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The Seal Death in Danish Waters 1988

2. Virological Studies

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Have, P., J. Nielsen and A. Bøtner: The seal death in Danish waters 1988. 2. Virological studies. Acta vet. scand. 1991, 32, 211–219. – Mass abortions and high mortality were observed in harbour seals in Danish waters during 1988. Severe pneumonia and emphysema were typical clinical and post-mortem findings. Virological studies were carried out to identify the cause of the epidemic. Although seal herpesvirus (SeHV) was isolated in 23 of 114 animals this virus was subsequently found not to be the primary cause of the disease. Following the observation of seroconversion against canine distemper virus (CDV) in diseased seals (*Osterhaus & Vedder* 1988) a CDV-like morbillivirus (phocine distemper virus, PDV) was identified in organs of diseased animals. It is concluded that the epidemic was caused by introduction of PDV into a highly susceptible population presumably free from morbillivirus infection. The origin of PDV remains unknown but evidence of prior morbillivirus infection has been found in arctic and antarctic seal populations.

virus infection; pneumonia; epizootic.

Introduction

During the spring and summer 1988 mass abortions and high mortality were observed in harbour seals (*Phoca vitulina*) in Danish waters (*Heide-Jørgensen et al.* 1988). Typical post-mortem findings were those of acute, severe pneumonia and emphysema (*Heje et al.* 1991). The pattern of the disease was clearly epidemic, i.e. pointing towards a sudden introduction of 1 or more external factors (probably contagious) into the population. During 1988 the disease spread to most harbour seal populations in the Baltic Sea and North Sea.

Several virus infections of harbour seals have been described in the literature. Thus, pox-virus have been associated with skin lesions (*Wilson et al.* 1972). Caliciviruses have been isolated from several species of pinnipeds, including aborted northern fur seal pups (*Callorhinus ursinus*), and it is believed that these species constitute a natural reservoir of caliciviruses for other marine and terrestrial mammals (*Smith et al.* 1986). In 1979–80 influenza A virus was involved in mass mortality of harbour seals primarily showing pneumonia at the coast of New England (*Geraci et al.* 1982). A seal herpesvirus was described as the primary cause of fatal pneumonia in harbour seal pups in a sanctuary in the Netherlands (*Osterhaus et al.* 1985, *Borst et al.* 1986).

A canine distemper-like virus (seal or phocine distemper virus, PDV) has now been shown to be the primary cause of the mass mortality seen in 1988 (*Osterhaus & Vedder* 1988, *Osterhaus et al.* 1988, *Kennedy et al.* 1988a, *Hofmeister et al.* 1988, *Liess et al.* 1989). Interestingly, a similar virus has been identified in an epidemic occurring simultaneously among Baikal seals (*Phoba siberica*) in Lake Baikal (*Grachev et al.* 1989). The present paper describes the virological

studies that have been conducted on the Danish epidemic so far.

Materials and methods

Seal kidney cell cultures (SeKC)

Abandoned premature harbor seal pups were used as kidney donors. In total, 4 pups without any signs of disease were euthanized. Using aseptic techniques, the kidneys were exposed by abdominal midline incision and the renal artery was flushed with 0.5 to 0.75 litres of a solution consisting of 0.25 % trypsin in Ca^{++} and Mg^{++} free PBS (PBS-A) preheated to 37°C. The kidneys were removed and placed in 37°C warm PBS-A for approximately 15 min. Adjacent tissues and the pelvis were removed by dissection and the remaining kidney tissue was chopped. Preheated trypsin solution was added and the mixture was placed under magnetic stirring at 37°C until disaggregation of the tissue was completed. The harvest was diluted with Eagle's MEM (Glasgow mod.) containing 0.1 g streptomycin and 0.05 g neomycin per litre (hereafter called Eagle's MEM) and filtered through 1 layer of sterile gauze. After centrifugation (220 g for 20 min at 4°C), the cell sediment was resuspended in Eagle's MEM supplemented with 10 % fetal calf serum (FCS, Biochrom KG, W. Germany). The cell suspension was distributed into 200 cm^2 plastic tissue culture bottles (Nunc, Denmark). The pH in the cell cultures was adjusted by short-time exposure to a 5 % CO_2-air gas flow. For subcultures, confluent SeKC monolayer cultures were trypsinized by a solution consisting of 0.025 % trypsin and 0.02 % EDTA in PBS-A and resuspended in Eagle's MEM supplemented with either 10 % FCS or 15 % newborn calf serum (NCS, Gibco, Scotland).

Material from seals

Organ material from 120 harbor seals and serum samples from 72 harbor seals that were found dead or dying along the Danish coasts were examined. Furthermore material from 4 dead harbor seals from the Faroe Islands were submitted for virological examination.

Lung tissue was represented in all the submitted organ material whereas samples of liver, spleen, kidney and brain tissue were obtained from a limited number of the seals only.

Virus isolation

Tissues were ground to make a 20 % suspension in Eagle's MEM containing penicillin, dihydrostreptomycin and mycostatin. The preparations were lowspeed centrifugated and the supernatants were used immediately or stored at – 80°C until used in virus isolation procedures. The supernatants were inoculated in 1–2 ml volumes onto primary or secondary SeKC monolayer cultures in 25 cm^2 plastic tissue culture bottles. Following incubation at 37°C for 2 h on a rocking platform the cell cultures were washed twice and supplied with Eagle's MEM containing 2 % FCS. The cultures were incubated at 37°C and examined daily for cytopathic changes (CPE). Cell cultures revealing CPE were harvested by a single freeze/thaw procedure.

Initially, organ suspensions were also inoculated into embryonated chicken eggs (intraamniotic) and newborn mice (intraperitoneally and intracerebrally) to test for influenza virus and other putative viruses. Amniotic fluid was tested for hemagglutinin activity using chicken erythrocytes and newborn mice were observed for a period of at least 1 week. Isolation of PDV was attempted by inoculation of susceptible mink. Details of these procedures have been reported elsewhere (*Blixenkrone-Møller et al.* 1989).

Infectivity test
For infectivity titrations, 50 µl volumes of each ten-fold dilution of virus in Eagle's MEM were dispensed in 5 wells of microtiter plates (NUNC, Denmark) followed by 100 µl of a SeKC suspension (200.000 cells/ml) in Eagle's MEM supplemented with 7.5 % NCS. The plates were sealed and incubated for 4 days at 37°C in a 5 % CO_2-air atmosphere. The cultures were examined microscopically for CPE and titres were calculated according to the method of *Reed & Muench* (1938).

Ether sensitive test
Equal amounts of ether and virus suspension were thoroughly mixed by shaking. The mixture was left at room temperature for 10 min, low speed centrifugated and the aqueous phase was aspirated and tested for infectivity as described. As a control the titre reduction of feline calicivirus on CRFK (Crandell feline kidney) cells was determined to be less than 0.5 $\log_{10}$ $TCID_{50}$.

Iodo-deoxyuridine inhibition test
Infectivity titrations were performed using 5-iodo-2'deoxyuridine at a final concentration of 50 µg/ml in the maintenance medium. As a control the titre reduction of calicivirus on CRFK cells was determined to be less than 0.5 $\log_{10}$ $TCID_{50}$.

Purification of virus
A crude viral harvest (250 ml) was centrifugated for 1 1/2 hour at 20000 rpm and 5°C (Beckman, rotor JA 20). The pellet was resuspended in the supernatant (1/70 of the original volume) overnight at 5°C on a magnetic stirrer. The resuspended material was sonicated (4×15 sec, 12 µ) and clarified by low speed centrifugation From this suspension 0.5 ml was loaded on a sucrose gradient prepared by freezing and thawing a 17.5 % (w/w) solution of sucrose in 0.04 M sodium phosphate with 0.2 M NaCl pH 7.6. The gradient was spun at 20000 rpm and 5°C for 20 min (Beckman, rotor SW 50). The gradient was scanned and the UV-absorbing band appearing approximately in the middle of the gradient was collected.

Electron microscopy
For negative contrast examination, small drops of sucrose gradient purified virus were placed on formvarcoated grids, and contrasted with 2 % uranylacetate in H_2O.

Cryostat sectioning and immunofluorescent staining
Lung, liver, spleen, kidney and lymph node specimens were cut into 6 µ sections, air dried and fixed in acetone for 10 min. Immunofluorescent staining was performed by reacting with monospecific rabbit antisera, diluted 1/20 in PBS, for 30 min at 37°C followed by a FITC-conjugated anti-rabbit IgG (DAKO F 205) diluted 1/50 in PBS. Slides were mounted in a permanent polyvinyl alkohol mounting fluid and examined by fluorescence microscopy.
The following monospecific rabbit antisera were used:
1. Rabbit antiserum raised against seal herpesvirus by immunizing rabbits twice with sucrose-gradient purified seal herpesvirus capsids emulsified in incomplete Freund's adjuvant.
2. Rabbit antiserum against lapinized rinderpest virus (Nippon Institute for Biological Science, Japan).
3. Rabbit antiserum against influenza A virus A/swine/Als/4744/82(H_1N_1).

SeHV antibody blocking ELISA
Sucrose-gradient purified SeHV-capsids were coated onto microplates (NUNC, Immunoplate I) overnight at a dilution of 1/50

in carbonate buffer pH 9.6. Test sera were applied at a dilution of 1/4 in PBS-0.1 % Tween 20 (PBST) for 1 h at 37°C. Following washing, rabbit anti-SeHV diluted 1/400 in PBST-10 % bovine serum was added and left for 1 h at 37°C. Finally horseradish-peroxidase conjugated anti-rabbit IgG (DAKO P 217) diluted 1/400 in the same buffer was added and left for 30 min at 37°C. Sera reducing the optimal density more than 50 % compared to blank controls were considered positive.

CDV IFAT

Antibodies against canine distemper virus (CDV) were detected in an indirect fluorescent antibody test using acetone-fixed VERO cell monolayers infected with the Onderstepoort strain of canine distemper virus (CDV). Sera were tested at a dilution of 1/5 in PBS for 30 min at 37°C followed by incubation with a 1/50 dilution of FITC-protein A (Pharmacia) in PBS for 30 min at 37°C.

CDV neutralization test

Neutralizing antibodies against CDV were assayed in a micro neutralization test using 100 $TCID_{50}$ of the Onderstepoort strain of CDV in VERO cells. Sera were tested in 2-fold dilutions starting at 1/5. Serum-virus mixtures were incubated for 1 h at 37°C prior to addition of cells. Plates were read after 3–5 days and the highest dilution completely inhibiting CPE was taken as the neutralizing titre.

Antibody tests for influenza A virus

The following 3 methods were used for detection of antibodies against influenza A virus:

1. Hemagglutination-inhibition test using A/equil/Prague 56 (H_7N_7).
2. Antibody blocking ELISA as described for SeHV using A/equil/Prague 56 as antigen and a pooled rabbit antiserum against A/swine/Zealand/6019/82(H_1N_1) and A/swine/Als/4744/82 (H_1N_1).
3. IFAT testing as described for CDV using monolayers of swine kidney cells infected with A/swine/Als/4744/82.

Results

Virus isolation

Organ suspensions from 114 seals were inoculated onto primary or secondary SeKC monolayer cultures supplied with Eagle's MEM as maintenance medium. Cytophatic changes suggestive of a herpesvirus infection developed after 2–4 days in the SeKC cul-

Table 1. Summary of virological and serological examinations carried out on seal specimens.

Geographic location	Isolation of SeHV in SeKC	PDV IFAT cryostat sections	Serology	
			SeHV	CDV
Kattegat	16/82*		39/47	38/58
Waddensee	1/6			1/1
Limfjorden	1/10			
Western Baltic	5/15	6/8	8/9	5/11
Faroe Islands	0/1	0/2		1/2
Total	23/114	6/10	47/56	45/72

* no. positive/total

tures inoculated with suspensions from 23 of the examined seals. The cytophatic changes consisted of a focal rounding of cells and syncyticum formation. Intranuclear inclusion were observed in hematoxylin-eosin stained cultures. The results have been summarized in Table 1. Inoculation of embryonated eggs and newborn mice yielded no evidence of influenza virus or other viruses.

Ether sensitivity and inhibition with iodo-deoxyuridin

One cytopathogenic virus isolate was tested. This isolate was sensitive to ether treatment and inhibition by 5-iodo-2'deoxyuridin. After each treatment no residual infectivity or replication was found when 4.8 $\log_{10}$ $TCID_{50}$/ml was treated by either procedure.

Electron microscopy

Typical herpes virus capside, measuring approximately 96–104 nm were revealed in negative contrast preparations of sucrose-gradient purified virus (Fig. 1). Distinct enveloped particles were not seen.

Cryostat sectioning

No distinct immunofluorescent staining was observed in any of the organ sections using monospecific rabbit antisera against SeHV and influenza A virus. Ten animals have been examined using the rabbit antiserum against rinderpest virus. Of these, 6 animals showed extensive specific fluorescence in lung specimens and less so in spleen, liver and kidney (Table 1).

SeHV antibodies

Due to the generally poor quality of post-mortem seal sera it was usually not possible to perform neutralization tests.

A total of 56 sera were examined in ELISA for SeHV antibodies. Antibodies against SeHV were found in 47 of these sera (84 %). One strongly positive serum sample originated from a seal which had been kept since 1972 in Esbjerg in a sanctuary, where no clinical signs of infectious disease have been observed to date. This sample was strongly positive for SeHV antibodies, thus indicating, that SeHV was present in Danish seals before the present epidemic.

CDV antibodies

Antibodies against CDV were detected in 45 (63 %) of 72 seal sera by IFAT. In a few instances IgG fractions were prepared from IFAT-positive sera by ammonium sulphate precipitation and tested in the neutralization test. These preparations neutralized CDV (data not shown). Sera from the kidney donor seals were free from neutralizing antibodies to CDV.

Influenza A antibodies

Influenza A antibodies were not detected in any of the seal sera tested, using either of the 3 methods (data not shown).

Discussion

The rapid development and serious course of the epidemic of seal deaths in 1988 was strongly suggestive of an infectious (viral) agent being the primary cause of the disease. Considering the limited pre-existing knowledge of viruses infecting seals, this urged a broad approach in an attempt to identify putative viral agents.

Soon after the first observations of abortions, orphans were collected for preparation of stocks of primary kidney cells. These have been found consistently free from SeHV, PDV and other viruses.

In the present study a high number of the diseased seals were found to be infected with a virus causing cytopathic changes typical for herpesviruses in SeKC cultures. Extended investigations including electron micro-

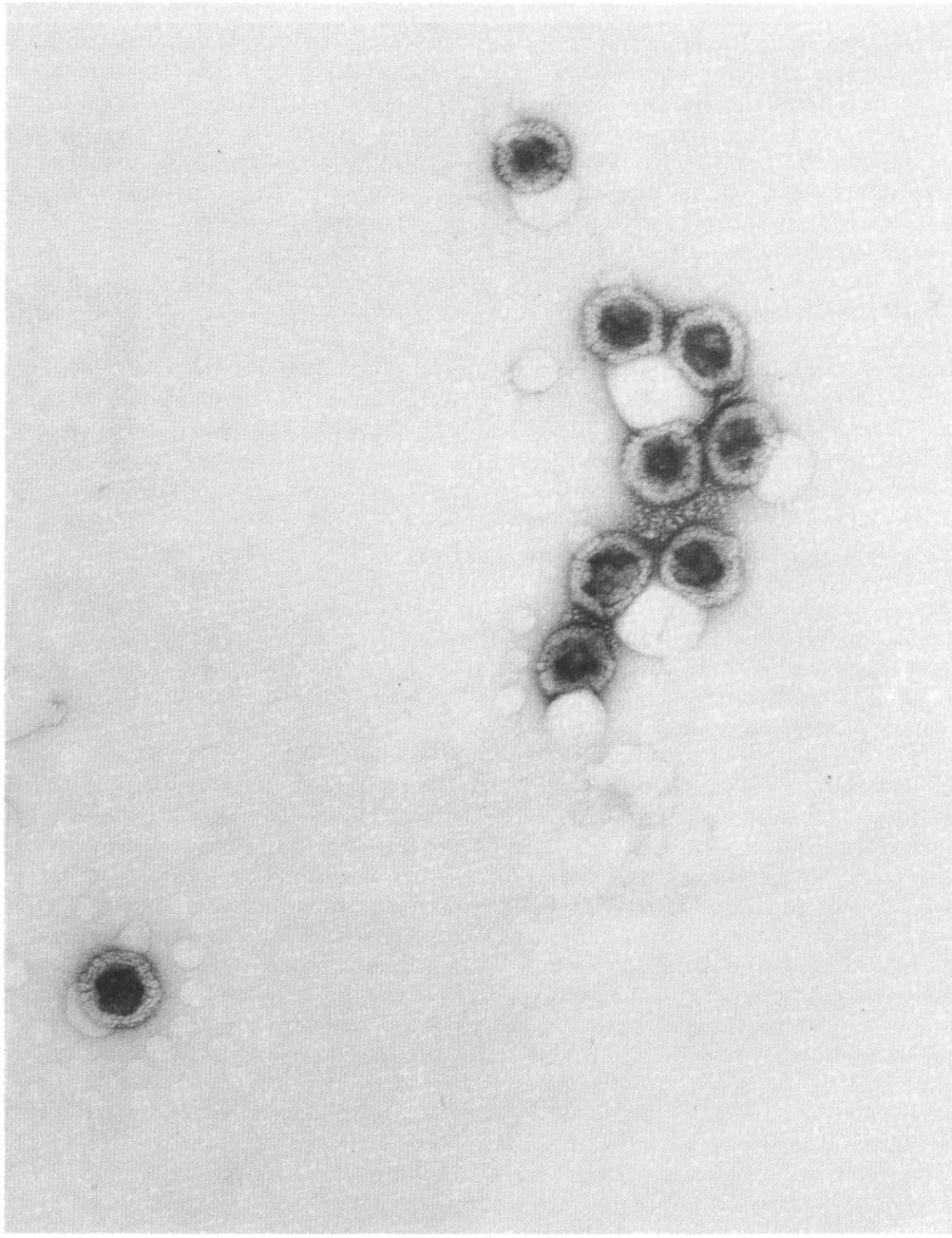

Figure 1. Electron micrograph of seal herpesvirus capsids (negative contrast, uranyl acetate). 100.000 ×.

scopy, ether sensitivity test and iodo-deoxyuridin inhibition test classified the isolated virus as an alphaherpesvirus.

While others have isolated picornaviruses from organs of diseased or dead seals using other sources of cells (*Grauballe PC* pers. comm., *Osterhaus* 1988), picornaviruses were not isolated in the present study. PDV has been isolated in kidney cell cultures of infected seals (*Kennedy et al.* 1988, *Liess et al.* 1989), in VERO cells and primary lung cells from a healthy seal (*Hofmeister et al.* 1988). Mink have also been found to be susceptible to PDV following experimental infection (*Blixenkrone-Møller et al.* 1989).

Tissue suspensions of seal lungs where PDV had been identified by IFAT were inoculated onto SeKC, but these attempts to isolate PDV remained unsuccessful. However, PDV has now been isolated in VERO cells by coculture with infected mink lung macrophages (data not shown).

Due to a relatively high isolation frequency and the unknown status of seals in Danish waters before the start of the epidemic regarding SeHV and picornavirus both of these viruses were considered likely candidates as the primary cause of the disease (*Osterhaus* 1988). However, SeHV had been shown to be prevalent in several species of pinnipeds prior to the present outbreak (*Vedder et al.* 1987) and growing serological evidence gradually made it less likely that any of these 2 viruses could be the primary cause of the epidemic (*Osterhaus & Vedder* 1988). The finding of the SeHV antibody positive seal in the Danish sanctuary indicated that SeHV was also present in Danish seals before the present epidemic.

The hint to look for another virus came from the pathologist Anders Bergman, Uppsala, Sweden, who found histopathological changes and intracytoplasmic inclusion bodies resembling canine distemper. Shortly after this observation the involvement of a canine distemper-like virus (PDV) was demonstrated by seroconversion (*Osterhaus & Vedder* 1988) and by cryostat sectioning and immunofluorescent staining of affected organs (our observations). The identification in several laboratories of PDV associated with the recent epidemic leaves little doubt that it plays a primary role in the development of disease. On the other hand, the relatively high isolation frequency of SeHV and picornavirus might indicate these viruses as aggravating factors. In spite of extensive studies other environmental factors (pollutants such as heavy metals, PCB's etc.) have not yet shown a significant link to the disease (*Heide-Jørgensen et al.* 1988).

The origin of PDV has not yet been resolved. The course of the epidemic in the Baltic and North Seas points towards an introduction into a highly susceptible population which in turn leads to the conclusion that the infection was not present prior to the start of the outbreak. Retrospective serological surveys have shown the presence of a PDV-like infection in harp seals at the westcoast of Greenland years before the present outbreak (*Dietz et al.* 1989) and in crabeater seals in the Antarctic (*Bengtson et al.* 1991). A high prevalence of PDV-infection has now been demonstrated in harp seals from the eastcoast of Greenland (*Markussen & Have,* in press) and the recent migration of these seals to Norwegian and Danish waters (*Larsen et al.* 1987) could constitute an epidemiological link to harbor seals in the Baltic See and the North Sea. Recently, porpoises have been shown to be affected by PDV and their possible role as vectors has been considered (*Kennedy et al.* 1988b).

PDV has been found to be very closely related to CDV on the basis of 2-way neutralization (data not shown) and comparison with monoclonal antibodies (*Cosby et al.*

1988). Others have by DNA/RNA hybridization found less clear relationship with CDV (*Mahy et al.* 1988) and have proposed that PDV is another independent member of the morbillivirus group. Thus, further studies in both epidemiology and molecular characterization will be needed before the exact origin of PDV can be determined. In this respect it will be interesting to compare European strains of PDV with those that occurred at the epidemic in Lake Baikal.

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Sammendrag

Sældøden i danske farvande 1988. 2.
Virologiske undersøgelser.

Der blev i 1988 observeret et stort antal aborter og massedødsfald blandt spættede sæler i danske farvande. De kliniske og patologiske undersøgelser viste primært bronchopneumoni og interstitielt emfysem. Der gennemførtes virologiske undersøgelser med henblik på klarlæggelse af årsagen til epidemien. Der påvistes et herpesvirus i 23 af 114 sæler, men retrospektive serologiske undersøgelser tydede på, at dette virus ikke var primær årsag til epidemien. Efter påvisning af serokonversion mod hundesygevirus blev der identificeret et hundesygevirus lignende morbillivirus (phocint distemper virus; PDV) i organer af døde sæler. Det konkluderes, at epidemien skyldtes introduktion af PDV i en højt modtagelig sælpopulation. Oprindelsen af dette virus er ukendt, men der er fundet tegn på PDV infektion i arktiske og antarktiske sælpopulationer.

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