding the calf with 5 ml of the virus. On the third day following the feeding of the virus, the animal refused food, and had diarrhea. The temperature was normal. The following day, the animal had a subnormal temperature and subsequently died.

The second animal was a 3 month old Aberdeen-Angus calf obtained from the Department of Animal Husbandry, Michigan State University. The animal appeared to be in good physical condition. The calf was observed for a period of one week during which the animal had a normal temperature. At the end of the observation period, stools and a blood serum sample were collected before the calf was injected intravenously with 2 ml of the virus. Temperatures were taken daily and a stool sample was collected each day starting the fifth day after inoculation. Samples were collected for 15 days and were stored in the freezer for subsequent evaluation.

Preparation of Stool Suspension

The frozen fecal specimen was allowed to thaw at room temperature. A 10 per cent suspension of fecal material was prepared in phosphate buffered solution at pH 7.6. Penicillin and streptomycin, 500 units and 500 ugms respectively, were added to each milliliter of the suspension. A portion of the suspension was then centrifuged at 4000 rpm for 30 minutes at room temperature. If the supernatant fluid was still turbid, centrifugation was continued for another 30 minutes. The supernatant fluid constituted the virus suspension.

Serum Neutralization Tests

The serum neutralization tests were performed on the pre- and post-inoculation sera from dogs, cats, chickens, calf 2, and pre-inoculation serum from calf 1 that died before obtaining the post-feeding serum sample. The post-inoculation sera were obtained at the end of the 21 day observation period. The test was also carried out on the pre- and post-inoculation sera from a calf infected with "virus diarrhea" virus.

The concentration of the virus contained in the allantoic fluid was determined immediately prior to use. The LD50, as determined via the Reed-Muench method (1938), was found to be $10^{6.6}$ i.u. per 0.1 ml of allantoic fluid.

To perform the neutralization test, serial 10 fold dilutions of the virus were made in phosphate buffer. The unknown serum was inactivated at 56 C for 30 minutes. From each dilution tube, equal parts of diluted virus and the test serum were thoroughly mixed and incubated at room temperature for 30 minutes prior to inoculation. In order to be within the range of 100 per cent mortality in the lower dilutions and 100 per cent survivals in the higher dilutions the 10^{-3} through 10^{-7} dilutions were employed .

From each dilution, five 9 day old embryonate eggs were inoculated via the allantoic sac, each with 0.1 ml of the virus serum mixture.

Virus and serum controls were also included. From each dilution tube, five 9 day old embryonate eggs were inoculated via the allantoic sac, each with 0.1 ml of the virus serum mixture, and incubated at 37 C for 10 days.

The end point of virus activity was considered to be the highest dilution of the virus in which 50 per cent or more of the inoculated embryos were killed. Assuming the end point dilution to contain one infectious unit, the reciprocal of the dilution would indicate the concentration of infective doses or the virus titer in the original undiluted sample. The end point of viral activity in the virus-serum mixtures was the lowest dilution of the virus in which 50 per cent or more of the embryos survived. The neutralization index, which is the measure of reduction of viral activity by neutralizing antibody, was expressed as the difference between the virus titer and the virus-serum mixture titer. The average neutralization index was computed by dividing the sum of neutralization indexes of either the pre- or post-inoculation sera by the number of serum samples from each respective group of animals.

PART IV

RESULTS

The first virus passages in embryonate eggs were made by way of the allantoic sac in White Leghorn eggs in order to obtain enough of the virus suspension to complete this work. The original virus, as received in this laboratory, had been adapted to the amnionic cavity of Barred Rock embryonate eggs. Of 48 - 9 day old embryonate eggs, inoculated via the allantoic cavity, each with 0.1 ml of a 10^{-1} dilution of the original amnionic fluid, only 15 embryos (31.2%) died within 10 days. The allantoic fluid from these eggs was harvested and after sterility tests, was pooled and stored in the frozen state for further passage.

In contrast to control eggs inoculated with buffered solution, but no virus, the infected embryos were dwarfed and showed less development. Edema of the chorioallantoic membrane, congestion and focal hemorrhages were the most consistent findings. Cutaneous and subcutaneous hemorrhages, edema and malformation of the toes were seen in many embryos. It was observed that the allantoic fluid from the White Leghorn eggs did not contain the melanin particles which were present when this virus was harvested from the Barred Rock eggs (Kunin and Minuse, 1957). On the contrary, the allantoic fluid was invariably clear except for traces of blood or excess of urates in some of the harvested eggs.

A sample of the harvested virus in the allantoic fluid was

serially passed through eggs from White Leghorn hens. After 10 serial passages, this virus was found to produce high mortality when passed by the allantoic route of White Leghorn embryos. Changes, as described, in the morphology of the embryos continued. The titer of virus in the pooled, frozen, allantoic fluid harvested from the tenth serial passage was determined. Again, White Leghorn eggs were used for the titration and 0.1 ml of the allantoic fluid was introduced into the allantoic cavity. The LD50 titer of the virus $10^{7.0}$ in the allantoic fluid. These data are represented in Table I.

To determine if the virus in the allantoic fluid would infect the embryos by the amnionic sac, 12 eggs were inoculated, each with 0.1 ml of the pooled allantoic fluid into the amnionic cavity. Nine of the 12 eggs embryos became infected and died (Table I). Five control eggs were inoculated by the same route with 0.1 ml of phosphate buffer solution (pH 7.6) and all survived the 10 days incubation period.

The pooled allantoic fluid containing the virus (10th serial passage) was used as source of virus for the experimental animal inoculations.

When adult Swiss mice were inoculated with the virus, all inoculated mice remained free of symptoms of disease. Table IV shows the routes of inoculation of the adult mice.

To determine if this virus could be propagated in suckling mice, a sample from the allantoic fluid was injected intracerebrally into a litter of 7 suckling mice. Five days after inoculation (Table II) 2 of the suckling mice were found paralyzed. One of the two mice developed paralysis of both hind legs and was sacrificed. The second animal

showed paralysis of the right front leg, or a "wrist drop" type of paralysis, quite similar to that seen in adult Swiss mice after being injected intracerebrally with the type II poliovirus. This mouse was also sacrificed.

The brains were removed from these two suckling mice and a 10^{-1} buffered suspension (pH 7.6) was made by grinding the brains with a mortar and pestle. The suspension was then centrifuged at 3000 rpm for 30 minutes to remove coarse particles. A sample of the supernatant fluid was used to inject 12 suckling mice intracerebrally and 7 intraperitoneally. Two of the 12 mice (Table II) died without observed symptoms. However, 10 were seen to be paralyzed. All seven intraperitoneally inoculated mice died after developing paralysis (Table II). The first evidence of infection was sluggishness and impaired movement of the affected limb. Paralysis usually developed in the animals one day later and was progressive. The paralysis developed in either the front or hind legs but no evidence of complete paralysis in all four limbs was observed in mice. The animals were sacrificed at the height of paralysis to harvest the brains. A second passage of this mouse brain material was introduced intraperitoneally in seven suckling mice all of which developed paralysis and died (Table II).

To compare infectivity by the intracerebral and the intraperitoneal injection, the virus was titrated by both routes in one or two day old mice. Paralyzed mice brains were aseptically harvested and finely ground. A 10 per cent suspension was prepared in phosphate buffer solution (pH 7.6). Penicillin and streptomycin, 500 units and 500 ugms, respectively, were added to each ml of the suspension.

The suspension was spun at 4000 rpm for one hour. The clear supernate was then removed from the sediment and serial ten fold dilutions ranging between 10^{-1} and 10^{-8} were made in phosphate buffer solution (pH 7.6).

Four suckling mice per dilution were inoculated either intracerebrally or intraperitoneally. Mice inoculated by the first route received 0.01 ml, while those inoculated by the second route had 0.05 ml of the respective dilution.

The virus titers in both cases were determined according to Reed and Muench's formula. Following intracerebral injection the virus titer was LD50 $10^{4.6}$, and LD50 $10^{5.5}$, as a result of intraperitoneal inoculation (Table III).

The guinea pigs did not show any evidence of paralysis or disease during the test period of 21 days. Temperatures were recorded on each injected animal twice each day. None of the animals developed abnormal temperature.

White rats were injected with the virus by various routes (Table IV). None of the 16 animals injected showed symptoms of disease during the 21-day holding period.

Three cats ranging in age between 2 and 4 months old, but of unknown breed, were given a 0.5 ml intracerebral injection of the virus contained in allantoic fluid. After recovery from the anesthesia, none developed evidence of infection, during the 21 day observation period.

The two dogs used in this study were 3 and 4 months old and of unknown breed. Each was given 1.5 ml of the virus intracerebrally. During a 21 day observation period the temperature of these animals was

recorded twice daily. At no time during this period did either animal have a rise in temperature or have symptoms of illness.

Six White Leghorn chickens, about 6 months of age, were given 0.1 ml of the virus intracerebrally. Thirty-seven 1 day-old chicks of the same breed were given 0.03 ml of the virus intracerebrally. As can be seen in Table IV, none of these animals developed any signs of illness during the 21 day holding period.

Like the adult mice, adult cotton rats and hamsters did not develop any evidence of disease after being injected with the virus. In Table V the amounts of virus given and the routes are shown. All animals remained symptom-free during the 21 day observation period.

Suckling hamsters were found to be susceptible to the virus (Table VI). All suckling hamsters receiving the virus intracerebrally and intraperitoneally died. Of the eight animals receiving the virus intracerebrally, two died without symptoms being observed, while six were seen paralyzed before death. Of those eight animals receiving virus intraperitoneally, seven were seen to have paralysis. The paralysis seen in these animals differed from that seen in the mice. In the suckling hamsters some had complete paralysis of all four limbs. The animals' limbs were extended posteriorly and paralysis appeared to be of the spastic type.

Table VI also shows the results of exposing young calves to this virus. The first animal received orally 5 ml of the virus contained in allantoic fluid. Three days after receiving the virus this animal developed diarrhea. No blood or mucus was observed in the feces. The temperature of calf 1 remained within normal limits. On the fourth

day after receiving the virus, the animal was weak and could not stand alone. The diarrhea continued and the animal's temperature was found to be subnormal. The animal died during the night and was autopsied. The emaciated animal had patches of lung congestion.

The major pathological changes were found in the digestive system. The omasum, abomasum, intestines, and cecum all had patchy inflammation of the mucus membrane. The intestinal contents were liquid, but did not contain blood. The liver and the kidneys were congested and the gall bladder was distended. Congested areas of the omasum, abomasum and segments of the small and large intestines were frozen in sterile glass tubes and held for isolation of the virus.

Calf 2 received 2 ml of the virus, in allantoic fluid, intravenously. The temperature of the animal remained within normal limits throughout the experimental period. During the 15 day observation period no clinical evidence of infection was observed except some mucus in the feces. The mucus was minimal but was not observed in the feces prior to inoculation. The animal remained well throughout the test period.

To determine whether either calf was shedding virus in the feces, the pre- and post-inoculation fecal samples and tissue samples were tested. The stool and tissue frozen specimens were thawed at room temperature. A 10 per cent suspension was made in phosphate buffered solution (pH 7.6). Pooled samples of tissue from the alimentary tract of calf 1 were also made into a suspension by the same method. The suspension was centrifuged at 3000 rpm for 1 hour. The supernatant fluid was removed from the sediment and the former was treated with 500

units of penicillin and 0.5 mg streptomycin per ml to destroy the bacteria; cultures were also made to determine sterility. The specimens were finally inoculated into the allantoic cavity of White Leghorn embryonate eggs (Table VI). Control eggs were inoculated with the phosphate buffer solution. The eggs were incubated at 37 C and candled daily. Embryos which died within 24 hours of inoculation were not considered in the results.

The results of inoculating eggs with the fecal samples and tissues from calf 1 are given in Table VII. The pre-exposure sample produced death in one embryo of 12 inoculated. The post-exposure fecal sample killed half of the embryos inoculated. Only 2 of 12 embryos inoculated with tissue of the gastrointestinal tract were killed. None of the control embryos was killed by the procedure. A pool was made of the allantoic fluid from those embryos that died subsequent to injection with the fecal sample and another pool from those dying after inoculation with tissue sample preparations. The allantoic fluid was then injected intraperitoneally into suckling mice.

Five of 7 mice receiving the allantoic fluid from eggs inoculated with post-inoculation fecal sample were either paralyzed or dead within 4-7 days. The mice receiving the allantoic fluid of tissue sample apparently were not affected. (Table VIII).

In Table IX the results given are of eggs inoculated with the fecal samples from calf 2. There was no evidence of virus in the feces of the animal prior to inoculation, as the pre-inoculation feces did not kill any of the 5 eggs inoculated with this material. The post-inoculation fecal samples at first did not contain virus, as was

evidenced by survival of the eggs receiving these samples. The embryos receiving the fecal sample of the seventh day following inoculation began to die. This pattern continued throughout the 15 day period that the feces where collected and tested for infectivity of the virus.

To make certain that the embryos were being killed by the virus, the allantoic fluid from embryos that died following inoculation with post-inoculation fecal sample pools of the allantoic fluid from the eggs previously receiving the fecal preparations was combined in five pools. From each pool a number of suckling mice was inoculated intraperitoneally. Each mouse was injected with 0.05 ml. All five pools (Table X) caused paralysis and death in the mice. The type of paralysis seen in the mice, after re-isolation of the virus, was identical to that already described. Control animals in all cases remained in a normal condition and were disposed of at the end of the experiments.

Serum Neutralization Tests

Neutralization tests were completed with pre- and post-inoculation sera from the two dogs tested. The neutralization index of the dog 2 pre-inoculation serum sample was 0.4 and the post-inoculation increased to 0.6. The serum of dog 1 shows a negligible increase between the pre- and post-inoculation specimens (Table XI).

The results of 3 cats paired sera are presented in Table XI. Cat 2 is the only one that shows an appreciable increase in neutralization index in the post-inoculation serum when compared to that of the pre-inoculation serum.

The results of six neutralization tests on the pre- and post-inoculation sera of chickens are illustrated in Table XII .

The results on chicken 2 serum show that only in this one animal was there any rise in the serum neutralization index. The results on the five remaining birds do not show a significant increase in the serum neutralization index.

The results in Table XIII are those of the neutralization tests on calf 1 pre-inoculation serum and calf 2 pre- and post-inoculation sera, and both sera from a calf infected experimentally with a strain of "virus diarrhea" virus. The latter sera were kindly supplied by Dr. G. H. Gillespie, New York State Veterinary College. Only calf 2 post-injection serum exhibits a significant neutralization index of 2.1

TABLE I

RESULTS OF INOCULATING WHITE LEGHORN EMBRYONATED EGGS WITH THE VIRUS

Test animals	Dose in ml	Route	Results	Incubation period in days	Virus titer LD50
Embryonated Eggs	0.1	A.S.[a]	50/56*	5 - 6	$10^{7.0}$
Controls			0/14		
Embryonated Eggs	0.1	Am.S.[b]	9/12	3 - 4	
Controls			0/4		

(a) A.S.: Allantoic sac.

(b) Am. S.: Amnionic sac.

*Numerator: Number Affected
Denominator: Number Injected

TABLE II

RESULTS OF INOCULATING SUCKLING MICE WITH THE VIRUS

Amount in ml	Inoculum	Route	Results	Incubation period in days
0.01	A.F.[a]	I.C.[b]	2/7*	4-5
0.05	M.B.S.[c]	I.P.[d]	7/7	4-5
0.01	M.B.S.	I.C.	12/12	3-6
0.05	M.B.S.	I.P.	7/7	4-6
Controls			0/10	

[a] A.F.: Allantoic fluid.

[b] I.C.: Intracerebral.

[c] M.B.S.: Mouse brain suspension.

[d] I.P.: Intraperitoneal.

*Numerator: Number affected
Denominator: Number injected

TABLE III

COMPARISON OF INFECTIVITY BETWEEN INTRACEREBRAL AND INTRAPERITONEAL TITRATIONS IN SUCKLING MICE

Dose	Route	Dilutions								Virus Titer LD50
		10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	
0.01 ml	I.C.[(a)]	-	4/4*	3/4	2/3	1/4	1/4	0/4	0/4	$10^{4.6}$
0.05 ml	I.P.[(b)]	-	-	4/4	3/4	3/4	1/3	0/4	0/4	$10^{5.5}$

[(a)] I.C.: Intracerebral.

[(b)] I.P.: Intraperitoneal.

* Numerator: Number affected / Denominator: Number injected

TABLE IV

RESULTS OF INOCULATING ADULT WHITE MICE, GUINEA PIGS, AND WHITE RATS, WITH THE VIRUS

Test animals	Amount in ml	Route	Results
Adult White Mice	0.03	I.C.(a)	0/9
	0.5	I.P.(b)	0/9
	0.5	S.C(c)	0/9
	0.5	Oral	0/9
Controls			0/10
Guinea pigs	0.1	I.C	0/4
	0.5	I.P.	0/4
	0.5	S.C.	0/4
	0.5	Oral	0/4
Controls			0/4
White Rate	0.1	I.C.	0/4
	0.5	I.P.	0/4
	0.5	S.C.	0/4
	0.5	Oral	0/4
Controls			0/4

(a) I.C.: Intracerebral.

(b) I.P.: Intraperitoneal.

(c) S.C.: Subcutaneous

* $\frac{\text{Numerator: Number affected}}{\text{Denominator: Number Injected}}$

TABLE V

RESULTS OF INOCULATING CATS, DOGS, CHICKENS AND CHICKS WITH THE VIRUS

Test Animals	Amount in ml	Route	Results
Cats	0.5	I.C[(a)]	0/3*
Controls			0/2
Dogs	1.5	I.C.	0/2
Chickens	0.1	I.C.	0/8
Controls			0/3
Baby Chicks (1 day old)	0.03	I.C.	0/37
Controls			0/30

(a) I.C. Intracerebral.

*Numerator:Number affected.
Denominator:Number Injected.

TABLE VI

RESULTS OF INOCULATING ADULT COTTON RATS, ADULT AND SUCKLING HAMSTERS, AND CALVES WITH THE VIRUS

Test animals	Dose in ml	Route	Results	Incubation period in days
Adult Cotton Rats	0.1	I.C.[a]	0/4*	
	0.5	I.P.[b]	0/4	
	0.5	S.C[c]	0/4	
	0.5	Oral	0/4	
Controls			0/4	
Adult Hamsters	0.1	I.C	0/4	
	0.5	I.P.	0/4	
	0.5	S.C.	0/4	
	0.5	Oral	0/4	
Controls			0/4	
Suckling Hamsters	0.01	I.C.	8/8	3 - 5
	0.05	I.P.	8/8	4 - 6
Controls			0/7	
Calf (1)	5	Oral	1/1	
Calf (2)	2	I.V.[d]	0/1	

(a) I.C.: Intracerebral.

(b) I.P.: Intraperitoneal.

(c) S.C.: Subcutaneous.

(d) I.V.: Intravenous.

*Numerator: Number affected
Denominator: Number Injected

TABLE VII

RESULTS OF INOCULATING 9 DAYS OLD EMBRYONATED EGGS BY THE ALLANTOIC SAC, WITH FECAL AND TISSUE SPECIMENS FROM CALF I

Specimen	Amount in ml	Results
Pre-feeding feces	0.1	1/12*
Post-feeding feces	0.1	6/12
Pooled intestinal tissues	0.1	2/12
Controls		0/10

*Numerator: Number affected
Denominator: Number Injected

TABLE VIII

RESULTS OF INOCULATING SUCKLING MICE WITH ALLANTOIC FLUID FROM EGGS INOCULATED WITH FECAL AND TISSUE SPECIMENS FROM CALF I

Specimen	Inoculum	Amount in ml	Route	Results	Incubation period in days
Feces	A.F.	0.05	I.P.(a)	5/7*	4-7
Pooled Tissues	A.F.	0.05	I.P.	0/7	
Controls				0/7	

(a) I.P.: Intraperitoneal.

*Numerator: Number affected
Denominator: Number Injected

TABLE IX

RESULTS OF INOCULATING 9 DAY OLD EMBRYONATED EGGS, BY THE ALLANTOIC SAC, WITH FECAL SAMPLES FROM CALF 2

Specimen	Amount in ml	Days after inoculation of calf														
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Pre-inoculation feces	0.1							0/5*								
Post-inoculation feces	0.1	0/5	0/5	2/5	4/5	3/5	4/5	3/5	2/5	4/5	4/5	3/5	2/5	3/5	3/5	4/5
Total post-inoculation feces							41/57									
Controls																0/20

*Numerator: Number affected
Denominator: Number injected

TABLE X

RESULTS OF INOCULATING SUCKLING MICE WITH ALLANTOIC FLUID FROM EGGS INOCULATED WITH FECAL SPECIMENS FROM CALF II

Fecal samples on	Allantoic fluid pools	Amount in ml	Route	Results	Incubation
3-4	Pool I	0.05	I.P.*	5/8**	
5-6	Pool 2	0.05	I.P.	9/9	
7-9	Pool 3	0.05	I.P.	7/7	4-8
10-12	Pool 4	0.05	I.P.	6/8	
13-15	Pool 5	0.05	I.P.	8/9	
Controls				0/12	

*I.P.: Intraperitoneal.

**Numerator: Number affected
Denominator: Number Injected

TABLE XI

RESULTS OF SERUM NEUTRALIZATION TEST IN EMBRYONATED EGGS WITH DOGS AND CATS SERA

Test Animals	A* B**	Virus Dilutions 10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	Serum-virus mixture titer	Neutralization index	Average Neutralization index	
Dog (1)	A	5/5***	5/5	5/5	3/5	0/5	6.2****	0.4****		
	B	4/5	4/5	4/5	2/4	2/4	6.0	0.6	A	0.3****
									B	0.6
Dog (2)	A	4/4	5/5	4/5	3/5	1/5	6.5	0.1		
	B	5/5	3/5	3/5	2/4	0/5	6.0	0.6		
Cat (1)	A	5/5	4/5	4/5	3/5	2/5	6.5	0.1		
	B	3/3	4/5	4/5	3/5	2/5	6.5	0.1		
Cat (2)	A	5/5	4/5	3/4	3/4	1/5	6.5	0.1		
	B	5/5	3/5	3/5	2/5	2/5	6.1	0.5	A	0.1
									B	0.3
Cat (3)	A	5/5	4/4	5/5	3/5	1/5	6.4	0.2		
	B	5/5	4/5	3/5	3/4	2/4	6.2	0.4		
Virus Control		5/5	4/5	4/5	4/5	2/5	6.6			

*Pre-inoculation serum (serum control).

**Post-inoculation serum.

***Numerator: Number affected / Denominator: Number injected

****Approximation to one decimal.

TABLE XII

RESULTS OF SERUM NEUTRALIZATION TEST WITH CHICKEN SERA IN EMBRYONATED EGGS

Test Animals	A* B**	Virus Dilutions 10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	Serum virus mixture titer	Neutralization index	Average Neutralization index
Chicken (1)	A	5/5***	4/5	4/5	3/5	2/5	6.3****	0.3****	
	B	4/5	4/5	4/4	3/5	0/5	6.0	0.6	
Chicken (2)	A	4/5	4/5	4/5	3/4	2/4	6.6	0.0	
	B	5/5	4/5	4/5	2/4	2/5	6.0	0.6	
Chicken (3)	A	5/5	5/5	4/5	3/5	2/5	6.5	0.1	A 0.2
	B	5/5	5/5	4/5	3/4	1/5	6.5	0.1	B 0.4
Chicken (4)	A	3/3	4/4	3/4	3/5	2/5	6.5	0.1	
	B	5/5	4/5	3/4	3/5	2/5	6.2	0.4	
Chicken (5)	A	4/5	4/4	3/5	3/4	2/5	6.2	0.4	
	B	4/5	4/5	3/5	3/4	2/4	6.2	0.4	
Chicken (6)	A	5/5	4/5	4/4	3/5	2/5	6.5	0.1	
	B	5/5	5/5	4/5	3/5	2/5	6.5	0.1	
Virus Control		5/5	4/5	4/5	4/5	2/5	6.6		

*Pre-inoculation serum (serum control).
**Post-inoculation serum.
***Numerator: Number affected
Denominator: Number injected
****Approximation to one decimal.

TABLE XIII

RESULTS OF SERUM NEUTRALIZATION TEST WITH CALVES SERA IN EMBRYONATED EGGS

Test Animals	A* B**	Virus Dilutions 10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	Serum virus mixture titer	Neutralization index
Calf (1)	A	5/5***	5/5	4/5	3/5	1/5	6.6****	0.4****
Calf (2)	A	5/5	5/5	4/5	3/5	2/5	6.5	0.1
	B	5/5	3/5	1/5	0/5	0/5	4.5	2.1
Virus Diarrhea Sera	A	4/5	4/5	4/5	3/5	2/5	6.5	0.1
	B	5/5	5/5	3/5	3/5	2/5	6.4	0.2
Virus Control		5/5	4/5	4/5	4/5	2/5	6.6	

*A: Pre-inoculation serum (serum control).

**B: Post-inoculation serum.

***Numerator: Number affected
Denominator: Number injected

****Approximation to one decimal.

PART V

DISCUSSION

A group of agents producing various cytopathogenic effects in tissue culture have been discovered in cattle. These agents compare with the enteric viruses that have been isolated from human sources. Regardless of the source, the role which some of these viruses play in disease is, at present, unknown. In man, the poliovirus, Coxsackie groups A and B, and the ECHO viruses are examples of these cytopathogens. The first two groups are essentially responsible for either specific or shared syndromes. The ECHO viruses are considered to be without specific disease syndromes. However, in many instances, types 5, 6, and 9 have been found in close correlation with aseptic meningitis and, in some cases, as the only isolate observed from patients with this disease. Prior to recognition of the ECHO viruses, the poliovirus and the Coxsackie viruses were the only incitors of aseptic meningitis demonstrable.

In cattle, viruses of vesicular stomatitis, calf pneumonitis, bovine mucosal disease, and bovine rhinotracheitis are examples of enteric cytopathogens.

Hammon, Mack, and Reeves (1947) reported the discovery of neutralizing antibodies for the Lansing strain of poliomyelitis virus in bovine serum. That these antibodies were induced by the poliovirus or other related cytopathogens, was questioned by Bartell and Klein (1955). At any rate, search for the poliovirus in the alimentary tract of cattle

was due to the virus. Calf (2), three months of age, was given virus intravenously. No symptoms of disease were seen in this animal; however, the calf became an attractive issue. During the course of such a search, Kunin and Minuse (1957) discovered the virus used in this study. This agent, used as a prototype, was not neutralized by specific antisera for the three types of poliovirus, Coxsackie groups A and B, the 14 ECHO viruses, or by those for the known bovine cytopathogenic viruses (Kunin and Minuse, 1957).

It became evident that the virus isolated might be a member of a group analogous to the ECHO viruses in man. The fact that these cattle, from which the virus was isolated appeared to be symptom free, completes the analogy. Therefore, the term Enteric Cytopathogenic Bovine Orphan (ECBO) for this virus was suggested.

Considering the basic analogy between this virus and the ECHO viruses, and the aberrations of some strains of this group, it became of interest to determine the host range of this virus within the limits of practicality.

White Leghorn embryonate eggs, adult and suckling Swiss mice, white and cotton rats, guinea pigs, adult and suckling hamster, dogs, cats and calves were used in an attempt to evaluate the host range. Kunin and Minuse (1957) propagated the virus in the amnionic sac of 10 day old Barred Rock embryonated eggs, but the allantoic cavity failed to support growth of this virus.

In our work, after 10 serial passages from the original virus stock, the virus was adapted to the allantoic sac of 9 day old White Leghorn embryonate eggs. The adapted virus grew readily in the amnionic cavity of 12 day old embryonate White Leghorn eggs. Dwarfed embryos, malformation of the toes, cutaneous and subcutaneous hemorrhages, edema

of the embryo and of the chorio-allantoic membrane were common findings. In general, the pathological changes were similar to those reported in the Barred Rock embryo. Due to the differences between the host species, the melanin particles observed in the amnionic fluid of the Barred Rock egg did not develop in the amnionic fluid of the White Leghorn egg.

Adult Swiss mice, Syrian hamsters, cotton rats, guinea pigs and white rats were exposed to the virus by the following routes: intracerebral, intraperitoneal, subcutaneous and per os. None of these animals showed signs of systemic disturbance during the 21 day observation period. One day old chicks were inoculated intracerebrally with the virus and all of the birds remained normal throughout the observation period.

Suckling mice, following intracerebral or intraperitoneal inoculation with the virus, either developed paralysis or died. The paralysis affected either the front or hind limbs. A drop-wrist paralysis of the fore-limb, identical with that noted in suckling mice inoculated with Lansing strain of the poliovirus was seen in the experimental animals. Paralysis of hind limbs was of a spastic type. Limbs were extended posteriorly as seen following experimental inoculation with Coxsackie group A virus. A different picture was seen in suckling hamsters. Paralysis was generally complete. The four limbs were extended posteriorly and short rhythmic movements of the flanks indicated an abdominal type of respiration.

Cats, dogs, chickens and calves were also included in this study. These animals were observed for a one week period prior to use, for

clinical signs of infection and, in addition, pre-inoculation blood samples were taken to study the production of immune bodies in these groups. Kunin and Mimuse (1957) prepared specific antiserum for the virus by means of repeated intravenous inoculation in rabbits. In our study, a different procedure was followed. A single intracerebral dose of the living virus was inoculated into each cat, dog and chicken. Calf 1 was given a single dose *per os* and calf 2 also received only one intravenous inoculation. In every case, except calf 1 which died 4 days subsequent to inoculation, serum samples were collected before and after the virus administration.

All cats, dogs and chickens appeared to be in a normal condition during the 21 day observation period. Calf 1, which died four days after being given the virus orally, was either in a convalescent stage, or in general, debilitated. Adequate feeding did not improve the animals condition. Speculation regarding the improved feeding was based on the fact that the animal maintained a good appetite and normal temperature prior to exposure to the virus. Autopsy showed marked post-mortem changes but interest in the outcome of the experiment necessitated collecting and evaluation specimens.

It was doubtful whether or not the virus was responsible for the death of the calf. Attempts were made to recover the virus from the gastro-intestinal tissue and contents (*vide infra*).

The results of the embryonate egg inoculation (Table VII) show that only one embryo of 12 inoculated with the preparation from pre-exposure feces died (8.3%). When this result is compared with that of the post-exposure feces preparation which produced death in 6 of 12

(50%) inoculated embryos, the difference was marked. The post-feeding result infers that a factor was present which did not appear in the pre-feeding specimen. If the history of calf 1 is questioned, in the sense that it had had previous contact with the virus, a higher percentage of the embryos inoculated with preparations from the pre- and post-inoculation feces and tissue samples might have been expected to die. The post-feeding feces sample then tends to indicate that a lethal factor exists. Most probably, death of one of twelve embryos was due to trauma.

On the other hand, 6 of 12 (50%) embryos died as a result of inoculation with preparations from the post-feeding intestinal contents. Calf 1 died on the fourth day after being given 5 ml of the virus per os. Death of the inoculated embryos is, therefore, believed to be due to the amount of virus existing at that time in the intestine. Another outstanding result is the death of 2 of 12 (16.7%) embryos inoculated with the processed tissue samples. The probable answer that such a result may offer is that the virus did not actually multiply in the intestine, but that it was present, The tissue samples could not be completely freed from all the adherent virus particles originally contained in the loose feces, by rinsing the tissues several times with saline solution. Evidently, the virus content in the tissue samples was not sufficient to give a higher mortality rate in the inoculated embryos (Table VII).

It was of interest to learn whether or not the death of these embryos inoculated with preparations from the pre- and post-inoculation feces samples and tissue specimens was due to the activity of the virus

injected. The allantoic fluids were combined into the following pools: pre-feeding, post-feeding, and tissue sample pools. An equal number of 1 day old mice were inoculated intraperitoneally with 0.05 ml from the respective pool (Table VIII). Results of the egg inoculation procedure were supported by those results of the mouse inoculation (Table VIII). The allantoic fluid from eggs, originally injected with the supernate of the prepared post-feeding feces was inoculated into 7 suckling mice. Within 4-7 days, 5 mice collected for study were either paralyzed or dead. The paralysis was identical to that previously observed in mice inoculated with the ECBO virus. Pooled allantoic fluid, from the embryos which had died following injection with supernate of processed pooled tissue samples, apparently did not contain enough virus to initiate either paralysis or death in the inoculated mice. The minimal amount of virus that might have killed 2 of 12 embryos inoculated, did not affect any of the seven mice injected with the harvested allantoic fluid (Table VIII).

Calf 2, was 3 months of age, in good physical condition and was discarded at the end of the 21 day experimental period of observation.

None of the five embryonate eggs inoculated with preparations from the pre-inoculation stool sample died. Comparing this result with those of the post-inoculation sample (Table IX), it might appear that the calf was exposed to a factor, lethal for the egg embryo, with which the animal had had no previous contact. However, inoculating the calf with this virus did not produce any apparent symptoms other than the shedding of some mucus with the feces. This mucus was not seen during the observation period prior to injecting the calf with

the virus.

A different picture was apparent in the egg embryos inoculated with the post-inoculation fecal sampled (Table IX). Preparations from fecal samples collected on the fifth and sixth day of inoculation did not kill the embryos. Preparations from the stools specimens collected during the period between the seventh and the fifteenth day, which was the end of the observation period, caused in the inoculated embryos, a mortality rate ranging from 2-4 of 5 embryos (40-80%). The bacteriologically sterile allantoic fluid harvests from eggs injected with the 13 stool samples were combined into 5 pools. Each pool was inoculated intraperitoneally into a number of suckling mice. The egg inoculation results were supported by the results of mouse inoculation (Table X). Within 4-8 days most of the mice inoculated from each pool were either paralyzed or dead. Paralysis in every case was of the same type as previously described. In calf 2, it was assumed that the infection was limited to the multiplication of the virus in the intestinal tract. Excretion of the virus in the stools continued over a period of two weeks which was the end of the experimental period.

In general, responses to the ECBO virus elicited in the various host representatives, were absent or inapparent in adults. Definite response was noted in the egg embryos, suckling mice and suckling hamsters.

Random representatives of the following host species were selected for the serum neutralization study: cats, dogs, chickens and calves. The test was performed on pre- and post-inoculation serum samples. The post-inoculation samples were collected at the end of the observation period. The results indicate that none of the inoculated animals had

previous contact with the virus. These indexes, either individual or within the group, are practically insignificant. The post-inoculation serum samples of dog 2 and cat 2 (Table XI), chicken 2 and chicken 4 (Table XII), show a rise in their respective neutralization indexes. If these animals had responded to the large dose of the virus inoculated intracerebrally, an increased neutralization index might have been obtained. This increased index, however, was not observed.

On the other hand, the difference between the neutralization indexes of the post-inoculation serum samples of calf 2 for the ECBO virus, and that for the virus of "diarrhea disease" is significant. While the calf 2 specimen showed a definite rise in index compared with that of the pre-inoculation sample (2.1), the "virus diarrhea" serum sample showed little, if any, rise in this index (0.2).

The cross-neutralization test performed on the post-injection serum received from Dr. Gillespie, New York State Veterinary College, indicate that the ECBO virus under study and that of "virus diarrhea" appear to be antigenically unrelated. It appears, however, that the virus used in this study, is more like the human enteric viruses than some enteric cytopathogens isolated from cattle. The virus reported by Olafson et al., (1946), was not lethal to the chicken embryo. The virus of Moll and Finlayson (1957) did not paralyze the suckling mouse.

This thesis work, was concerned with an evaluation of a selected host range for an ECBO virus. In general, the results indicate that the virus is virulent for the embryonated egg, suckling mice and hamsters. Neutralization studies of sera from host species no response to this virus were practically insignificant. One exception was noted, the

intravenous inoculation of calf number 2 showed a rise in the post-inoculation titer as compared with the pre-inoculation index.

Further evaluation of this host-parasite relationship is indicated, and a great deal of study is yet to be done before the enteric viruses of animal origin can be characterized to the same extent as the ECHO viruses from human sources.

PART VI

SUMMARY

Kunin and Minuse (1957) isolated the ECBO virus, used in this study, from the intestinal contents of "healthy" dairy cattle. They propagated the virus in the amnionic sac of Barred Rock embryonated eggs, but not in the allantoic sac of Barred Rock or White Leghorn embryonated eggs. They also found that white suckling mice either became paralyzed or died following intraperitoneal inoculation with this virus. Tissue cultures from bovine and monkey kidney, chick embryo tissues, supported virus growth. HeLa cell culture was observed to be an exception to this method of progapagion.

In this work, after several passages, the virus was adapted to the allantoic cavity of White Leghorn embryonate eggs. The adapted virus was observed to multiply in the amnionic sac of I2 day old White Leghorn embryonate eggs.

In an attempt to determine the host range of this virus, a number of adult laboratory animals was screened. The virus, following intracerebral inoculation, was found to be non pathogenic for the adult white mouse, white and cotton rats, guinea pigs, cats, dogs, chickens and one day old chicks. In suckling mice and suckling hamsters, intracerebral or intraperitoneal inoculation resulted in either paralysis or death. Paralysis in suckling mice and suckling hamsters was similar, in type, to that induced by the Coxsackie group A virus and the Lansing type of poliovirus.

Calf I, less than week old, died four days after receiving 5 ml of the virus orally. There was no evidence that the animal's death

was due to the virus. Calf (2), three months of age, was given virus intravenously. No symptoms of disease were seen in this animal, however the calf was found to be excreting virus in the feces for at least 13 days subsequent to inoculation.

Serum neutralization tests were performed on the pre- and post-inoculation sera of the cats, dogs, chickens, calf (2) and on the pre-inoculation serum of calf (I). Cross serum neutralization tests were also carried out on the pre- and post-infection sera from a calf infected experimentally with "diarrhea disease" virus. That these animals had had previous access to the virus was not demonstrable, only the post-inoculation serum of calf (2), showed a significant rise in the neutralization index. The virus, which was the material of this study, and that of the "diarrhea disease" virus appear to be antigenically dissimilar.

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