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ISOLATION AND CLASSIFICATION OF FELINE PICORNAVIRUS AND HERPESVIRUS IN DENMARK*

By

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FLAGSTAD, A.: *Isolation and classification of feline picornavirus and herpesvirus in Denmark.* Acta vet. scand. 1972, *13*, 462—471. — Seventeen viral isolates cytopathic for kitten kidney cells were isolated from 23 cats with symptoms of the respiratory disease feline viral rhinotracheitis or feline influenza. Five of these are classified tentatively as calicivirus, a member of the picornavirus group, and 12 have the properties of the herpesvirus. Classification is based on the cytopathic effect in cell cultures and physico-chemical characteristics.

The five isolates which are classified tentatively as calicivirus produced a quick cytopathic effect without formation of intranuclear inclusion bodies. The isolates were resistant to chloroform, were not inhibited by IDU, were labile at pH 4 and were not stabilized against thermal inactivation by molar $MgCl_2$. The 12 isolates which seem to belong to the herpesvirus produced intranuclear inclusion bodies in cell cultures. They were sensitive to chloroform, and multiplication was inhibited by IDU. Antisera were produced by inoculating rabbits with calicivirus and cats with herpesvirus. The five isolates classified tentatively as calicivirus belong in one serotype, and the 12 isolates which seem to belong to the herpesvirus also belong in one serotype.

feline picornavirus; feline calicivirus; feline herpesvirus; feline viral rhinotracheitis; feline influenza; isolation; classification; Denmark.

A study has been carried out on a number of cats with symptoms of the respiratory disease known as feline viral rhinotracheitis or feline influenza (*Crandell & Mauer* 1958, *Piercy & Prydie* 1963, *Flagstad* 1968).

Feline viral rhinotracheitis is a highly contagious disease characterized by pyrexia, anorexia, conjunctivitis, lacrimation,

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and nasal discharge accompanied by sneezing. Ulcerative glossitis and salivation may be observed.

Two distinct viruses, a herpesvirus and a picornavirus, have been shown to have the ability to produce upper respiratory diseases in the cat (*Crandell & Mauer; Bürki* 1965). The symptoms of the diseases caused by the two viruses are identical and thus clinical differentiation is not possible. Distinction of the diseases must be based on isolation and identification of the virus.

The aim of this study was to ascertain the types of virus responsible for upper respiratory infection in cats in Denmark. The study is based on a material of 23 cats with upper respiratory disease. From nasal excretion and saliva from nine cats with symptoms of feline viral rhinotracheitis picornavirus was isolated in the two cases and herpesvirus in seven. In organ specimens of eight dead or euthanized cats picornavirus was found in three cases and herpesvirus in five.

An attempt was made to identify the agents isolated by means of their cytopathic effect in cell cultures and physico-chemical characteristics. Neutralization tests were made to examine the serological relationship of the isolates within each group of viruses.

MATERIAL AND METHODS

Cell cultures

Primary monolayer cultures of kidney tissue from kitten were prepared according to the method of *Madin et al.* (1957). Ordinary tube cultures and Leighton tube cultures were used.

The maintenance medium for the cultures was Earle's BSS containing 0.5 % lactalbumin hydrolysate, 0.01 % yeast extract, 1 % calf serum. G-penicillin 100 i.u./ml, dihydrostreptomycin 100 µg/ml and mycostatin 100 i.u./ml were used in all media.

Isolation of virus strains

The material used for virus isolation was nasal excretion and saliva from live cats with symptoms of rhinotracheitis and necropsy material from respiratory organs from affected cats. Samples from diseased cats were collected in Hanks' BSS by swabbing or flushing the nose and throat. Necropsy material was prepared as 10 % suspension in Hanks' BSS. All samples were centrifuged at 10,000 r.p.m. for 10 min. (5000 $\times$ g) in order to

obtain sedimentation of organic materials and bacteria. 0.1 ml of supernatant was used as inoculum for tube cultures. Before inoculation G-penicillin, dihydrostreptomycin and mycostatin were added to the concentration given above. The cell cultures were subjected to daily microscopic examination. When a cytopathic effect was observed in a tube culture, the agent was cloned by three to five passages in cell cultures after the limit dilution procedure. The cloned isolate was used for further classification.

Examination of isolates for cytopathic effect and physicochemical characteristics

The cytopathic effect of the isolates was examined in tube cultures and in cultures grown on coverslips in Leighton tubes. These latter cultures were fixed in methanol 24—48 hrs. after inoculation and stained with haematoxylin-eosin. Titrations were made of the isolates in tenfold dilutions using three tubes per dilution, and titres were calculated by the method of *Reed & Muench* (1938).

Infectious culture fluid without serum was used for the physico-chemical tests. These are described in the following.

The chloroform sensitivity of the virus was examined by shaking 20 % by volume mixture of chloroform and virus for 10 min., after which the sample was inoculated in tube cultures. If no cytopathic effect developed, the virus was regarded as chloroformsensitive.

For study of the inhibition by 5-iodo-2-deoxyuridine (IDU), the virus sample was titrated in tube cultures to each of which 100 µg IDU was added. If a cytopathic effect was observed in control tube cultures but not in cultures containing IDU, the virus was considered to be inhibited.

The pH stability was examined in the pH range 2—9. Stock solution of 0.2 M secondary sodium phosphate (Na_2HPO_4,H_2O) was adjusted to pH values 2, 3, 4, 5, 7 and 9, with 0.1 M citric ($C_6H_8O_7,H_2O$) and 0.1 M sodium hydroxide (NAOH). A 1:20 dilution of the virus was made in each of the buffer solutions. After 3—4 hrs. at room temperature, pH measurements and inoculation for infectivity titrations were carried out.

For study of the thermal inactivation, the virus was incubated in water bath at 50 °C for 30 min., after which it was transferred to ice bath prior to infectivity titrations. The stabilizing effect of molar concentrations of divalent cations on thermal inactivation

was examined. A stock solution of 3 M magnesium chloride ($MgCl_2$,$6H_2O$) was made. The virus was diluted 1:3 in the $MgCl_2$ solution and the mixture incubated in water bath at 50 °C for 30 min. After incubating and cooling in ice bath, infectivity titrations were carried out.

Preparation of immune serum

Immune serum against picornavirus was produced by inoculating rabbits with the first isolated virus of this type (No. 1). The rabbits were injected intravenously with 4 ml (TCID50 6.0) infective fluid four times at 48 hrs. intervals, on the 12th day and after a further 30 days. They were given a booster injection five months later. Immune serum against herpesvirus was produced by inoculating cats with that virus (No. 10485). Intravenous injections were made with 4 ml (TCID50 4.5) infective fluid four times at 48 hrs. intervals, on the 12th day and after a further 30 days. The cats were given a booster injection one month later.

Blood samples were taken 10 days after the primary immunization and also 10 days after the booster injection. The recovered serum was inactivated at 56 °C for 30 min. and stored at — 20 °C.

Neutralization test

Serial twofold dilutions of serum were tested against 100 TCID50 of virus. Equal volumes of serum and virus dilutions were mixed and incubated at 37 °C for 1 hr. After incubation, 0.2 ml of each mixture was inoculated into each of three tube cultures. Simultaneous virus control titration was carried out.

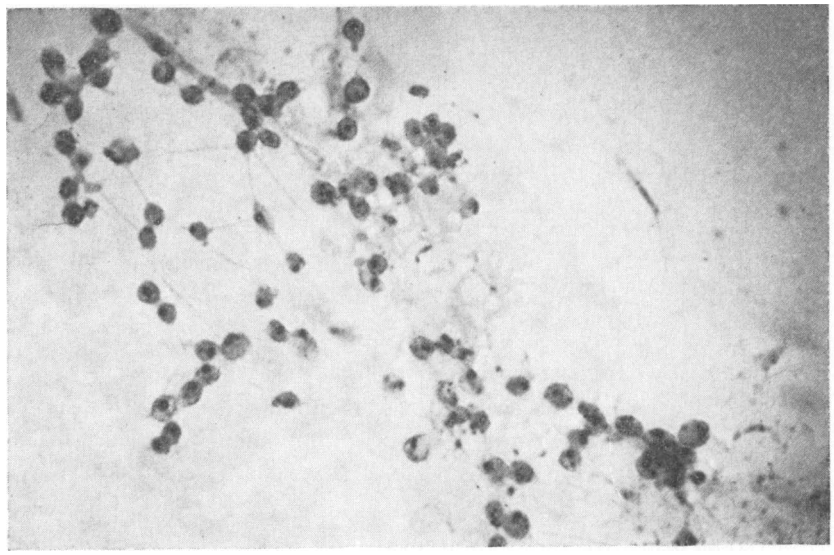
The tubes were incubated at 37 °C and examined daily by microscopy. The antibody titre was expressed as the highest serum dilution which inhibited the cytopathic effect in at least two out of three tubes.

RESULTS

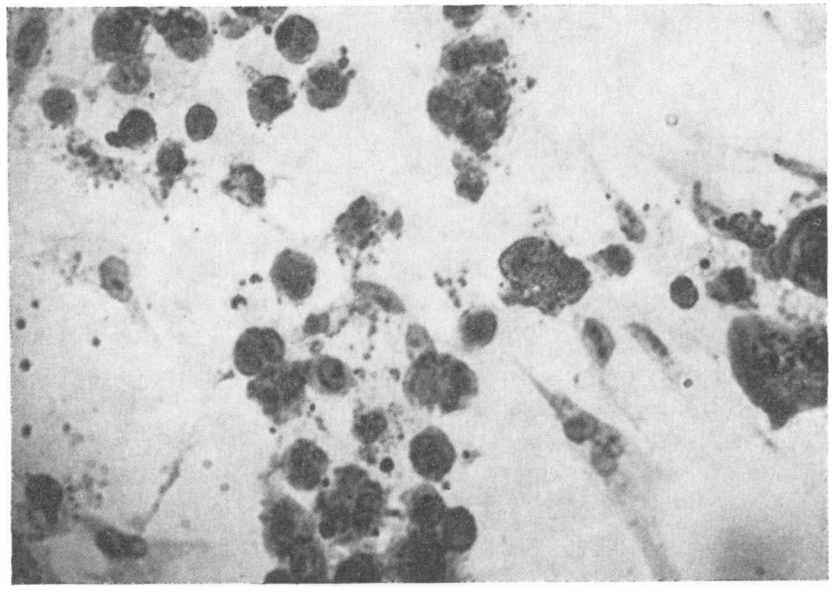
Isolation and propagation of the virus in cell cultures

Agents which produced cytopathic effect in feline kidney cell cultures were isolated from 17 out of 23 cats.

Five of the 17 isolates (1, 8977, 8980, A 317, A 318) produced similar cytopathic effect. About 24 hrs. after inoculation, the cytopathic effect was observed in the form of small scattered foci



F i g u r e 1. Cytopathic effect of feline picornavirus in feline kidney cell culture. Haematoxylin-eosin stain.



F i g u r e 2. Nuclei of the affected cells either pyknotic or karyolytic with intensely stained basophilic chromatin. No inclusion bodies are seen. The cytoplasm is dark red and has a somewhat granular structure.

of degenerative cells. During the next 24—48 hrs. the entire monolayer was totally destroyed. The infectivity titre was $10^{5.0}$—$10^{7.0}$ TCID50/0.1 ml.

Changes similar to those seen in unstained culture were observed on haematoxylin-eosin stained coverslip preparations (Figs. 1—2).

It was found that the five isolates were resistant to chloroform and therefore did not possess envelopes. Multiplication was not inhibited by IDU, thus indicating that the nucleic acid is probably RNA. These properties, in connection with the cytopathic effect, tentatively classify the isolates as picornavirus or reovirus. It was shown by neutralization tests that the five isolates belong in the same serotype. Further examination of one of the five isolates (No. 1) showed lability at pH 4.0 (Table 1) and inactivation at 50 °C for 30 min. The isolate was not stabilized by 2 M-$MgCl_2$ (Table 2). These properties indicate that one isolate and all the five isolates probably belong to the calicivirus, a subgroup of the picornavirus group (*Wildy* 1971).

T a b l e 1. Titre after 3 hrs. at room temperature at various pH values.

Isolate	pH 2.0	pH 3.0	pH 4.0	pH 5.0	pH 7.0	pH 9.0
No. 1	< 0.5	< 0.5	< 0.5	4.5	4.5	< 0.5

T a b l e 2. Titre after thermal inactivation and stabilization against heat by $MgCl_2$.

Isolate	Control 30 min. 4°C	H_2O 30 min. 50°C	$MgCl_2$ 30 min. 50°C
No. 1	6.5	< 0.5	< 0.5

All the remaining 12 isolates (10485, 10528, 10559, 10981, 11026, 11049, 11050, Fys.inst. I, Fys.inst. II, Lyngby hosp., A 807, A 809) produced a cytopathic effect which differed from that produced by the five picornavirus isolates. In tube cultures the first change was seen about 48 hrs. after inoculation in the form of vacuoles in the monolayer, bordered by large round cells with homogenic refraction. The infectivity titre of these isolates ranged from $10^{4.5}$—$10^{6.5}$ TCID50/0.1 ml.

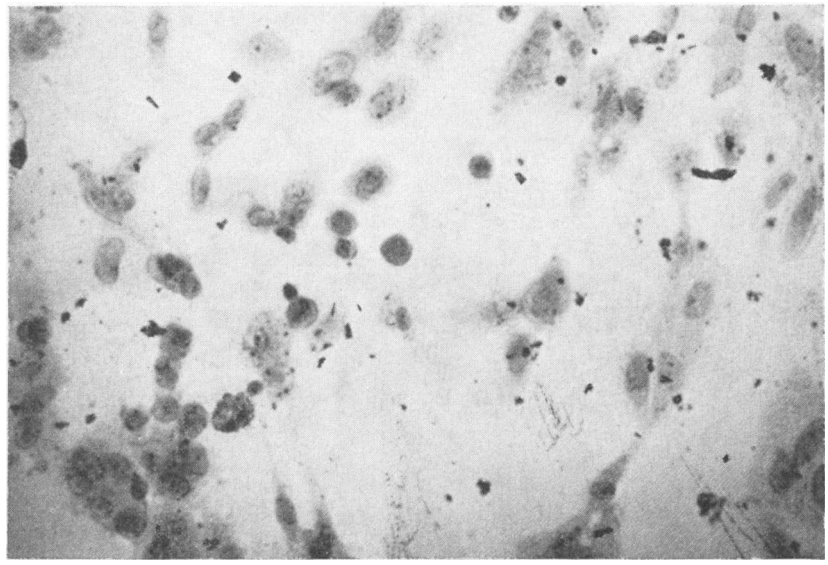


Figure 3. Cytopathic effect of feline herpesvirus in feline kidney cell culture. Haematoxylin-eosin stain.

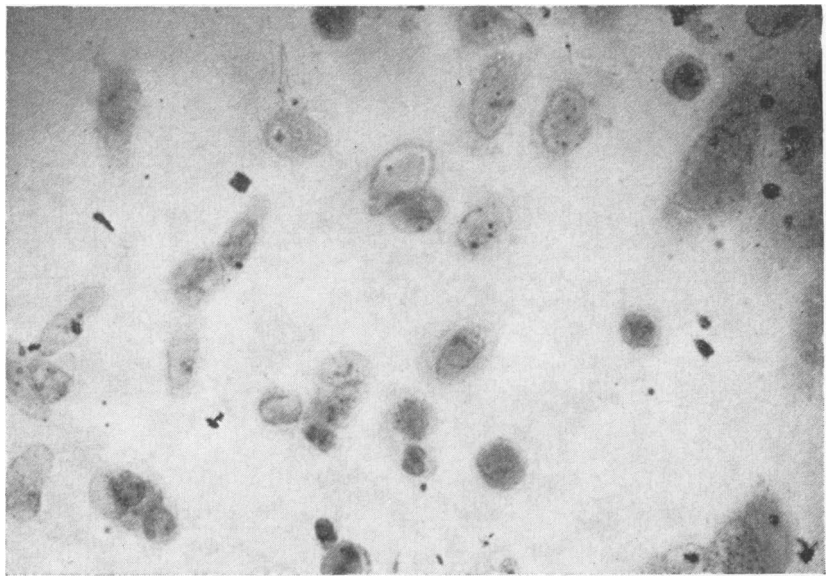
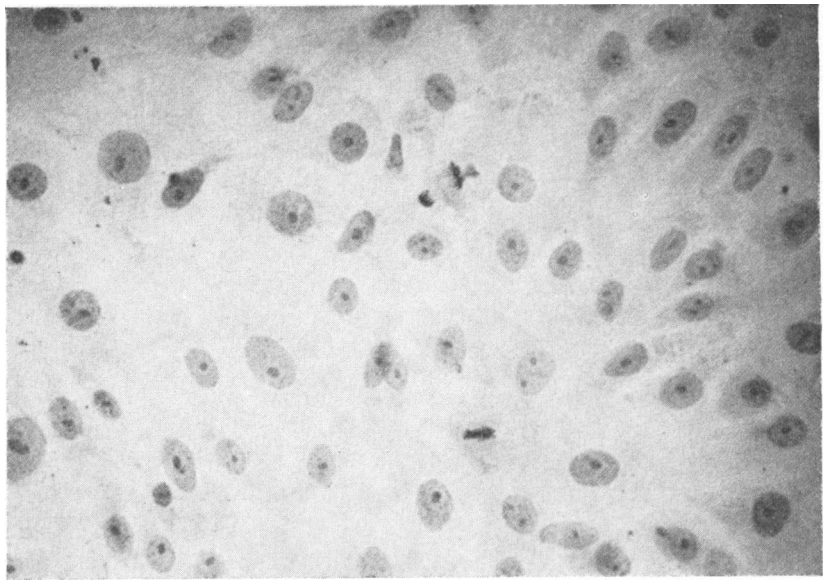


Figure 4. Intranuclear inclusion of feline kidney cell culture. The affected cells show nucleus and the chromatin, with possible disappearance of the nucleolus. The intranuclear inclusion is surrounded by a clear halo.



F i g u r e 5. Noninoculated feline kidney cell culture. Haematoxylin-eosin stain.

Intranuclear inclusion bodies could be recognized on haematoxylin-eosin stained coverslip preparations. The inclusion bodies were characteristic of herpesvirus (Figs. 3—4).

The 12 isolates were found to be sensitive to chloroform and multiplication was inhibited by IDU. This demonstrates the presence of envelopes of the virus and probably DNA as the nucleic acid. These findings, in connection with the ability of the isolates to produce type A intranuclear inclusion bodies, make it probable that the virus belongs in the herpesvirus group.

Serological examination

No neutralizing antibodies were found in the preinoculation serum of rabbits inoculated with the picornavirus isolate (No. 1). Serum had a titer of 1:160 after the primary immunization. Following the booster injection five months later the titre was 1:640. This immune serum neutralized the other picornavirus isolates.

No neutralizing antibodies were found in the preinoculation serum of cats inoculated with the herpes isolate (No. 10485). After the primary immunization the serum had a titre of 1:32,

and after the booster one month later the titre was 1:64. This immune serum neutralized the other herpes isolates. It has been attempted to produce immune serum in rabbits with a herpes isolate, which, however, has not been possible. Therefore, cats were used for immunization experiments. No serological relationship between the two groups of virus could be detected in cross neutralization tests.

DISCUSSION

From the 23 cats with the respiratory disease feline viral rhinotracheitis or feline influenza, 17 virus isolations were made. Five of the isolates were found to belong to the calicivirus, a subgroup of the picornavirus group, the remaining 12 to the herpesvirus.

The observations on the calicivirus isolates are compared with the recognized criteria for the classification of rhinovirus, enterovirus, calicivirus and reovirus (*Wallis & Melnick* 1962, *Ketler et al.* 1962, *Tyrell & Chanock* 1963, *Wildy* 1971). The virus is labile at pH 4 as calicivirus and is not stabilized against heat by $MgCl_2$, which stabilizes enterovirus and reovirus at high temperature.

Bürki (1965) and *Crandell* (1967) have reported feline picornavirus holding a position between enterovirus and rhinovirus. The isolates reported by *Bürki* were resistant to low pH in the same way as the enterovirus (pH tested was 4), and the isolates reported by *Crandell* were regarded as being less stable against lower pH than the enterovirus but more resistant than the rhinovirus (pH tested was 3). The isolates reported by *Bürki* and *Crandell,* like those in the present study, were not stabilized against heat by $MgCl_2$. The properties of these isolates are in agreement with those of the genus for which the international committee on nomenclature of viruses recommends the name calicivirus (*Wildy*). The five isolates are identified as belonging in only one serotype, whereas those reported by *Bürki* belonged in five different serotypes, and those reported by *Crandell* in eight serotypes.

The 12 isolates seem to belong to the herpesvirus and all the 12 strains could be placed in one serotype in the same way as the herpes isolates reported by *Crandell* and *Bürki.*

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SAMMENDRAG

Isolering og klassificering af picornavirus og herpesvirus hos kat i Danmark.

I et materiale på 23 katte med symptomer på luftvejslidelsen rhinotracheitis eller influenza er der isoleret 17 virusisolater, der har cytopatisk effekt på nyrecellekulturer af killinger. Fem af disse er klassificeret som calicivirus tilhørende familien picornavirus, og 12 har egenskaber karakteristiske for herpesvirus. Klassifikationen er baseret på de cytopatiske forandringer i cellekulturer og fysisk-kemiske forhold.

De fem calicivirusisolater frembragte en hurtig cytopatisk forandring i cellekulturer uden dannelse af intranukleære inklusionslegemer. Disse isolater var resistente overfor kloroform, blev ikke hæmmet af IDU, var labile ved pH 4 og blev ikke stabiliseret af $MgCl_2$ mod varmeinaktivering. De 12 herpesvirusisolater dannede intranukleære inklusionslegemer i cellekulturer. De blev inaktiveret af kloroform og hæmmet af IDU. De fem calicivirusisolater tilhørte én serotype og de 12 herpesvirusisolater én serotype.

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