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A Description of Eight Feline Picornaviruses and an Attempt to Classify Them. (32412)

ROBERT A. CRANDELL* (Introduced by S. S. Kalter)
USAF Epidemiological Laboratory, Aerospace Medical Division, Lackland Air Force Base, Texas

The isolation of 6 distinct serotype viruses from the oropharynx(1,2) and 2 from visceral organs(3,4) of the domestic cat has previously been reported. Although some of their biologic properties are known, the viruses remain unclassified. These viruses are currently grouped as to the type of cytopathology induced in cultures of feline renal cells. The characteristic feature of their cytopathic effect is the rapidity of destruction of the monolayer without the formation of inclusion bodies or giant cells. Since other workers in the United States(5,6) and Europe(7,8,9) have isolated similar agents from the domestic cat, it appears that this group of viruses may comprise a large but undetermined number of serotypes.

The present study of some of their biochemical and biophysical properties was undertaken in an attempt to characterize these viruses. The 8 serotypes were examined with respect to: host range, plaque production, sensitivity, acid stability, nucleic acid type, ability to be stabilized against thermal inactivation by molar magnesium salts and size.

Materials and methods. Viruses: The 8 viruses are designated as California feline isolate (CFI)(2), kidney cell degenerating virus (CKD)(3), Bolin's virus (FPL)(5) and the feline respiratory isolates (FRI) 6, 12, 14, 29, and 278(1). They were used in their 6 to 13 feline cell culture passage. A feline herpesvirus, Poliovirus I and Reo I viruses were used as controls. Virus pools were prepared in cell cultures grown in 32 oz. prescription bottles. The cells and fluid were frozen and thawed once after maximum cytopathic effect (CPE) occurred. The virus suspensions were clarified at 2000 rpm for 15 minutes.

Tissue culture. Four types of cell cultures were employed. Bovine, feline and monkey† renal cell cultures, and HeLa cells. Primary bovine (BKC) and feline (FKC) renal cell cultures were prepared by the method of Madin *et al*(10). The BKC were used both as primary cells in culture tubes and subcultures prepared from bottles. The medium used for the BKC at the time of inoculation was 0.5% lactalbumin hydrolysate in modified Hank's balanced salt solution (HBSS) containing 0.1 M Tris buffer with the addition of 3% lamb serum. FKC and MKC cultures were maintained on 0.5% lactalbumin hydrolysate in Earle's balanced salt solution with 3% calf serum. HeLa cells were maintained with Ginsberg's medium. All media contained 500 units of penicillin and 0.5 mg of streptomycin per ml. All cells were incubated stationary at 36°C.

Plaque production. Plaque studies were performed by the prescription bottle technique. Culture medium was aspirated and 0.3 ml of virus was inoculated. Adsorption was carried out at 37°C for 1 hour with gentle agitation at 15-minute intervals. The cell sheet was overlaid with 10 ml of melted agar (1.5%) in Earle's salt solution containing

* Present address: AFSECT-JBUSMC APO, N. Y. 09676.

† Obtained from commercial source.

2% lamb serum and 0.0017% neutral red. The bottles were incubated at 37°C, with the cell layer uppermost.

Mice. Litters of 1 to 2 day old suckling Swiss mice were inoculated with each virus as follows: 0.03 ml intraperitoneally, 0.03 ml subcutaneously and 0.02 ml intracerebrally. The mice were observed for a 21 day period.

Embryonated eggs. 8-9 day old embryonated eggs were inoculated with 0.1 to 0.2 ml of a 10^{-1} and a 10^{-3} dilution of each virus by the amniotic and allantoic routes. After 4-5 days incubation at 37°C the eggs were chilled at 4°C for 1 hour prior to harvesting the amniotic and allantoic fluids. One blind passage was made with each virus by each route of inoculation. Harvested fluids were tested for virus by inoculation into FKC.

Infectivity titrations. Infectivity titrations were performed by inoculation of 0.1 ml of virus in serial 10-fold dilutions in Hank's balanced salt solution. Four cultures were used per dilution. End points were based on CPE and were calculated by the method of Reed and Muench(11). Titers are expressed as the 50% tissue culture infective dose per 0.1 ml ($TCID_{50}$).

Histologic preparations. Coverslips were fixed with Bouin's fluid and stained with hematoxylin and eosin (H & E).

Ether sensitivity test. Undiluted virus was mixed with ethyl ether (20% by volume) and held at 4°C for 18 hours. Ether was removed by evaporation in an open petri dish. Control specimens without ether were tested simultaneously.

Nucleic acid determination. Indirect evidence for determining the type of nucleic acid was obtained by using 5-bromo-deoxyuridine (BUDR) as described in detail for the feline herpesvirus(12).

Stability. Aliquots of virus were incubated in a water bath at 50°C for 30 minutes in stoppered 13 × 100 mm tubes. After incubation the tubes were placed in an ice bath prior to performing virus infectivity titrations.

Acid stability. The pH stability was tested according to the method of Ketler *et al*(13). Each virus was diluted 1:10 in Eagle's medium adjusted to pH 3.0 and to pH 7.0 with tris buffer. After 3 hours incubation at room temperature, infectivity titrations were carried out in FKC.

Size determination. Prior to filtration each filter was "satisfied" with 20 ml of sterile beef heart infusion broth. Undiluted virus preparations were first passed through a HA 0.45 mμ millipore filter. These filtrates were then filtered through gradocol membranes of 200, 95, 69, 51, 29 and 10 mμ APD. Each filtrate was collected in an ice bath and assayed by inoculation of 0.8 ml into each of 8 FKC cultures.

Stabilization by magnesium salts. Viruses were diluted 10-fold in distilled water, 1M $MgSO_4$ and 1M $MgCl_2$(14) and each sample was adjusted to pH 7.0 with HCl or NaOH as indicated. Virus solutions were heated at 50°C for 30 minutes as described by Wallis and Melnick(15) and titrated in FKC.

Results. All 8 viruses propagated well and induced marked cytopathic changes in feline renal cell cultures. With 100 $TCID_{50}$ doses focal lesions characterized by rounding and shrinking of the cells with a tendency to clump were first observed about 18 hours following inoculation (Fig. 1). These lesions progressed rapidly with the occurrence of secondary foci. The cytopathic changes continued until the entire monolayer was affected and the cells were completely released from the glass surface within 24-36 hours. At this

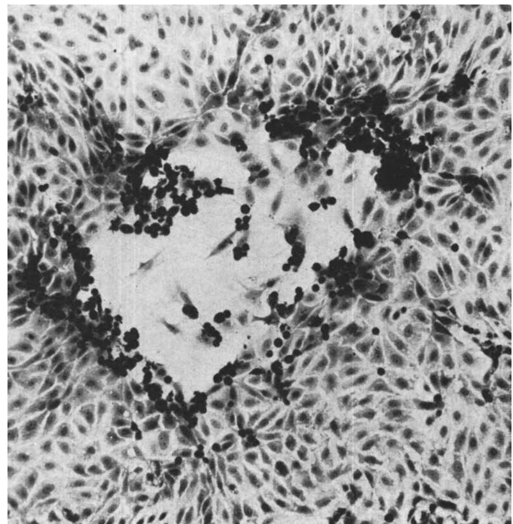

FIG. 1. Cytopathic effect of feline picornavirus in feline kidney cell culture. H & E stain; × 80.

time the infectivity titer of the viruses ranged from $10^{-6.5}$ to $10^{-8.0}$. In H & E stained coverslip preparations no inclusion bodies or elementary bodies were demonstrated. The affected cells contain either pyknotic or karyolytic nuclei, with intensely staining basophilic nuclear chromatin and dark red, somewhat granular cytoplasm (Fig. 2).

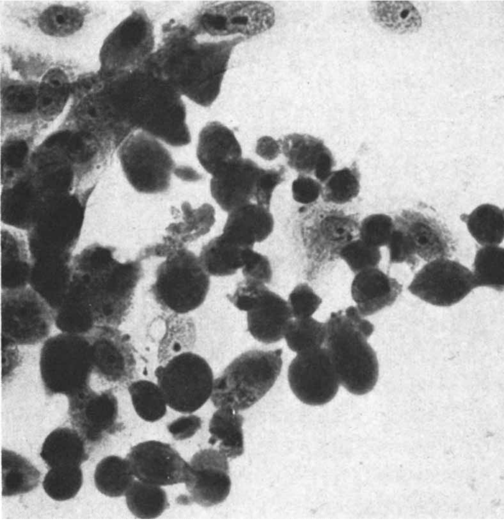

FIG. 2. Cytopathology of feline picornavirus in feline kidney cell culture. H & E stain; × 424.

The BKC, PMK and HeLa cells were inoculated with 0.1 ml of 10^{-1} and 10^{-3} dilutions of each virus. Cells and fluid were passed into respective cell systems at 3 and 6 day intervals. No cytopathic effects were observed. Residual virus was detected in FKC after 3 days but not after 6 days. No virus was demonstrated following the second passage. An attempt to adapt one virus to the 3 cell systems by alternating passages to FKC was unsuccessful.

With the exception of KCD virus, clearly defined plaques measuring 2 to 4 mm were evident in 24 hours. By 72 hours they ranged in size between 4 to 7 mm in diameter. Morphologically the plaques were clear, circular with somewhat irregular edges (Fig. 3). Plaques produced by KCD were demonstrable at 72 hours and measured 1 to 22 mm in diameter. These plaques increased in size to 2 to 4 mm with an additional 24 hour incubation time. Their morphology was similar to the other viruses.

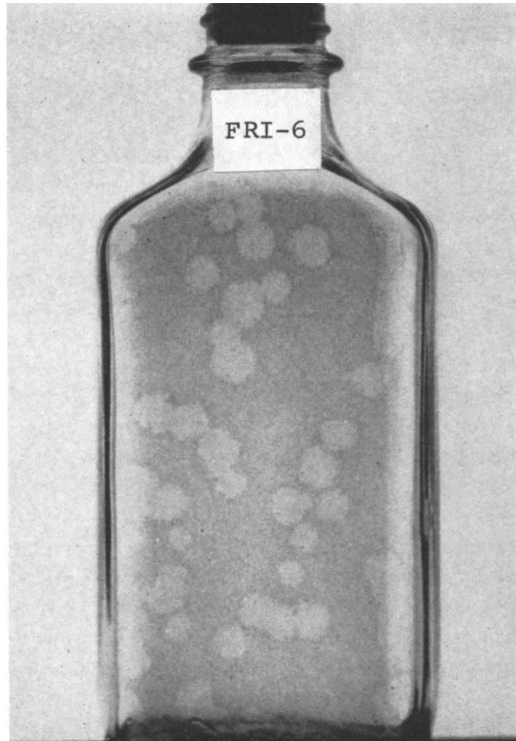

FIG. 3. Plaque morphology of feline FRI-6 virus in feline renal cell culture 3 days after inoculation.

The 8 feline viruses failed to produce death or evidence of illness in suckling mice inoculated intraperitoneally, subcutaneously and intracerebrally during a 21 day observation period.

Attemps to cultivate the viruses in the allantoic and amniotic cavities were unsuccessful. Virus was not recovered in FKC from the allantoic or amniotic fluids 4-5 days after inoculation. Some viruses were recovered in FKC after 3 days incubation but not after 2 consecutive egg passages.

None of the viruses was inactivated by ether. Infectivity titrations for individual viruses ranged between $10^{-6.7}$ and $10^{-7.6}$ and differences between ether treated and control specimens for each virus did not exceed 0.33 of a $\log_{10}$ unit.

Results of the nucleic acid, thermal stability and acid lability tests are summarized in Table I. Lack of inhibition of virus multiplication by BUDR is indirect evidence that these viruses are of RNA type. Similar results were also obtained with IUDR. With the ex-

TABLE I. Chemical and Biological Properties of the Feline Picornaviruses.

	Indirect evidence for Nucleic Acid type			Influence of temp at 50°C for 30 min			Acid lability 3 hr at room temp		
Virus	With BUDR	Without BUDR	Nucleic acid	Control	Heated	Titer reduction	pH 3.0	pH 7.0	Titer reduction
FRI-6	7.8*	7.9	RNA	8.0	3.5	4.5	5.3	7.0	1.7
FRI-12	7.6	7.5	RNA	7.6	4.0	3.6	5.5	7.0	1.5
FRI-14	7.6	7.5	RNA	7.5	6.3	1.2	6.0	7.3	1.7
FRI-29	7.8	7.7	RNA	7.0	4.6	2.4	5.5	7.6	2.1
FRI-278	7.5	7.6	RNA	7.6	4.5	3.1	5.5	7.2	1.7
CFI	7.1	7.2	RNA	7.6	6.3	1.3	5.0	7.6	2.6
FPL	7.6	7.5	RNA	7.5	4.3	3.2	4.8	7.5	2.7
KCD	7.3	7.4	RNA	7.0	2.6	4.4	5.6	7.3	1.7
Controls									
Feline herpes	0.0	6.1	DNA	NT†			<1.0	>5.0	>5.0
REO 1	NT			5.5	5.5	0.0	NT		
Polio	NT			NT			7.5	7.6	0.1

* Log_{10} $TCID_{50}$/0.1 ml.
† NT — Not tested.

ception of the viruses (FRI-14 and CFI) the feline viruses were sensitive to heat and showed a minimum loss in infectivity titer of log 2.4 on heating. Although FRI-14 and CFI were less sensitive to heat for 30 minutes than the other viruses, more than 99% of their infectivity was lost when heated for 1 hour. By contrast the REO 1 was very stable to heat. Exposure of the viruses to pH 3.0 for 3 hours and resulted in a reduction of infectivity ranging between log 1.5 to 2.7. In comparison to polio virus, the group is acid labile, with three viruses (FRI-29, CFI, and FPL) exhibiting a hundred fold or greater in infectivity reduction.

All viruses except two (FRI-29, KCD) passed through the 51 or 49 mμ gradocol membrane, with FRI-12 passing the 29 mμ membrane. FRI-29 and KCD passed the 69 mμ but not the 51 mμ membrane. Estimation of size, using Black's formula(16) each virus is calculated to measure between 31 to 43 mμ and FRI-12 might be considered to be as small as 20 mμ.

The effect of 1M $MgCl_2$ and 1M $MgSO_4$ on the infectivity of the viruses is shown in Table II. Incubation at 50°C for 30 minutes in the presence of 1M $MgCl_2$ enhanced inactivation of all feline viruses. Although the viruses were not stable in 1M $MgSO_4$, some sparing effects were demonstrated. The inactivation of FRI-14 and 29 were actually enhanced in presence of 1M $MgSO_4$.

TABLE II. Effect of $MgCl_2$ and $MgSO_4$ on Feline Viruses.

	Log_{10} decrease in titer at 50°C for 30 min		
Viruses	H_2O	$MgCl_2$	$MgSO_4$
FRI-6	3.3*	4.1	2.8
FRI-12	3.0	7.0	2.7
FRI-14	1.7	5.0	2.7
FRI-29	3.9	4.9	5.0
FRI-278	2.7	5.8	1.8
CFI	1.5	5.3	1.8
FPL	5.4	6.0	3.4
KCD	4.2	6.5	2.9
Control			
Polio	1.7	.3	.3

* $TCID_{50}$ per .1 ml.

Discussion. No significant differences were observed in the properties of the 6 respiratory isolates and the two isolated from visceral organs of the domestic cat.

The 8 feline viruses studied were shown to be ether resistant, small in size and to contain ribonucleic acid (RNA) core. These characteristics indicate that the 8 serotypes should be classified as picornaviruses of lower animals(17). Further investigations of their properties did not provide a satisfactory basis for including them in a single subgroup of the picornaviruses. The observations are compared to the recognized criteria for the classification of the enteroviruses(18), rhinoviruses(19), and reoviruses(20).

They appear less stable at pH 3.0 than the enteroviruses but not as labile as the

rhinoviruses. The enhancement of inactivation of the feline viruses by $MgCl_2$ at 50°C is in sharp contrast to the stabilizing effect it has on the enteroviruses, reoviruses(21) and most human rhinoviruses (19,22,23). Also, the feline viruses were not stabilized in solution of $MgSO_4$ as are some rhinoviruses(14,23) and a few enteroviruses(14). The feline viruses, unlike the rhinoviruses(2) are not subject to a wide variation in conditions necessary for cultivation *in vitro*. Although they have been shown to multiply only in tissue of feline origin, high titers are easily achieved in stationary cultures at 35-37°C.

The feline viruses differ from the reoviruses in their small size, absence of hemagglutinins(1), rapidity of cytopathic effect in cell culture without formation of cytoplasmic inclusions, host specificity, paucity of isolation from the intestinal tract and in their sensitivity to heat.

The properties of the feline viruses reported here are similar to 5 feline serotypes isolated in Switzerland by Burki(24). However, the European strains were found to be more stable towards low pH. Like the present viruses, they were classified as picornaviruses holding a position between the enteroviruses and the rhinoviruses. Recently, Zwillenberg and Burki (25), by electromicroscopy, found their feline picornaviruses to measure about 40 mμ and to differ significantly in morphology from the picornaviruses of other species.

Because these viruses cannot be placed into one of the presently defined subgroups of the picornaviruses, their definitive classification must await additional information concerning their shape and subunits. An analysis of the purified viruses would also be helpful for comparative purposes.

Comparative serological studies with all feline respiratory isolates are necessary before the actual number of distinct serotypes of the feline group is known. This is particularly important so that a simple numerical system for classifying can be applied to this apparently large group of agents.

Summary. Eight feline viral serotypes are classified as picornaviruses. They are ether resistant, less than 51 mμ in diameter, and their essential nucleic acid is RNA. As a group, they are heat sensitive and moderately labile at pH 3.0. The viruses are not stabilized in solution of magnesium salts. Their position within the picornavirus group is yet to be determined.

Experimental Research with animals has been conducted according to the National Society for Medical Research "Principles of Laboratory Animal Care."

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Interferon Production by Germfree Mice.* (32413)

GERALD R. FITZGERALD† AND MORRIS POLLARD
Department of Microbiology, University of Notre Dame, Notre Dame, Ind.

Since the initial studies of interferon by Isaacs and Lindenmann(1), ample evidence has been accumulated which has suggested that interferon plays a significant role in host-resistance to primary viral infections(2-4). Germfree animals have played important roles in investigations which otherwise would have been complicated by the presence in them of a microbial flora(5). Because bacteria and bacterial products have been shown to induce the formation of interferon-like substances *in vivo*(6), it seemed likely that the germfree animal might again provide a more refined test animal for investigations involving the induction and production of interferon *in vivo*. Mice respond to intravenous inoculation of Newcastle Disease virus (NDV) with high levels of serum interferon (7). Therefore, the same system was employed to determine the general capacity of germfree mice to produce interferon in response to viral stimulation.

Materials and methods. Animals. Germfree CFW mice were reared and maintained by the routine procedures developed at Lobund Laboratory for germfree animals(8-10). The conventional control mice were derived originally from germfree stock which had been adapted to the microflora of the animal quarters. The mice were 5 weeks of age and consisted of approximately equal numbers of males and females.

Viruses. The Asian strain of Newcastle Disease virus (NDV) was propagated in embryonated chicken eggs and demonstrated a titer of $10^{9.7}$ PFU/ml. A large plaque variant of encephalomyocarditis virus (EMC-r) was kindly provided by K. Takemoto of National Institutes of Health, Bethesda, Md. Vesicular Stomatitis virus (VSV) was obtained through the courtesy of B. Postic, Graduate School of Public Health, University of Pittsburgh, Pa. The latter 2 viruses were propagated in MCN mouse fibroblast cell cultures and were used for assay of interferon.

Tissue cultures. A continuous mouse fibroblast cell line(11), designated as MCN cells, kindly provided by F. Dienhardt, Presbyterian-St. Luke's Hospital, Chicago, Ill., was used for virus propagations and titrations, and for assay of interferon. Primary cell cultures were prepared from trypsinized chicken embryos and used for NDV titrations. All cultures were grown in Eagle's Minimum Essential Medium (MEM) with non-essential amino acids and glutamine added, and supplemented with 15% heat-inactivated calf serum. Penicillin and streptomycin were included at concentrations of 100 units/ml and 0.1 mg/ml, respectively. Sodium bicarbonate was added to give a concentration of 0.04%. The plaque medium recipe and the procedures for plaque assays were those described in a personal communication from B. Casto, Graduate School of Public Health, University of Pittsburgh, Pa.

Interferon production. Interferon was induced by intravenous inoculation of mice with NDV *via* the lateral tail veins. The blood was collected by incision of the axillary blood vessels, and stored overnight at 4°C. Spleens and livers were placed in vials and immediately frozen in an alcohol-dry ice bath.

* From a dissertation submitted by G. R. Fitzgerald in partial fulfillment of the requirements for the Ph.D. degree to Dept. of Microbiology, Univ. of Notre Dame, Notre Dame, Ind.

† Present address: Nat. Cancer Inst., Nat. Inst. Health, Bethesda, Md.