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ISOLATION AND IDENTIFICATION OF HEPATITIS CONTAGIOSA CANIS VIRUS (HCC VIRUS) FROM AN ENZOOTIC OUTBREAK IN A DOG KENNEL

By

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SAXEGAARD, FINN: *Isolation and identification of Hepatitis contagiosa canis virus (HCC virus) from an enzootic outbreak in a dog kennel.* Acta vet. scand. 1972, *13*, 372—380. — Using a continuous cell line of canine kidney (MDCK-cell line) cells, HCC virus was isolated from 4 of 6 puppies affected in an outbreak of the disease in a dog kennel. The virus was most easily, and rapidly, isolated in primary cultures from the liver samples, but also from other organs such as the spleen and lung, although isolation from these organs could require 1 or 2 passages in addition to the primary culture. Attention is therefore drawn to the necessity of using a sufficient number of passages when organs other than the liver are available. Among the bacteria identified in addition to the virus infection, were Staphylococcus aureus, Bordetella bronchiseptica and β-hemolytic streptococci. Emphasis is made on the significance of isolation and identification of the virus in pure culture, thus enabling its various properties to be studied in detail, and possible new serotypes to be recognized.

canine hepatitis virus; canine kidney cell line.

According to *Rubarth* (1947) and *Bruner & Gillespie* (1966) HCC usually occurs sporadically but may assume an enzootic character in dog kennels with a high mortality rate, especially in young puppies. Although *Green & Shillinger,* as early as in 1934, described infectious canine hepatitis in detail in experimentally infected dogs, *Rubarth,* after extensive investigation of 190 naturally infected dogs, first introduced the name "Hepatitis contagiosa canis (HCC)", by which the disease is now generally

known. Since then, *Rubarth*'s pathological and serological investigations have primarily formed the basis for the diagnosis of the disease. Macroscopically, the most characteristic findings are a swollen, light-coloured liver with a distinct lobular structure, and a marked subserous edema of the gall bladder. Microscopically, the salient lesions are centrolobular liver necrosis and intranuclear inclusions in the liver cells, the endothelial cells of the sinusoids and the van Kupffer cells. These histopathological lesions are considered pathognomic for HCC.

In order to confirm the diagnosis of HCC serologically *Rubarth* (1947) developed the complement fixation test (CFT), using the liver from infected dogs as antigen. He also found the CFT to be useful in detecting antibodies in dog sera, an investigation which was extended by *Lehnert* (1948) and later confirmed by *Mansi* (1955). In 1957 *Mansi* developed the gel precipitation test for the diagnosis of the disease.

In 1954 *Müller & Thordal-Christensen* cultivated the virus in explants of testicular tissue from puppies, and *Cabasso et al.* (1954) cultivated it in dog kidney cell cultures. In 1957 *Fieldsteel & Yoshihara* also cultivated the virus in ferret and swine kidney cell cultures. The above authors succeeded in producing a characteristic cytopathogenic effect (CPE) in tissue cultures. Furthermore, *Cabasso et al.* found the fluid from infected cultures to be most suitable as an antigen in the CFT, a finding which represented an improvement on the laborious and time-consuming preparation of the antigen from infected dog liver.

The isolation and identification of the virus from an enzootic outbreak among puppies in a kennel is described in the following. Although the disease has previously been diagnosed on a pathological and serological basis, the virus has not been isolated from cases of HCC in Norway, except for 1 sporadic case in an adult dog in 1970.

MATERIALS AND METHODS

Animals

Altogether organs from 6 puppies aged 3 weeks to 3 months were examined microbiologically during a period of 2 months*.

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* Necropsy of the animals was performed at the Department of Pathology at the College, which also carried out the microscopic pathological examinations.

Preparation of specimens for tissue culture

A 20 % suspension of the various organs was prepared in phosphate buffered saline (PBS), pH = 7.4, to which 100 i.u. penicillin, 100 i.u. polymyxin, 100 µg streptomycin and 10 µg fungizone were added per ml. The tissue was immersed in alcohol, flamed and minced for 10 min. in a mechanical mixer. After centrifugation for 15 min. at 1350 × g, the supernatant was used for the inoculation of tissue cultures.

Tissue cultures

The Madin and Darby canine kidney (MDCK) cell line (*Gaush et al.* 1966), supplied by the American Type Culture Collection, Registry of Animal Cell Lines, was used. The cells were inoculated into 2 oz. prescription bottles, each containing 5 ml Hanks' medium with 0.5 % lactalbumin hydrolysate (Difco), 0.01 % yeast extract (Difco), 10 % calf serum and antibiotics in the above concentrations. As virus medium, Earle's medium with 2 % inactivated calf serum was used.

Isolation of virus

One bottle was used per sample, and the bottles were washed with 5 ml virus medium before inoculation. The bottles were then inoculated with the sample (0.5 ml per bottle) and the bottles left at 37°C for 30 min. for absorption of virus. One uninoculated bottle was included as a cell control. Five ml virus medium was added and the bottles incubated at 37°C. In order to avoid any cytotoxic effects of the inoculum, the medium was replaced after 3 hrs. The bottles were incubated for 6 days, and by means of a low power tissue culture microscope, observed daily for any CPE. The cultures were harvested as soon as the CPE was complete and the culture fluid used as virus. If no CPE occurred after 6 days, the cultures were frozen and thawed twice, centrifuged immediately and passaged. If no CPE occurred after 3 passages in addition to the primary culture, the sample was regarded as negative.

Tests for contamination with bacteria and PPLO were performed as follows: Ten ml beef heart infusion broth was inoculated with 1 ml virus culture and incubated for 2 days at 37°C. Blood agar plates were then streaked with the broth and incubated aerobically for another 2 days. Ten ml PPLO broth (Difco), enriched with 20 % fresh horse serum and 10 % of a

Table 1. Results of the microbiological examinations.

Puppy No.	Material	Virological examination: first appearance of CPE in tissue culture, number of passages: p.c.*	1	2	Bacteriological examinations
1	liver	+			coliform bacteria, α-hemolytic streptococci, micrococci
	lung		+		coliform bacteria, Staphylococcus aureus
2	liver	+			coliform bacteria
	spleen	+			„
	lung			+	Bordetella bronchiseptica
3	liver	+			coliform bacteria
	spleen	+			„
	lung	+			coliform bacteria, β-hemolytic streptococci
4	liver	+			coliform bacteria, proteus bacteria
5	lung		negative		coliform bacteria, proteus bacteria
6	spleen		negative		negative
	lung		„		coliform bacteria

* p.c. = primary culture.

10 % solution of yeast extract, was inoculated with 1 ml virus culture. After incubation for 5 days PPLO agar plates (Difco) with the same additions were streaked with the broth, and incubation continued for another 5 days.

Identification of virus isolates

The virus neutralization test was performed, and for this purpose anti-HCC serum (kindly supplied by Behringwerke AG, Marburg-Lahn, Germany*) was used. The serum was diluted 1:10, and 0.5 ml serum and 0.5 ml undiluted virus were mixed and left for neutralization at room temperature for 1 hr. One bottle was inoculated with the virus-serum mixture and 1 bottle with virus only.

Cytology

Coverslip cultures of MDCK cells, prepared in Leighton tubes were inoculated with 0.2 ml of undiluted, and a tenfold dilution,

* Representative in Norway: Norske Hoechst A/S.

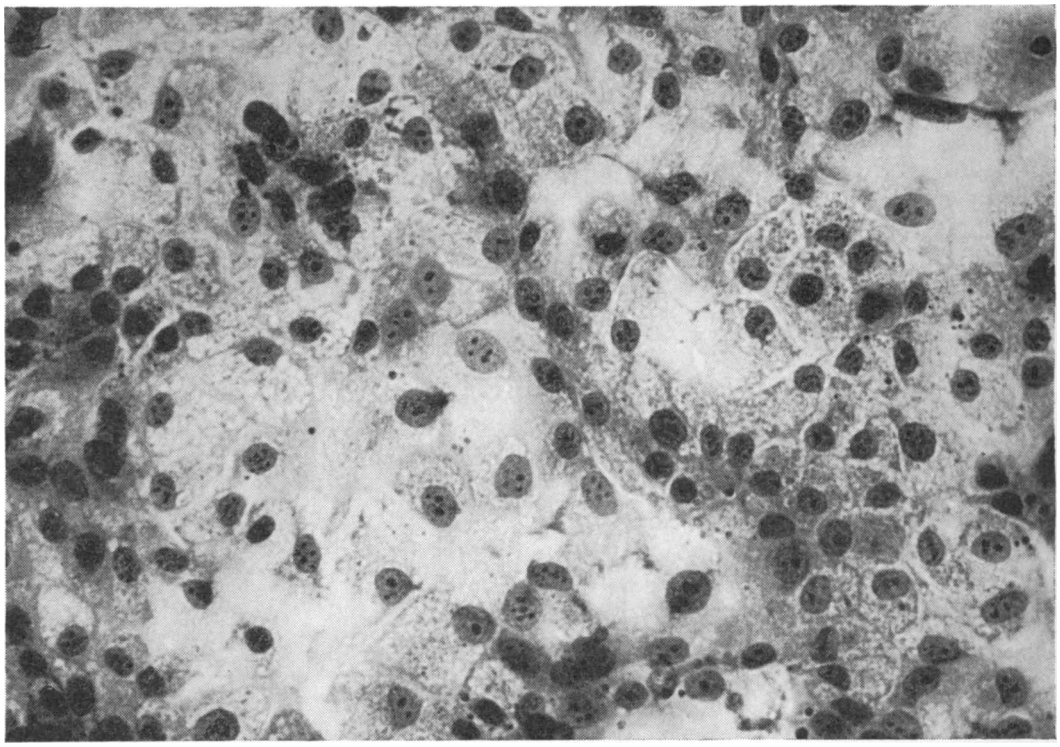

Figure 1. Uninoculated culture of MDCK cells. H.E. 105 ×.

of the virus isolates. Uninoculated tubes were included as controls. When the CPE was about 50 %, the coverslips were fixed in Bouin's fluid, stained with hematoxylin and eosin, and examined microscopically for cytological changes.

Bacteriological examinations

These were performed according to standard bacteriological methods.

RESULTS

The results of the virological and bacteriological examinations are shown in Table 1.

HCC virus was isolated from all organs tested from puppies Nos. 1 to 4, while puppies Nos. 5 and 6 were negative. The CPE was characterized by shrinking and rounding of affected cells which aggregated into grapelike clusters. All the virus isolates

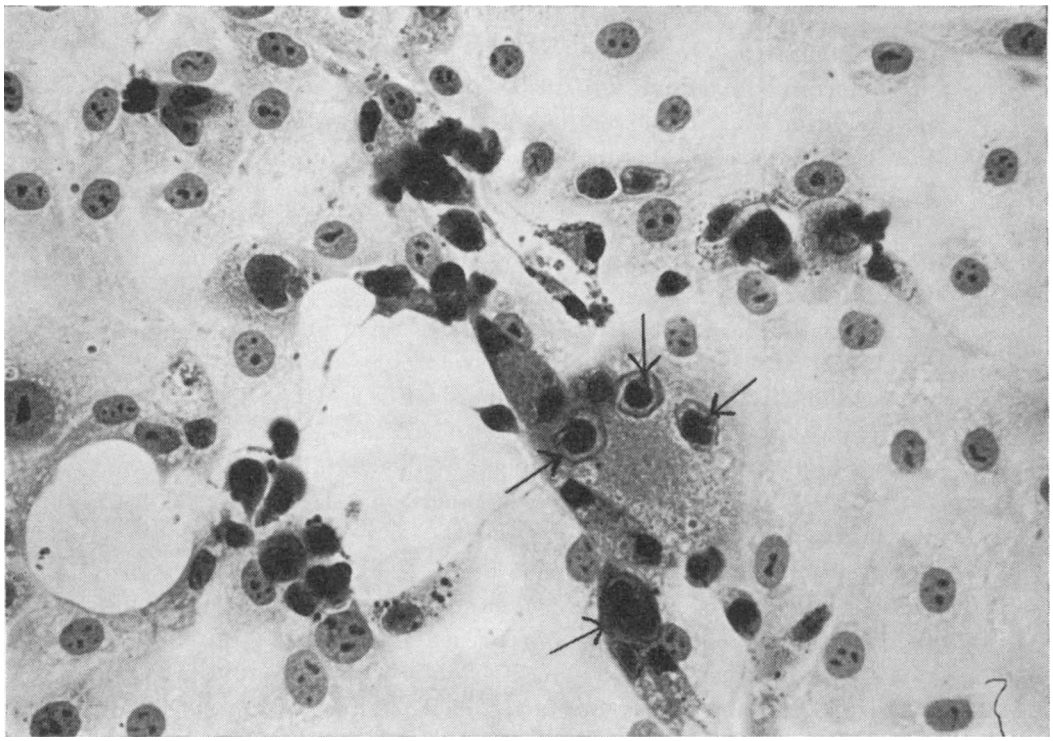

Figure 2. MDCK cells 2 days after inoculation with HCC virus isolated from the liver of puppy No. 1.
Note intranuclear inclusions (arrows). H.E. 105 ×.

were specifically neutralized by anti-HCC serum. Cytological examination of infected coverslip cultures revealed characteristic basophilic intranuclear inclusions (Fig. 2).

The time which elapsed before the CPE appeared depended on the kind of tissue used for inoculation. When liver tissue was inoculated, the CPE was usually complete 4 days after inoculation of the primary culture (p.c.). When other tissues such as spleen and lung were inoculated the CPE did not usually appear until 2—6 days after inoculation of the p.c. or even later, and did not become complete until the second or third passage. This is best illustrated by the results for the lungs of puppies Nos. 1 and 2 where the CPE did not appear until the first and second passage, respectively.

It may be seen from Table 1 that the bacteria which were isolated were quite diverse.

DISCUSSION

Although the concentration of virus in various organs was not determined exactly by titration, it is evident from the present investigation that the liver had the highest content of virus, a finding which corresponds to previous pathological and serological investigations (*Rubarth* 1947, *Lehnert* 1948, *Mansi* 1955, 1957). The liver should therefore be considered as the major organ for virus isolation. By using this organ the virus may be harvested 4 days after inoculation of the p.c. and identified by neutralization after another 4 days. However, the isolation of virus from the spleen and lung proved that a viremia had occurred. These, and other organs, should therefore be used when the liver is not available. The failure to isolate virus from puppies Nos. 5 and 6 may be due to advanced degenerative changes or cadaverosis, and the importance of obtaining fresh material for the investigations should be emphasized.

Despite the value of the direct pathological and indirect serological diagnosis of HCC it is evident that the isolation and identification of the causative virus represents the only definite diagnosis of the disease. The value of isolation of the virus in pure culture is emphasized by the fact that it enables various properties such as the morphological, physico-chemical, cultural, serological, immunological and biological properties to be studied in detail. *Rubarth* draws attention to the possibility of more than 1 serotype of HCC virus, a supposition which is not surprising when one considers that there exist 31 serotypes of human adenoviruses (*Andrewes & Pereira* 1967). The control of possible new serotypes of HCC virus will inevitably require continuous virological examination of material from affected animals. The recognition of different serotypes will be of major importance in an effective vaccine production, which can only be performed with a pure culture of the virus.

The isolation of Staphylococcus aureus, Bordetella bronchiseptica and β-hemolytic streptococci from the lungs of puppies Nos. 1, 2 and 3, respectively, was probably due to secondary infection. This may also be the case for the coliform bacteria, the growth of which, in most cases, was abundant, although advanced cadaverosis could not be precluded. The occurrence of α-hemolytic streptococci, micrococci and proteus bacteria was considered to be due to external contamination.

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SAMMENDRAG

Isolasjon og identifikasjon av Hepatitis contagiosa canis-virus (HCC-virus) fra et enzootisk utbrudd i en kennel.

Med bruk av en cellelinje av hundenyreceller (MDCK-celler) ble HCC-virus isolert fra 4 av 6 hvalper under et enzootisk utbrudd i en kennel. Viruset ble isolert i primærkultur fra lever, men også fra andre organer som milt og lunge, selv om isolasjon fra sistnevnte organer ofte krevet 1 til 2 passasjer i tillegg til primærkultur. En fremhever derfor betydningen av et tilstrekkelig antall passasjer i cellekultur når andre organer enn lever anvendes for undersøkelse. Som

sekundær bakterieinfeksjon forekom Staphylococcus aureus, Bordetella bronchiseptica og β-hemolytiske streptokokker. En påpeker betydningen av isolasjon av viruset i renkultur for at dets forskjellige egenskaper kan studeres i detalj og eventuelle nye serotyper påvises.

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