ISOLATION AND CHARACTERIZATION OF A VIRUS FROM A BOVINE WITH LYMPHOMA

by

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INTRODUCTION

Until relatively recent times viruses have been classified primarily according to their location in the animal body. It has become increasingly apparent that classification by means of stable biophysical and biochemical properties is the most logical means of characterization. Such properties are mutationally stable and afford a more suitable way to place a virus taxonomically. For example, the Committee on Enteroviruses (1962), states that a virus should exhibit the following characteristics to be considered an enterovirus: Particle size of approximately 28 mu. in diameter, ribonucleic acid core, resistance to ether and cationic stabilization to thermal inactivation. Other workers have suggested additional criteria. The ability of enteroviruses to resist a low pH was proposed as a valuable means to provide a way of distinguishing a rhinovirus from an enterovirus. Heat stability is said to offer a method for separating certain subgroups, such as differentiating reovirus from enterovirus. (Hamparian et al., 1963) Immunologic characters have also been advocated as being of importance in characterization. (Cooper, 1961; Andrews et al., 1961) Cytopathic effect is yet another means utilized to aid in preliminary characterization. Although there are numerous reports of isolations of bovine enteroviruses in the literature, few have been characterized completely.

It was the purpose of the following investigation to isolate and characterize a bovine virus by the several accepted chemical and physical means. Serological studies were not carried out because they were beyond the scope of this work.

Samples were collected from a number of Holstein dairy cattle, part of a larger herd that had a high incidence of lymphoma, and which were purchased by and maintained at the College of Veterinary Medicine, Kansas State University. It was felt that isolates obtained from these animals might prove to be more interesting than those obtained from normal cattle.

From the isolates obtained, one was arbitrarily selected and subjected to the following tests: plaque purification, ether sensitivity, cationic stabilization, acid sensitivity, nucleic acid determination, hemagglutination characteristics, heat lability, nuclease and trypsin sensitivity, virus purification, electron microscopy and histology of the cytopathic effect. The results of the several tests carried out on a plaque purified virus strain should enable one to appreciate their value collectively in placing a virus in a modern scheme of classification.

REVIEW OF THE LITERATURE

The introduction of tissue-culture methods to virological research by Enders et al. (1949), whereby he demonstrated the susceptibility of non-nervous-tissue cells to polioviruses and the production of a cytopathic effect (CPE) by such viruses, led to rapid and epoch-making discoveries. Further studies with tissue culture systems have resulted in the discovery of great numbers of cytopathogenic agents in many tissues and from many species examined. Dalldorf and Sickles (1948), contributed an important finding by demonstrating that the suckling mouse was required

for isolation of the coxsackie viruses.

The isolation of agents from fecal material examined for evidence of poliovirus was an extremely rewarding source of isolates and it soon became evident that although many of these agents were isolated from cases of polio-like disease (aseptic meningitis), similar agents were also isolated from normal persons. The term "orphans" came to be commonly used for these agents when they could not be associated with disease. The numbers of such agents isolated increased rapidly and a conference entitled, "Viruses in Search of Disease" (New York Academy of Sciences, 1957), was arranged in order to decide which agents were responsible for recognized clinical disease.

Association of those organisms with which no disease was associated with the alimentrary tract of man and their ability to produce a cytopathic effect (CPE) in tissue culture led to the term, "ECHO virus" (enteric cytopathogenic human orphan virus). (Committee on the ECHO viruses, 1955) The ECHO viruses, recognized to have certain properties in common with polioviruses and Coxsackie viruses, were grouped with the polioviruses and Coxsackie viruses as enteroviruses. (Committee on the Enteroviruses, 1957)

The existence of these enteric cytopathogenic human orphan (ECHO) viruses, and their association with clinical disease in some cases, stimulated interest in a search for such agents in animals. Such agents in the animal population could conceivably pose a serious threat to both human and animal health.

Animal diseases, their existence and possible transmission to man, have been studied by numerous investigators for many years. Rabies, vaccinia-variola, arthropod-borne disease, to cite only a few.

Such agents have been found in the alimentary tract of animals, and it now appears that we have enteroviruses for a number of different animal species, such as enteric cytopathogenic monkey orphan virus (ECMO); enteric cytopathogenic bovine orphan virus (ECBO); and enteric cytopathogenic swine orphan virus (ECSO). Isolates from other species have been similarly named.

Classification

The International Enterovirus Study Group proposed in 1962, the term Picornavirus Group, (pico, small) RNA- containing viruses, in an attempt to classify major groups of viruses according to common biochemical and biophysical properties. (International Enterovirus Study Group, 1963) Table 1 indicates the classification.

The picornaviruses are small (15-30 mu. in diameter) viruses insensitive to ether, containing ribonucleic acid (RNA). Enteroviruses are isolated from the intestinal tract, but may be found in the nasopharynx. The Committee on the Enteroviruses (1962), states that viruses within this subgroup should exist as multiple antigenic types and possess the following characteristics: (a) A particle size of approximately 28 mu. in diameter, as determined by electron microscopy, gradocol membrane filtration, or other acceptable methods; (b) ribonucleic acid core as indicated by susceptibility of the infectious nucleic acid to ribonuclease and

Table 1. Classification of the Picornaviruses.*

PICORNAVIRUSES
A. Picornaviruses of human origin
1. Enteroviruses
a. Polioviruses
b. Coxsackie viruses, group A
c. Coxsackie viruses, group B
2. Rhinoviruses
3. Unclassified viruses
B. Picornaviruses of lower animals
1. Enteroviruses--subhuman primates, bovine, porcine, avian, and other animal species
2. Rhinoviruses
3. Unclassified viruses

*International Enterovirus Study Group, 1963

resistance to deoxyribonuclease, red staining with acridine orange, and failure of 5-fluoro 2-deoxyuridine to inhibit viral synthesis; (c) resistance to ether as evidenced by the failure of 20 percent ethyl ether to reduce infectivity after contact for 18 hours at 4 C.; and (d) cationic stabilization to thermal inactivation. (no loss of infectivity at 50 C. for one hour in the presence of molar MgCl2)

The ability of many of the enteroviruses to resist low pH (pH 2.5-3.0) appears to be a stable basic property of animal viruses which provides a simple, certain means to distinguish rhinoviruses from enteroviruses. (Hamparian et al., 1963) The enteroviruses are all resistant to low pH which is reasonable since this property may be essential

for any virus which reproduces in the gut and needs to survive passage through the acid conditions of the stomach. As can be seen from the preceding means of classification, the ways of classifying viruses are based on mutationally stable criteria. Earlier systems of classification were based mainly on habitat of the virus in the intact animal. This means of classification at fault since obviously different viruses (Foot-and-mouth disease and cowpox viruses) can produce similar lesions. Also, since adenoviruses have been found in stools they might have been called enteroviruses. Grouping by hosts would also be unsuitable since viruses can often be adapted to unusual hosts without much problem.

Cooper (1961) and Andrews et al. (1961), emphasized the importance of stable characteristics in classification. Cooper used nucleic acid type as the first criterion, ether sensitivity as the second criterion, with size and immunologic characters as tertiary characters.

Hamparian et al. (1963) studied several kinds of viruses to determine how consistently the various tests for classification actually proved useful. These workers presented a useful classification scheme for certain viruses based on Cooper's system, but using acid lability as the third criterion after nucleic acid type. Hamparian et al. (1963) mentioned that measurement of heat stability or lability may prove valuable as a criterion for separating certain subgroups such as for differentiating reovirus from enterovirus. The reoviruses are stable on heating at 50 C. for one-half hour. The enteroviruses are relatively labile to heating at 50 C. for one-half hour. (Ketler et al., 1962) The property of cationic stabilization of enteroviruses has proved to

be a valuable aid in characterizing viruses. (Wallis and Melnick, 1962) Enteroviruses and reoviruses are stabilized by magnesium ions when heated at 50 C. for one hour. Adenoviruses, papovaviruses, herpes viruses, myxoviruses, arboviruses and pox viruses are not.

Figure 1 is the schematic position of several animal virus families or groups as proposed by Hamparian et al. (1963).

Structure

Estimates of particle size for members of the picornavirus group vary from 15 mu. to 30 mu. There has probably been a variation in size reported due to different techniques being used and the same technique being used by different workers. Detailed studies on all the picornaviruses have not been made by one worker, thus it is not possible to decide if there really is a variation in size, and if so, if this variation can be correlated with other properties of the viruses such as host and pH sensitivity. Crick and Watson (1956), postulated that the viral nucleic acid of all small plant and animal viruses was surrounded by protein subunits. X-ray diffraction studies by Finch and Klug (1959), of crystals of poliomyelitis virus showed possession by the virus particles of icosahedral symmetry. The crystals were demonstrated to be built up out of 60 structurally equivalent asymmetric units or a multiple of 60, with a diameter of the spherical shell of about 300 A., close to that of the virus particle. Icosahedral structure means that the virus possessed the form of a polyhedron which had 20 faces, each taking the shape of an equilateral triangle. If this structure (the icosahedron)

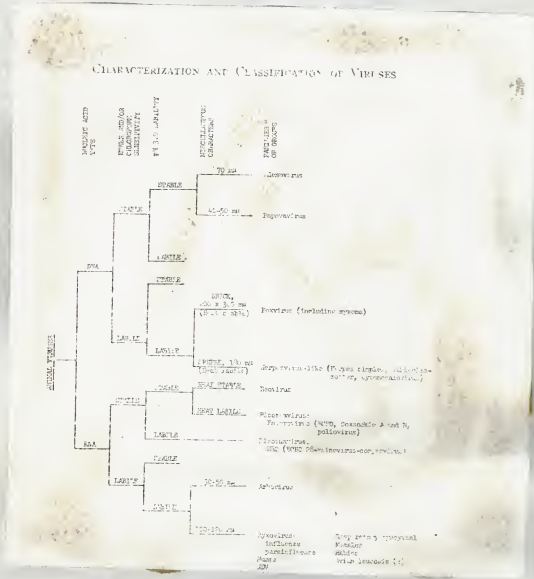

Fig. 1. Schematic position of several animal virus families or groups. (Hamparian et al., 1963)

were viewed from any corner, it could be rotated in five positions about that corner without changing its appearance. If the structure were viewed looking at the center of any face, the structure could be rotated in three positions without changing its appearance. If the structure were viewed from the center of the edge of any triangle it could be rotated in two positions without a change in appearance. The regular icosahedron is thus said to exhibit 5:3:2 symmetry. Horne and Nagington (1959) demonstrated by negatively stained preparations and electron micrographs that the subunit arrangement of the protein shell appeared to be consistent with the icosahedral symmetry (5:3:2) predicted by x-ray diffraction investigations. All three antigenic types of poliovirus appear to be 300 A. in diameter with protein subunits approximately 60 A. in diameter. The exact structural arrangement of the protein subunits within the icosahedron was not established.

Cultivation of Picornaviruses

A great deal of the work with the human viruses has been carried out utilizing monkey kidney cells and continuous lines of human cells. Picornaviruses of other animal species will, for the most part, proliferate in cells derived from kidney cell cultures derived from the kidney of the animal species from which the isolate was made. Animals, in some instances are essential for the isolation and cultivation of Coxsackie viruses.

Cytopathic Effect

That picornavirus is recognized to be proliferating in a tissue culture depends on its cytopathic effect. The CPE produced, although not specific for picornavirus, is very characteristic. Usually a CPE manifests itself within 24 to 72 hours after seeding with virus contaminated material, although some types require six to seven days. Infected cell cultures initially develop a granular appearance followed by a rounding and increased transparency of the cells. Disintegration and degeneration of the cells occur rapidly and they fall away from the glass and float free.

Picornaviruses of Man

The name ECHO virus (Enteric Cytopathogenic Human Orphan) was adopted in 1955. (Committee on the ECHO viruses, 1955) This name was given to the group at a time when little was known of its pathogenic potential. It was proposed that a member of the orphan group would be removed from this classification as soon as it could be associated with a disease. In the last several years it has become clear however, that few specific diseases are involved. Few of the agents are associated with specific syndromes, but members of the group may cause a variety of different illnesses. For this reason the syndrome is designated with the virus type, e.g. aseptic meningitis due to echovirus type 6, or to whatever other virus type happens to be involved. A large number of non-polio enteroviruses have been found in the intestinal tract of man. Twenty

three group A, six group B Coxsackie viruses and 30 echoviruses have been identified.

The Committee on Enteroviruses (1957) suggested the following criteria for establishing an etiologic association between an ECHO virus and a disease. The virus must have a much higher prevalence among patients with a disease than in healthy individuals of the same age and socioeconomic status living in the same area at the same time as the patients. Antibodies against the virus must develop during the course of the illness. Virologic or serologic evidence must be negative for concurrent infection with other agents that already are known to cause the same clinical syndrome. Etiologic probability is increased if the virus is isolated in significant concentration from body fluids or tissues manifesting the lesion, as from the cerebrospinal fluid in cases of aseptic meningitis, or the heart muscle in cases of myocarditis. Table 2, taken from Kibrick (1964), points out the number of syndromes which have been attributed to Coxsackie and ECHO viruses. The virus etiology in each case was linked to the syndrome by the above stated criteria. Many of the syndromes occurred in outbreaks involving several individuals rather than just sporadic cases.

A comparison of the clinical conditions with which enteroviruses of animals and man can be associated is not easy, for little is known about the animal viruses.

Many of the syndromes listed in Table 2 exhibited themselves as relatively mild transient conditions and many with only subjective symptoms leading to their discovery. Only when a relatively severe illness is

Table 2. Clinical associations of ECHO and Coxsackie viruses.*

Syndrome or Clinical feature	Coxsackie and ECHO virus types	
	isolated in feces	isolated parenterally
Aseptic Meningitis	Coxsackie A-1-11, 16-18 22 and 24 ECHO 4, 6, 9, 11, 16, 30 1-3 5, 7, 12-15 17-23, 25	Coxsackie A-1, 2, 4-7 9, 10, 14, 16, 22, 24 ECHO 2, 4, 5, 6, 7, 9 11, 14-20, 23, 30, 31
Paralytic Disease	Coxsackie A4, 7, 9, B1 ECHO 1, 2, 4, 6, 7, 9, 11, 16, 18, 30	Coxsackie A-4, B-2, 3 ECHO 2, 6
Encephalitis, Ataxias	Coxsackie A-2, 5, 6, 9 B-1, 2, 3, 5 ECHO 3, 4, 6, 7, 9, 11, 14, 18, 19	ECHO 2, 9
Guillain-Barre' Syndrome	Coxsackie A-2, 5, 6	Coxsackie A-2, 6
Exanthematous Disease	Coxsackie A-9, 2, 4, 5,16 ECHO 1-7, 9, 11, 14, 18, 19	Coxsackie A-5
Epidemic Myalgia (Epidemic Pleurodynia, Bornholm Disease)	Coxsackie B-1-5 ECHO 6, 8, 9	
Pericarditis and Myocarditis	Coxsackie A-2, 9, 13, 16 ECHO 8, 8, 5	Coxsackie B-3, 4, 2, 16
Orchitis		Coxsackie B-5
Severe generalized disease in newborns	Coxsackie B-1, B-t, A-4 ECHO 9	Coxsackie B-1, B-5, A-4 ECHO 9
Diarrheal disease and gastroenteritis	ECHO 7, 14, 19 Coxsackie strains also implicated	
Respiratory-enteric disease	ECHO 11, 20. 1, 8, 19	

Table 2 (Continued)

Syndrome or Clinical feature	Coxsackie and ECHO virus types	
	isolated in feces	isolated parenterally
Lymphadenitis and Splenomegaly	Coxsackie A-5, 6 ECHO 9, 4	Coxsackie A-5
Hepatitis	Coxsackie B-5 A-4, 9 ECHO 4, 9	Coxsackie A-4, 9
Respiratory Syndromes	ECHO 19, 28, 20, 6, 1, 3, 11 Coxsackie B-5 (Isolated from throat and feces)	
Ocular disease	ECHO 4, 6, 9, 16, 20 Coxsackie A-9, 16 B-5	

*Kibrick, 1964

produced in a wild or domestic animal does it come to notice. Mild gut, febrile, or even central nervous system infections might escape attention.

It is highly probable that many conditions such as those previously listed have not been noticed or have led to a diagnosis with no etiology.

Since the Coxsackie viruses in man have been implicated as the etiologic agents in so many of the syndromes previously listed, it would seem that an attempt to at least isolate some of them in lower animals would be worth while. The complete lack of information about these viruses in lower animals is readily apparent.

Picornaviruses of Lower Animals

The presence of viruses in the intestinal tract is obviously not limited to man alone. Reviews which discuss non-human animal enteroviruses through 1964 are available. (Hsiung and Melnick, 1958; Kalter, 1960; Kalter, 1964) The numbers of viruses isolated and whether or not they were associated with clinical disease is illustrated in Table 3.

It is interesting to note that foot and mouth disease virus (FMD), one of the earliest of recognized viruses, is now regarded as having the characteristics of an enterovirus. The literature review by Shahan (1962) describes the various properties of FMD in detail.

Koprowski (1958) reported on the occurrence of poliovirus, type I, in calf feces and the subsequent development of neutralizing antibodies. This is the only report of this nature and Koprowski did not altogether rule out laboratory contamination.

Table 3. "Enteroviruses" isolated from animals other than man.**

Investigator	Tissue Culture System*	No Isolates	No Prototypes	Clinical Disease
Simian enteroviruses				
Hull et al. (1956,1957,1958)	Monkey	?	5	No
Malherbe and Harwin (1957)	Monkey	7	?	No
Cheever (1957, Hofferet et al. (1958)	Monkey	187	13	Yes, no
Hsiung and Melnick (1958a, 1958b)	Monkey	51	3+	?
Fuentes-Marins et al. (1963)	Baboon	5	2	No
Hillis (1965)	Chimpanzee, monkey	26	?	No
Bovine enteroviruses				
Kunin and Minuse (1958)	Bovine, monkey	8	1	No
Klein and Earley (1957)	Bovine	70+	6+	No
Moll and Finlayson (1957) Moll and Davis (1959), Moll and Ulrich (1963)	Bovine, human amnion, HeLa, Pig, monkey	16	2	Yes
Moscovici et al. (1958,1961)	Bovine, monkey	11	2	No
Luginbuhl and Black (1961)	Calf, monkey	26	3	No
McFerran (1962)	Bovine, monkey	121	6	No
Inaba et al. (1962a, 1962b)	Bovine (embryonic)	6	1	No
Loeffler and Frosch (1897)	X#	?	7	Yes
Porcine enteroviruses				
Treffny (1930)	X#	X#	1	Yes
Moscovici et al. (1956)	Pig, monkey	1	1	?
Beran et al. (1958)	Pig	376	?	No
Webster (1959)	Pig	5	1	No
Bohl et al. (1960) Hancock et al. (1959), Singh et al. (1959, 1960)	Pig	30+	4	No
Betts et al. (1961)	Pig	?	9	Yes, no
Morimoto et al. (1961)	?	?	2	?
Mitchell et al. (1961) Greig et al. (1961)	?	?	?	Yes
Izawa et al. (1962a, 1962b)	Pig	4	2	Yes, no
Szent-Ivanyi (1963a, 1963b)	Pig	152	14	No
Sibalin and Lannek (1960)	Pig	?	1	Yes

Table 3 (Continued)

Investigator	Tissue Culture System*	No Isolates	No Prototypes	Clinical Disease
Miscellaneous enteroviruses##				
Burke et al. (1959a, 1959b)--Avian	Chicken, chick embryos	45	?	Yes, no
Clemmer (1965)--Avian	?	33	?	No
Fastier (1957)--Feline	Kitten	1	1	No
Theiler (1937)--Rodent	X#	?	1	Yes
Kalter (1965)--Rodent	Rabbit	6	?	No

*Kidney cells unless otherwise indicated.

#Isolated in animals rather than tissue culture.

##Limited studies with dog and hamster feces have failed to demonstrate the presence of enteroviruses

**Kalter, Basic Medical Virology, 1966

Kunin and Minuse (1957) and Klein and Earley (1957) proposed the term ECBO (enteric cytopathogenic bovine orphans) in keeping with the ECHO terminology suggested for human enteric cytopathogenic orphans. (Committee on the ECHO viruses, 1955)

Other than the viruses of foot and mouth disease and Teschen disease, it may be stated that the animal enteroviruses have not definitely been associated with disease. The role of human enteroviruses in producing disease is, however, well recognized. The multitude of clinical syndromes that these viruses are associated with has already been discussed. Possibly the failure to associate a recognized disease with the different isolates might explain this contradiction. Thus, merely lack of information may be the explanation.

There appears to be universal agreement as to what constitutes an enterovirus. An agent, to be considered as an enterovirus must fulfill the criteria proposed by the Committee on the Enteroviruses (1962).

Agreement is not so complete with regard to nomenclautre. The International Enterovirus Study Group (1963), has indicated that the enteroviruses of animals will be included with the picornaviruses. No recommendation was made for the naming of different isolates from the various animals. Cattle isolates have in some instancea been referred to as ECBO (enteric cytopathogenic bovine orphans) viruses, or given a laboratory code number.

Reference serums and prototype viruses would do much to eliminate tha present confusion.

In conclusion it appears that the enterovirus problem in animals may parallel that reported for man. Many of the animal "orphan" viruses may, in the future, be linked with diseases if findings concerning the animal orphan viruses continue to parallel those of human "orphan" viruses.

MATERIALS AND METHODS

Samples were collected from 7 Holstein dairy cattle which were purchased by the College of Veterinary Medicine, Kansas State University, and were part of a larger herd which had a high incidence of lymphoma.

Collection of Specimens

Nasal and rectal swabs were collected from each animal. The swabs were immersed in 5 ml. of veal broth and the collected material was expressed from the swabs. A penicillin and streptomycin solution (1,000 units of penicillin and 1,000 ug. of streptomycin/ml.) was added to the veal broth suspension in equal volume. The final concentration of antibiotics in the sample was thus 500 units of penicillin and 500 ug. of streptomycin/ml. Five ml. aliquoits of each sample were then centrifuged at 4,500 RPM (300-350 g) one and one-half hours in an International refrigerated centrifuge.

Cell Culture

Primary culture calf kidney cells were used for isolations. Kidneys from bovine fetuses were obtained from a slaughterhouse and kept at 4 C. until ready for use. The trypsinization procedure employed was that described by Youngner (1954) with certain variations. The kidneys were placed in sterile pans and as much fat was removed as possible. The capsule was removed and the cortex cut from the lobes. The pieces were minced with scissors and washed with phosphate buffered saline (PBS) until the supernatant was clear. The tissue was transferred to a 500 ml. Erlenmeyer flask and approximately 15 volumes of 0.25 per cent trypsin solution in PBS added. The flask was placed in a constant temperature water bath at 37 C. for one hour with occasional swirling of the flask. At the end of one hour the trypsin solution was discarded and fresh trypsin was added. The contents were then trypsinized overnight at 4 C. with constant stirring by means of a magnetic stirrer. At the end of this time the cell suspension was filtered through four layers of cheesecloth and centrifuged at 1,000 rpm for 10 minutes. The supernatant was then poured off and a 0.5 per cent suspension of the cells was made in the L-E medium of Melnick and Riordan. (Melnick and Riordan, 1952) Penicillin (500 units/ml. of medium), streptomycin (500 ug./ml.) and Amphotericin B (50 ug./ml.) were added to the medium.

When the cells grew to confluency the L-E growth medium was replaced with a medium in which the serum was replaced with 0.1 per cent Difco yeast extract and 0.1 per cent bovine albumin, a medium referred to as H-M 1X. (McClain and Hackett, 1959)

After virus isolations were made, Madin Darby Bovine kidney cells (MDBK)*, a continuous cell line was used for all further titrations and growth of virus.

Virus Isolation and Identification Procedures

Two-tenths ml. of the supernatant of each specimen was inoculated into each of 3 tubes of cell culture. The inoculated tubes were incubated at 35 C. and examined daily for seven days. Ten to twenty per cent of the uninoculated tubes of tissue culture were maintained as controls. Viral isolates were determined by the appearance of a cytopathic effect. Virus isolates were maintained for at least one passage in cell culture.

Virus Characterization

Plaque Purification. Virus isolates were purified by the plaque purification method (Dulbecco and Vogt, 1954) using an overlay medium containing 25 mM $MgCl_2$. (Wallis et al., 1962)

MDBK monolayers were grown in 50 mm. Petri dishes by the addition of approximately 1.5 X 10^6 cells in 5 ml. of L-E to each Petri dish. When the cells had grown to confluency the L-E medium was poured off and the monolayers were washed twice with phosphate buffered saline, pH 7.2 (PBS). Serial logarithmic dilutions were made in H-M medium from 10^{-1} thru 10^{-8} from tissue culture fluids and cells which had undergone a cytopathic effect. The virus suspension and cells were frozen and thawed rapidly

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*American type Culture Collection Cell Repository

three times before the dilutions were made. Two-tenths ml. of each dilution was delivered to the center of each of three Petri dishes. A total of 30 Petri dishes were used for each step, six serving as controls. The controls were inoculated with 0.2 ml. of H-M medium without virus.

The inoculated monolayers were incubated one and one-half hours at 37 C. to allow adsorption of virus. At the end of this period the monolayers were washed twice with PBS and the agar overlay at a temperature of 43 C. was added. The Petri dishes were then inverted and incubated in a CO_2 incubator at 37 C. for 36 hours in the dark.

Plaques were visible at the end of this period and picked according to the following procedure. Plaques were selected from cultures having less than 10 plaques and they were at least 10 mm. from adjacent plaques. Several stabs were made into each plaque and transfers made to 3 ml. of H-M medium. The resultant H-M, virus suspension was divided among three tubes containing MDBK monolayers. When a CPE occurred in these tubes the fluids were frozen and thawed three times rapidly and again plaqued. The virus was plaqued 3 times to assure purification.

Ether Sensitivity. The effect of ether was tested by the method of Andrews and Horstmann (1949). Tissue culture suspensions of virus with 20 per cent by volume pure anesthetic ether were placed in screw capped tubes and were taped tightly with adhesive. The tubes were shaken and placed at 4 C. for 24 hours. Controls consisted of virus suspensions in the same concentration as the test materials. At the end of 24 hours the mixtures were placed in a Petri dish from which the ether was allowed

to evaporate. The suspension was then titrated in tissue culture using 6 tubes per dilution and the virus titer calculated according to the method of Reed and Muench (1938). This technique of titration was used throughout except as otherwise indicated.

Cationic Stabilization. Stabilization by cations was determined utilizing the procedure of Wallis and Melnick (1962). As recommended by the Committee on the Enteroviruses, $MgCl_2$ was used and the virus was tested at 50 C. for 1 hour. (Committee on the Enteroviruses, 1962)

Two ml. of H-M medium, 0.5 ml. virus suspension and 2.5 ml. 2 M $MgCl_2$ were added to one tube. To another tube was added 4.5 ml. H-M and 0.5 ml. virus suspension. This resulted in a final dilution of 1:10 for the virus suspension in both tubes and 1 M concentration in the $MgCl_2$ test tube.

The tubes were stoppered and placed in a water bath at 50 C. for one hour. At the end of one hour the tubes were dipped in ice water a few seconds, appropriate dilutions made and the virus titer determined by the method of Reed and Muench (1938).

Acid Sensitivity. Virus fluids were diluted 1:10 in H-M medium and the pH was adjusted to 2.8 with 1 N HCl. The control virus was diluted 1:10 in H-M medium and the pH was adjusted to 7.2. Determinations of pH were made by means of a Coleman pH meter. (Hamparian et al., 1963)

The two samples were transferred to two tubes, stoppered tightly and allowed to stand at room temperature for three hours. At the end of this time the fluids were titered by the method of Reed and Muench (1938).

The pH of the fluids was again tested at the end of the three hour period.

Test for Nucleic Acid Type. The nucleic acid (RNA or DNA) was determined by the use of 5-fluoro 2-deoxyuridine (FUDR). (Salzman, 1960) This technique was shown in a later paper to be a reliable method equally as good as other more complex procedures. (Hamparian et al., 1963)

MDBK tissue cultures were prepared in tubes. When the cells in the tubes had grown to confluency the L-E medium was poured off and the cell sheets were rinsed with PBS six times. One ml. of Eagles MEM medium containing 10^{-4} M FUDR was added to one set of tubes, one ml. of Eagles MEM medium containing 10^{-3} M thymidine and 10^{-4} FUDR was added to another set of tubes and one ml. plain Eagles MEM medium was added to a third set of tubes. The cultures were inoculated immediately with tenfold serial dilutions of virus, 0.2 ml. per tube. Six hours postinoculation the culture medium was discarded, the cell sheets were washed six times with PBS and fresh medium, the same type as was previously in the tubes, was added.

Infectious Bovine Rhinotracheitis Virus (IBR) was titrated in a manner exactly as described above.

Hemagglutination Characteristics. Red blood cells from cattle, horse, mouse, sheep, chicken and guinea pig were collected for use in hemagglutination tests (HA) as described by Salk (1944).

Serial twofold dilutions, ranging from 1:2 to L:1024 were made in PBS using tissue culture virus suspensions. To 0.5 ml. of each dilution was added 0.5 ml. of a 0.25 per cent suspension of red blood

cells. The mixture was shaken and set aside until the cells settled. The test was performed at 4 C. and room temperature. The control wells contained 0.5 ml. of PBS and 0.5 ml. of a 0.25 per cent suspension of red blood cells.

Heat Lability. One ml. of virus containing fluids was delivered directly to the bottom of each of seven screw capped tubes. Six of the tubes were placed in a constant temperature water bath at 56 C. and one was titered immediately. At intervals of 30 minutes and 1, 2, 4, 6 and 8 hours a sample was taken and virus titrations were carried out. (Rovozzo et al., 1965a)

Nuclease Sensitivity. One ml. of virus fluids were added to each of 4 screw capped tubes. Deoxyribonuclease (DNAase) was added to one tube. Ribonuclease (RNAase) was added to one tube. One ml. virus fluids served as a control. The enzymes were added such that the final concentration in each tube was 10 gamma/ml. (Consigli et al., 1966)

The tubes were allowed to stand one hour at room temperature with occasional shaking. At the end of this time virus titrations were carried out and $TCID_{50}$'s determined.

Trypsin Sensitivity. Purified crystalline 5-A trypsin was added to virus fluids so that the final concentration of trypsin was 0.1 mg./ml. This mixture was allowed to stand at room temperature for one hour with occasional shaking. At the end of this time soybean trypsin inhibitor was added to a concentration of 0.1 mg./ml. A control tube of virus fluid was allowed to stand at room temperature for one hour. Appropriate

dilutions were made from the two tubes, virus titrations were carried out and $TCID_{50}$'s determined. (Consigli et al., 1966)

Virus Purification. Twenty 32-ounce Roux tissue culture bottles with heavy monolayers were infected with virus, the bottles were scraped and the fluids collected at the height of the CPE. The resultant fluids were frozen and thawed rapidly 3 times, followed by centrifugation at 8,000 X g in a Sorvall (SS-3 superspeed) centrifuge for 15 minutes to remove the cellular debris. The infected tissue culture fluid was concentrated by centrifugation in a Spinco ultracentrifuge (Model L, SW 25 rotor) at 80,000 X g for 2 1/2 hours. The supernatants were poured off and the viral pellets were resuspended in 0.01 M Tris buffered saline and the suspension was recentrifuged at 1,000 rpm for five minutes. The supernatant was collected. The virus was further purified by using a pre-set CsCl density gradient (1.20 to 1.40 g/ml.) and centrifuging (Spinco, SW 39 rotor) at 156,000 X g for 18 hr. (Consigli et al., 1966) The partially purified virus was layered on top of the preset gradient before centrifugation. A Buchler piercing unit was utilized to collect fractions of 20 drops each from the bottom of the tubes after centrifugation. From each of the 20 drop fractions .02 ml. was delivered into tubes containing 1.98 ml. of H-M medium. The fractions containing the virus were determined by making appropriate dilutions of the 1:100 H-M medium dilutions. Plaquing was carried out to more accurately assay the virus content of those fractions which showed the highest titer in the tube test. The buoyant density of that fraction which contained the

highest virus titer was determined by means of a Bausch and Lomb refractometer* by the method of Weigle et al. (1959).

The fractions containing the highest titer were pooled and dialyzed against PBS overnight to remove the CsCl. The dialyzed sample was pervaporated to one-half its original volume and submitted for electron microscopy.

Electron Microscopy. The sample was dialyzed 18 hours in 0.5 per cent tryptone in deionized water and again pervaporated to one-half the original volume. A small drop was further concentrated by agar filtration. (Kellenberger and Arber, 1957) The concentrated sample on the collodion was then floated in 1 per cent uranyl acetate and the specimen was sandwiched between the collodion and carbon membranes on a 200 mesh netted grid. (To, 1967) The specimen was observed by means of a Hitachi HU 11 B-1 electron microscope at 75 K. V. and utilizing an objective aperture of 25 u.

Histology. MDBK cells at a concentration of approximately 3.5×10^5 cells/ml. of L-E media were put in 60 mm. Petri dishes containing coverslips. In 24 hours the L-E medium was poured off and the coverslips washed with PBS. The coverslips were each inoculated with approximately 1.5×10^6 PFU of virus suspension and incubated for one and one-half hours in a CO_2 incubator. At the end of this period 5 ml. of H-M medium was added to each Petri dish. Coverslips were harvested at varying intervals, fixed in Bouins fluid and stained with hematoxylin and eosin.

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*Bausch and Lomb Model ABBE-3L Refractometer

RESULTS

Virus Isolation

A cytopathic effect (CPE) was observed in the culture tubes from 3 of the 7 cattle sampled. A CPE was observed only in those tubes inoculated with the fecal sample material. The nasal specimens failed to produce a CPE. The CPE was observed in 24 hours after inoculation of the culture tubes in all three cases.

The CPE was characteristic of an enterovirus. The cells developed a granular appearance followed by rounding, shrinking and increased opacity of the cells. Degeneration of the cells followed rapidly and they floated away from the glass.

Virus Characterization

Plaque Purification. One of the tubes showing a CPE was selected to be used for further characterization procedures. The virus was plaqued 3 times to assure that only one virus strain was present. The plaques were evident in 36 hours and were observed to be small circular clear areas with clear centers and regular borders, 1---2 mm. in diameter.

Ether Sensitivity. The virus was shown to be insensitive to ether. The $TCID_{50}$ of the ether treated and the untreated virus was calculated to be $1 \times 10^{6.5}$. (Table 4)

Cationic Stabilization. The virus was stabilized by divalent cations. The virus, stabilized by divalent magnesium ions exhibited a $TCID_{50}$ of $1 \times 10^{5.4}$. The virus suspension unstabilized exhibited a

$TCID_{50}$ of 1 X $10^{1.7}$. (Table 5)

Table 4. Effect of 20 per cent ether (final concentration) on a virus suspension held at 4 C. for 24 hours.

	24 hr.	Conclusion
Virus plus ether	6.5*	insensitive
Virus control	6.5*	--

*$TCID_{50}$ Infectivity titer, neg. Log_{10}

Table 5. Effect of a 1 M solution (final concentration) of $MgCl_2$ on a virus suspension held at 50 C. for one hour.

	50 C 1 hr.	Per cent loss in titer*	Conclusion
Virus plus $MgCl_2$	5.4**	--	stabilized
Virus plus H-M medium	1.7**	99.95	--

*Per cent loss in titer of virus suspension unstabilized by $MgCl_2$ as compared to stabilized virus.

**$TCID_{50}$ Infectivity titer, neg. Log_{10}

Acid Sensitivity. The virus was insensitive to pH 2.8 for 3 hours at room temperature. The virus $TCID^{50}$ was 1 X $10^{4.5}$ in the tube at pH 2.8 and in the tube at pH 7.2 at the end of the 3 hour period. The pH of the virus suspensions was demonstrated to be 2.8 and 7.2 in the respective tubes at the end of the 3 hour period. (Table 6)

Table 6. Effect of pH 2.8 on a virus suspension held at room temperature for three hours.

Virus suspension	3 hr.	Conclusion
pH 2.8	4.5*	stable
pH 7.2	4.5*	--

*$TCID_{50}$ Infectivity titer, neg. Log_{10}

Test for Nucleic Acid Type. The fact that the DNA virus (IBR) was inhibited by as much as 4 logs while the unknown virus was unaffected by FUDR shows indirectly that the unknown virus is of the RNA type with respect to its nucleic acid content. The reversal of the inhibition of the DNA virus in FUDR by the addition of thymidine to the medium offers further credibility to this technique. (Table 7)

Table 7. Results of test for nucleic acid type.

	Eagles Medium	Eagles & FUDR	Eagles FUDR & Thy*
IBR	5.7	1.7	5.0
Unknown virus	4.7	4.7	4.8

*Thymidine

Hemagglutination Characteristics. The virus did not hemagglutinate red blood cells of any of the species tested. (Table 8)

Table 8. Results of hemagglutination studies with a virus suspension carried out at 4 C. and room temperature utilizing red blood cells of various species.

Species	HA* titer (room temp.)	HA* titer (4 C.)
Cattle	Neg.	Neg.
Horse	Neg.	Neg.
Mouse	Neg.	Neg.
Sheep	Neg.	Neg.
Chicken	Neg.	Neg.
Guinea pig	Neg.	Neg.

*Results of serial twofold dilutions of a virus suspension from 1:2 to 1:1024 to which red blood cells (0.25 per cent) of the various species were added.

Heat Lability. The virus was completely inactivated by 56 C. for one-half hour. The control tube (unheated) had a $TCID_{50}$ of $1 \times 10^{5.5}$. The tubes titered at 30 min., 1 hour, 2 hours, 4 hours, 6 hours and 8 hours showed no evidence of a CPE. (Table 9)

Nuclease Sensitivity and Trypsin Sensitivity. The virus was trypsin and nuclease (DNAase and RNAase) insensitive as shown by comparisons with control tubes of the $TCID_{50}$ titer. (Table 10)

Table 9. Results of incubating virus fluids for varying periods of time at 56 C.

Prior to heating	30 min.	1 hr.	2 hr.	4 hr.	6 hr.	8 hr.
5.5*	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.

*$TCID_{50}$ Infectivity titer, neg. Log_{10}

Table 10. Results of incubation of virus fluids with RNAase, DNAase (10 gamma/ml. final concentration of each enzyme), and trypsin (0.1 mg./ml. final concentration) for 1 hour at room temperature.

	1 hr.	Conclusion
Virus (control)	4.5*	--
Virus plus DNAase	4.2*	stable
Virus plus RNAase	4.2*	stable
Virus plus trypsin	4.5*	stable

*$TCID_{50}$ Infectivity titer, neg. Log_{10}

Virus Purification. Figure 2 depicts the results obtained from the 22 fractions. The buoyant density from fraction number 7 (containing the greatest concentration of virus) was determined to be 1.358.

Electron Microscopy. The electron photomicrographs revealed spherical particles with a diameter of approximately 25 mu. (See Figure 3)

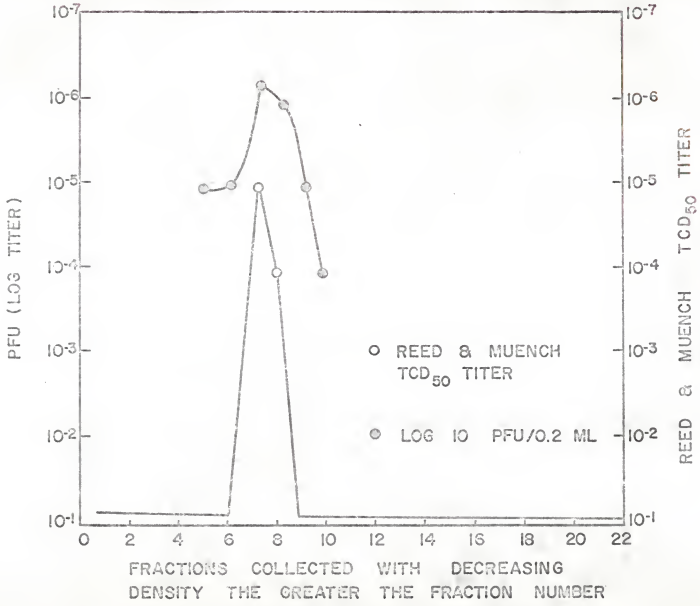
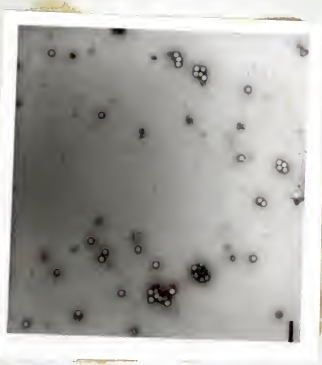
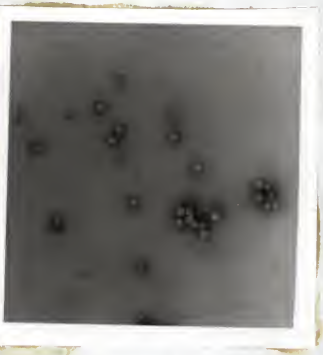


Fig. 2. Graph of results of CsCl density gradient depicting virus titers as demonstrated in the various fractions.



a. x 50,000



b. x 80,000

Fig. 3. Electron photomicrographs of material from fractions 7, 8, and 9 of the CsCl density gradient.

Histology. The following changes were noted in the cells.

Three hours postinoculation: Many cells were swollen and had assumed bizarre shapes. The cytoplasm was less acidophilic in the affected cells and the nuclei in many of them assumed a position at the edge of the cytoplasm. The cytoplasm of the affected cells contained variable numbers of clear vacuoles.

Four hours postinoculation: The cytoplasm of the swollen cells became more vacuolated and somewhat foamy in appearance. The chromatin material in the nuclei formed aggregates of deep staining basophilic clumps about the margins of the nuclei. The cytoplasm of some cells had disintegrated completely (cytoplasmolysis) as evidenced by the appearance of pyknotic nuclei surrounded by a small amount of condensed cytoplasm. At this stage it was difficult to differentiate the nuclei from the condensed cytoplasm in many of the cells, chromatolysis having taken place. Evidence of karyorrhexis was apparent in many nuclei also.

Five hours postinoculation: At this stage the majority of the cells had undergone cytoplasmolysis and the most striking observation was that of many nuclei which had undergone varying degrees of pyknosis and karyorrhexis. The degenerating nuclei were surrounded by a small amount of dense cytoplasm.

DISCUSSION OF RESULTS

The results of the various biophysical and biochemical tests indicate that the cytopathogenic agent subjected to these tests fulfills all the requirements proposed by the Committee on Enteroviruses (1962) as well as the additional property of acid insensitivity (Hamparian et al., 1963), necessary to classify it as a bovine enterovirus (ECBO agent) of the Picornavirus group.

Although no buoyant densities for bovine picornaviruses were found in the literature, the results of the buoyant density were comparable to those reported for human picornaviruses.

The results of the various tests performed as compared to those of known picornaviruses and other known viruses are illustrated in Tables 11, 12 and 13.

The treatment of partially purified virus concentrates with proteolytic enzymes and nucleases is frequently employed to hydrolyze non-viral protein and nucleic acid contaminants. Virtually all animal viruses are resistant to attack by nucleases if their protein capsids are intact. Most viruses except the large complex poxviruses are resistant to trypsin activity. (Schwerdt, 1965)

The resistance of the virus to nucleases and trypsin indicates that these enzymes could be used in the purification procedure.

Kunin and Minuse (1958) reported isolating eight agents in bovine kidney cultures from apparently healthy dairy cattle. The eight viruses were serologically related to one another, but unrelated to poliovirus

Table 11. Results as determined by the use of FUDR of nucleic acid type of a known picornavirus and a known adenovirus compared with the unknown virus.

	$TCID_{50}$ infectivity titer, neg. log_{10}		
Virus	FUDR plus thymidine	FUDR	Nucleic acid type
*Adenovirus 5	6.5	3.0	DNA
*ECHO 28	4.7	4.5	RNA
Unknown virus	4.8	4.7	RNA

*Hamparian, et al., 1963

Table 12. Results of determination of acid sensitivity of a known picornavirus, adenovirus, reovirus and rhinovirus compared with the results obtained with the unknown virus.

	$TCID_{50}$ infectivity titer, neg. log_{10}		
Virus	pH 7.2	pH 3.0	Conclusion
*Adenovirus 5	5.5	5.5	stable
*ECHO	7.0	7.0	stable
*Reovirus 1	6.5	6.5	stable
*Rhinovirus H-11	5.5	1.0	labile
Unknown virus	4.5	4.5	stable

*Hamparian, et al., 1963

Table 13. Results of several chemical and physical tests performed on known picornaviruses and known adenoviruses compared with the results obtained from tests performed on the unknown virus.

Test performed	Picornaviruses	Adenoviruses	Unknown virus
Cationic Stabilization	stabilized*	inactivated*	stabilized
Ether Sensitivity	insensitive**	insensitive**	insensitive
Size mu.	17-30**	65-85**	25
Buoyant density in CsCl Gm./ml.	1.33-1.38+	1.335-1.340++	1.358
Cytopathic effect	typical CPE	typical CPE	typical CPE
Heat Lability	labile	labile	labile

*Wallis and Melnick, 1962

**Green, 1965a

+Schaffer and Schwerdt, 1965

++Green, 1965b

I, II and III, or ECHO viruses 1-14. Ninety per cent of the herd had antibodies to these viruses. The eight agents did not hemagglutinate two-day-old chick or adult chicken cells. Studies with the ultracentrifuge indicated a sedimentation constant of the viruses in the range of those determined for known picornaviruses. There was no loss in titer when infected tissue culture fluid was shaken with ether for 26 hours at 4 C. The characteristic of size and resistance to ether was highly suggestive of enterovirus. These workers did not purify their virus strains by plaquing and considered all the isolates the same on

the basis of a similar CPE. One isolate was designated the LCR4 prototype strain. Klein and Early (1957), isolated 70 agents from the feces of apparently healthy cattle. These workers divided their isolates into six groups on the basis of CPE. Two strains were neutralized by human gamma globulin as well as by a large number of individual human sera. Two isolates from cattle feces were related, but not identical to human adenoviruses. These isolates were originally detected by a screening procedure with human gamma globulin and later shown to be adenoviruses by their characteristic CPE and possession of group specific complement fixing antigen. (Klein, et al., 1959; Klein, et al., 1960; Klein, 1962)

Moll and Finlayson (1957) and Moll and Davis (1959) reported on the isolation of enteric viruses from herds of cattle with a history of respiratory disease and abortion. Six isolates were characterized and separated into two serologic types. Plaque purification was not carried out prior to preparation of antisera. CPE was produced in cells derived from bovine kidney, human amnion, human kidney, HeLa cells, pig kidney and rhesus and patas monkey kidneys. The agents were ether resistant and heat stable. The agents passed ultrafilter membranes with pore sizes of 50 and 80 mu. A typical enterovirus CPE was observed. The prototype strains were neutralized by two different infectious bovine rhinotracheitis (IBR) virus convalescent serums and ECBO immune serum. Significant antibody responses were noted in cattle following recovery from illness indicating a connection of these isolates with the clinical disease. The respiratory disease did not, however, resemble IBR in that it was, "mild and inconspicuous." Intraperitoneal inoculation of this

virus into cortisone-treated, weaned mice did produce illness and extensive myocardial necrosis. Pneumonitis was produced in cortisone-treated calves following inoculation. Stillbirths and weakened offspring resulted when pregnant guinea pigs were inoculated with this virus.

Ten of the agents isolated were further characterized by Moll and Ulrich (1963). They were isolated from fecal samples from several cattle herds in Washington and Idaho and were cytopathogenic in primary bovine kidney cell cultures. All of these isolates passed filters with a pore diameter of 80-200 mu. Five of the agents passed filters with a pore diameter of 50-80 mu. and none passed filters with a pore diameter of 10-50 mu. The variation in size of these agents suggests the possibility that more than one class of virus was present. The use of additional means of characterization such as nucleic acid determination and stabilization by divalent cations would aid significantly in arriving at a correct classification. None of the viruses were completely inactivated within 30 minutes at 56 C. Three of the agents were not inactivated before 24 hours at 56 C. The viruses produced a typical enterovirus CPE. All produced macroscopic plaques within three days in cell culture and did not hemagglutinate guinea pig or sheep red blood cells.

Moscovici and Maisel (1958) reported agglutination of guinea pig red blood cells by three of 11 bovine enteroviruses. Five of the ECBO agents agglutinated bovine red blood cells. These reactions occurred in both cases only at 5-8 C. The reactions were not complete until one and one-half hours. Fairly rapid elution, complete within an additional one-half hour, was noted. It is apparent that the property of hemagglutination

is not possessed by all bovine enteroviruses.

An attempt was made by Moscovici et al. (1961) to characterize and classify the 11 viruses previously isolated with various recognized viruses. These workers plaqued each isolate three times before subjecting them to further studies. Plaque morphology indicated that the isolates could be subdivided into two distinct groups. One of the isolates was proposed as a member of the reovirus group by the results of finding cytoplasmic inclusions, ability to hemagglutinate human "O" red blood cells, no plaque production and by serologic tests. It would have been interesting to know if this isolate were heat stable or heat labile. Another one of the isolates produced intranuclear inclusion bodies and was not serologically related to any of the other isolates. It would have been interesting to know if this isolate could be stabilized by divalent cations. Among the other 9 isolates, 6 were studied by cross neutralization. They formed two distinct serologic groups. The first group included isolates 62/4, 64/4 and 76/2. All of these viruses hemagglutinated guinea pig and monkey red blood cells. Strains of this group were grouped with the BE1 and BE180 strains of Moll and Davis (1959) and the LCR4 virus of Kunin and Minuse (1958). The second group of viruses included strains 74/2, 79/2 and 87/1, because these strains did not appear to be related to any other known bovine enteroviruses.

Lunginbuhl and Black (1961) isolated 26 cytopathogenic agents from 111 fecal samples from normal cattle feces. Three distinct kinds of plaque morphology were observed. Type A plaques were 10 mm. in diameter and were hazy in appearance due to the presence of some living cells within them.

The plaques were visible after 10 days of incubation. The type B plaque was 5 mm. in diameter, a clear plaque which appeared in 10 days. The type C plaque was clear and 1 mm. in diameter in 7 days. It was demonstrated by gradocol membrane filtration that one agent had a diameter of less than 34 mu. and two of the agents had diameters of between 34 and 96 mu. It was shown by serological studies with the 3 strains that it was not possible to obtain completely type specific serum. Serum from monkeys and rabbits had prior titers to the viruses even after heating at 56 C. for one-half hour. These workers based their characterization on a typical CPE and plaque morphology. They did point out, however, that most young animals carried more than one type of agent and thus plaque purification was necessary before attempting neutralization procedures.

McFerran (1963) in summarizing his results with enteroviruses, suggested that bovine viruses be considered as enteroviruses only if they conform to the general criteria established for human enteroviruses. McFerran pointed out that the VG/5/27 strain isolated by him was closely related to the LCR4 strain of Kunin and Minuse (1958) which was similar to the BE1 and BE180 viruses of Moll and Davis (1959) and the 62/4 isolate of Moscovici and others (1961). McFerran (1963) further pointed out that his VG/5/27 virus was related to a virus received from Kipps in South Africa. McFerran isolated 121 agents from four herds of calves over a period of 9 months in Northern Ireland. The viruses fell into at least 6 serotypes. Difficulties were encountered in grouping the isolates because of considerable antigenic variation among the strains. McFerran

demonstrated also, that all cattle serums and the serum from calves at birth had neutralizing substance to his VG/5/27 strain. He pointed out the significance of this finding because of the way it could influence certain tests. The VG/5/27 virus caused a mild diarrhea in some experimentally infected calves and was recovered from a high percentage of normal cattle. McFerran stated, however, that it might be unwise to assume that this virus is not a pathogen, since if it were so capable of antigenic variation as well as variation in laboratory properties, it could possibly have variations in pathogenicity.

Yamada (1965), in Japan, reported on the isolation and characterization of 6 agents from fecal samples collected from apparently healthy cattle. Judging from their cytopathic effect, small size, stabilization by divalent cations and resistance to ether, these 6 viruses were considered as bovine enteroviruses.

Rovozzo and Lunginbuhl (1965a) described the characteristics of three strains of ECBO agents isolated from cattle feces. These agents were numbered 48, 93 and 97. These agents all passed millipore filters with a pore size of 50 mu. They were stabilized by divalent cations and were insensitive to ether. There was no loss of infectivity when they were incubated at room temperature for 1 hour at pH 3.2. They were completely inactivated when incubated at 56 C. for 30 minutes. They did not hemagglutinate the red blood cells of guinea pigs, calves, sheep, chicken, rat, mice or man. It would appear that these agents have met all the conditions required to be classified as bovine enteroviruses, except that of nucleic acid type. In a later paper Rovozzo and Lunginbuhl (1965b)

showed by cross-neutralization tests that these ECBO viruses were immunologically distinct. They further demonstrated that neutralizing substances were present in human gamma globulin and human serums to the 3 ECBO agents. The substances were heat stable and type specific. In a third paper, Rovozzo and Lunginbuhl (1965c) demonstrated that neutralizing activity for their 3 prototype strains was found in various mammalian serums. This activity was noted in 17 of 28 (60.7%) bovine serums, 6 out of 26 (23.4%) domestic-animal serums, and 11 of 131 (8.4%) wild animal serums.

Rovozzo and Lunginbuhl (1967) attempted to show a serological relationship between their bovine agents, 48, 93, and 97 and those of the human enteric group as well as some previously isolated enteric viruses. No relationship was found between their bovine agents, 48, 93 and 97, and those of the human enteric group as well as some previously isolated enteric viruses. No relationship was found between their 3 viruses and Coxsackie B-2 and B-5, ECHO viruses 1 to 25, or Klein's (1959), (1960), bovine adenoviruses, numbers 10 and 19, Kunin and Minuse's (1958), LCR4, and Moll and Finlayson's (1957), ECBO virus. Neutralizing activity for viruses 48, 93 and 97 was noted with rabbit prepared antiserums to certain salmonellae and slight neutralization was obtained between ECBO virus 48 and ECHO viruses 30 and 31 and between ECBO virus 97 and ECHO virus 27 in a one way cross.

From the above discussion of results obtained by various workers it is readily apparent that the stable physical and chemical characteristics

of the isolates have proved to be the most valuable in classification.

The presence of nonspecific neutralizing substances in animal serums and the incidence of cross neutralization reactions has led to considerable confusion in identification. It is evident that some of this confusion was the result of mixed viral populations.

In some instances agents have been classified as enteroviruses when they did not fulfill the criteria as set forth by the Committee on Enteroviruses (1962) or fulfilled only one or two of the criteria.

CONCLUSIONS

It has been demonstrated by various workers that at least three classes of viruses exist in the intestinal tract of the bovine animal. Reoviruses (Moscovici et al., 1961), adenoviruses (Klein et al., 1959; Klein, 1962) and picornaviruses (Rovozzo et al., 1965) have been isolated and identified. The chemical and physical tests described are sufficient to correctly classify a virus isolate. However, one or two of these tests are not enough for proper identification. Plaque purification, ether sensitivity, cationic stabilization, acid sensitivity, nucleic acid (RNA or DNA) determination, hemagglutination characteristics, heat stability and size determination are the technics which must be carried out to place a virus in the proper class.

The agent characterized in this investigation can properly be classified as a bovine enterovirus (ECBO agent) of the Picornavirus group.

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ISOLATION AND CHARACTERIZATION OF A VIRUS FROM A BOVINE WITH LYMPHOMA

by

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The purpose of the investigation was to isolate and characterize a bovine virus by the several chemical and physical means described in the literature. Physical and chemical properties of viruses are mutationally stable and are commonly accepted as the principal taxonomic criteria for a rational system of classification of viruses. The Committee on the Enteroviruses (1962), states that an enterovirus should exhibit the following characteristics: particle size of approximately 28 mu. in diameter, ribonucleic acid core, resistance to ether and cationic stabilization to thermal inactivation. Additional characteristics, useful in differentiating viruses have been proposed. The ability of enteroviruses to resist a low pH is said to be useful in distinguishing enteroviruses from rhinoviruses. Heat stability is said to offer a method for separating reoviruses from enteroviruses.

The agent characterized in this study was isolated by rectal swab from a Holstein dairy cow, one of a group of animals which had a high incidence of lymphoma. The presence of a virus in the sample (from which bacterial and fungal contaminants were removed by differential centrifugation and antibiotics) was determined by the appearance of a cytopathic effect (CPE) in primary culture calf kidney cells.

The following chemical and physical tests were carried out on the isolate after it was plaqued three times to assure the presence of a single virus strain: ether sensitivity, cationic stabilization to thermal inactivation, acid sensitivity, nucleic acid type (RNA or DNA), hemagglutination characteristics, heat lability, nuclease and trypsin sensitivity and electron microscopy.

The virus isolate was insensitive to ether, stabilized by divalent cations at 50 C., acid insensitive, nuclease and trypsin resistant, and heat labile. The nucleic acid content of the agent was ribonucleic acid (RNA). The virus did not hemagglutinate red blood cells of any of the species tested. The virus was 25 mu. in diameter. A typical enterovirus CPE was observed.

The buoyant density of the virus in CsCl was similar to that of human picornaviruses.

The results of characterization studies of enteric bovine viruses by other workers are discussed.

It is concluded that an agent must meet all of the criteria as set forth by the Committee on the Enteroviruses (1962), plus the additional property of acid insensitivity, to be classified as bovine enterovirus.

The agent characterized in this investigation, having met all the above criteria, could properly be classified as a bovine enterovirus (ECBO agent) of the picornavirus group.