location of the depopulated pig facility and/or the period for which the nursery remains empty may not be critical for success and this should enhance the applicability of the method to a wider range of farms. The results of this study provide the veterinary practitioner with a large database to which he can refer when considering nursery depopulation as a potential method for improving nursery pig performance. The duration of the effect was not measured beyond one year, but the process could be repeated if a decrease in performance should be detected.

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Age-related disease in recurrent outbreaks of phocid herpesvirus type-1 infections in a seal rehabilitation centre: evaluation of diagnostic methods

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Veterinary Record (1997) **140**, 500-503

The prevalence and clinical signs of phocid herpesvirus type-1 (PhHV-1) infections among harbour seals (*Phoca vitulina*) in a seal rehabilitation centre in the Netherlands were monitored between June and September 1993 and 1994 when 34 and 36 seals, respectively, were rehabilitated. In both years PhHV-1-related disease outbreaks occurred in the pupping season. PhHV-1 infections were diagnosed by the demonstration of a more than four-fold increase in virus neutralising serum antibodies in paired serum samples, by the isolation of the virus from swab samples in primary seal kidney cells, and by the detection of PhHV-1 DNA with a polymerase chain reaction (PCR) assay in swab samples. This assay targets a 290 bp fragment of the glycoprotein D (gD) gene equivalent of PhHV-1. The PCR assay when combined with Southern blotting (PCR-SB) was approximately 1000 times more sensitive than virus isolation when tested with serially diluted samples from PhHV-1-infected cell cultures. In contrast with virus isolation, the PCR-SB scored as positive all the animals with serological evidence of PhHV-1 infection. The majority of seals present in the centre during the outbreaks contracted the infection and developed benign upper respiratory disease. However, the severity of PhHV-1-related disease was inversely correlated with age and fatal generalised infections occurred only in neonates.

TWO types of pinniped herpesviruses have been distinguished. They were recovered from members of the *Phocidae* family and are referred to as phocid herpesviruses type-1 and type-2 (PhHV-1 and PhHV-2) (Osterhaus and others 1985, Frey and others 1989, Lebich and others 1994). PhHV-1 was shown to be antigenically closely related to felid and canid herpesviruses (Osterhaus and others 1985, Lebich and others 1994), whereas PhHV-2 appeared to be more distantly related to both PhHV-1 and herpesviruses of terrestrial carnivores. Recently, nucleotide sequence data have been obtained for PhHV-1 and PhHV-2 which suggest a taxonomic clustering of these viruses within the *Varicellovirus* genus of the α-Herpesvirinae subfamily and within the γ-Herpesvirinae subfamily, respectively (Harder and others 1995).

Infections with PhHV-1 in harbour seals (*Phoca vitulina*) have been associated predominantly with benign upper respiratory disease, but an enzootic outbreak with high mortality among newborn seals has been described in the population at the Seal Rehabilitation and Research Centre (SRRC) in Pieterburen, The Netherlands (Osterhaus and others 1985, Borst and others 1986). During the 1988 mass mortality of seals in north west Europe, caused by phocine distemper virus (PDV), generalised PhHV-1 infections were frequently detected in fatally diseased, PDV-infected adult seals (Osterhaus and Vedder 1988, Frey and others 1989, Zhang and others 1989). Upon experimental inoculation with a tissue culture-propagated PhHV-1 isolate, sub-adult harbour seals seroconverted but, apart from a moderate rise in temperature, no overt clinical signs developed (Horvat and others 1989). No association of PhHV-2 infection with disease in pinniped populations has so far been reported.

This paper describes investigations of the prevalence and clinical signs of PhHV-1 infection among harbour seals of different ages at the SRRC in 1993 and 1994, and compares the results of different diagnostic methods.

Materials and methods

Seals

The SRRC aims to rehabilitate weakened, orphaned or diseased seals which are found along the Dutch coast. The majority of the animals rehabilitated at the SRRC are newborn harbour seals or grey seals (*Halichoerus grypus*), which are admitted during the pupping seasons in June/July and December, respectively. During the present study, an additional group of 22 adult harbour seals, all seropositive for PhHV-1, was kept at the SRRC, for immunotoxicological investigations (De Swart and others 1994). However, the animals of this group were physically separated from the seals housed for rehabilitation. The survey was restricted to 52 harbour seals and one ringed seal (*Pusa hispida*), 34 of which were at the SRRC between June and September 1993 and 19 in the same period of 1994, and from which a complete set of samples (swabs from eyes, nose and throat, a heparin blood sample and paired serum samples) was available. The total numbers of seals at the SRRC at these times were 34 and 36, respectively.

Detection of PhHV infection by virus isolation and serological assays

Virus isolation procedures were applied to swab samples, using primary harbour seal kidney cells (SeKC) as described by Osterhaus and others (1985). Isolates were characterised antigeni-

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TABLE 1: Detection of active PhHV-1 infection in captive harbour seal populations

Season	PhHV-1 isolation	Seroconversion	Disease
June-August 1993	10*/34†	24/34	19/24‡
August-September 1994	14/19	19/19	18/19

* Number of positive animals
† Number of animals examined
‡ Number of animals with serologically confirmed active PhHV-1 infection

cally using a recently established panel of murine monoclonal antibodies (mAbs) in an immune-peroxidase monolayer assay (IPMA) (Lebich and others 1994).

Virus neutralising antibody titres against the PhHV-1 isolate PB84 were measured in paired serum samples as described by Osterhaus and others (1985). A more than four-fold increase of PhHV titre was scored as evidence for active PhHV-1 infection.

All swab samples were also checked for evidence of morbillivirus infections as previously described by Visser and others (1989).

Origin of clinical specimens for the evaluation of a polymerase chain reaction (PCR) assay

Nose, throat or eye swabs were collected from six harbour seals and one ringed seal which were hospitalised in 1993 or 1994, during outbreaks of upper respiratory disease. Retrospectively, these seals were shown to have experienced active PhHV-1 infection on the basis of seroconversion and/or virus isolation. In addition, throat swabs from 10 harbour seals which proved to be negative in virus isolation assays and did not seroconvert to PhHV-1 during the outbreaks were included. As controls, nasal and throat swab samples from six clinically healthy grey seal pups were sampled in 1995 when no signs of PhHV-1-related disease were observed in the seal population at the SRRC. These grey seals did not seroconvert to PhHV-1 during their stay at the SRRC.

The swabs were kept refrigerated in 4 ml of DMEM supplemented with penicillin (300 iu/ml) and streptomycin (300 µg/ml). An aliquot (1·0 ml) was inoculated into SeKC and Vero cell cultures for virus isolation within four days after sampling. The remaining fluid was frozen at –80°C for up to 15 months until used for DNA isolation as described below.

Preparation of DNA for PCR assay

DNA was recovered from diagnostic swab samples by adapting a silica-based technique described by Boom and others (1990). Briefly, 100 µl of a sample were mixed with 1 ml of lysis buffer (120 g guanidine isothiocyanate in 100 ml 0·1M Tris HCl [pH 6·4]), to which 22 ml 0·2M EDTA (pH 8·0), 2·6 g Triton-X 100 for 40 µl acidified Celite silica (Janssen Chimica) were added. After incubation for 10 minutes at room temperature, the silica was pelleted and washed twice in lysis buffer lacking EDTA and Triton-X 100. Additional washing steps in 70 per cent ethanol, and acetone, were carried out, after which the silica pellet was vacuum-dried. DNA was released by adding 100 µl of TE-buffer (10mM Tris HCl pH 8·0, 1mM EDTA) and incubating at 56°C for 10 minutes. For the PCR, aliquots of 20 µl were used.

As a control, PhHV-1 DNA (reference isolate PB84) was prepared from infected Crandell Rees feline kidney (CrFK) cell cultures as described by Harder and others (1995).

Primer selection and polymerase chain amplification

Primers were selected from conserved regions of the gD equivalent genes of felid and canid herpesviruses (Limbach and others 1994, Maeda and others 1994), which were found to be the closest relatives of PhHV-1 at the antigenic level (Lebich and others 1994). The DNA PCR, using primers gD_1 (5′-gAA gTT Cgg TAT gT[A/T] AC-3′) and gD_2 (3′-gA[C/T] gAT gAA TTg ggA TT-5′) was

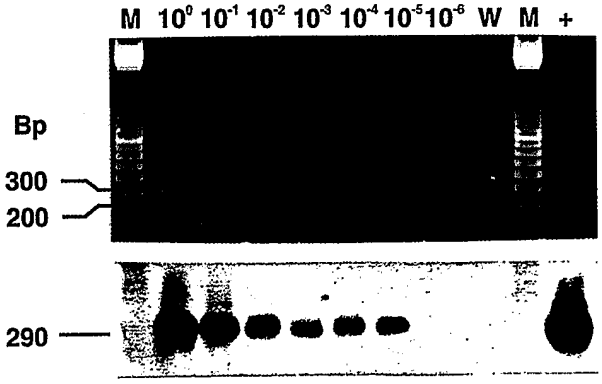

FIG 1: Detection limits of a PCR targeting a 290 bp fragment of the gD gene of PhHV-1 combined with Southern blotting. Template DNA was purified from 20 µl of serial 10-fold dilutions of a supernatant from a CrFK cell culture infected by PhHV-1 PB84. A parallel titration of viral infectivity (bottom line) revealed a titre of $10^{1.9}$ $TCID_{50}$/20 µl of the undiluted stock. W Cell culture medium control, + Corresponding gD fragment of PhHV-1 isolate PB84 amplified in a separate PCR reaction. Non-radioactive Southern blotting was carried out with a probe prepared from the cloned gD fragment of PhHV-1 PB84. [a] Number of virus-positive/total wells in a microtitration assay based on the development of cytopathic changes

essentially as described by Harder and others (1995), and produced specific amplificates of 290 bp from PhHV-1 but not from PhHV-2 templates.

The products were separated on agarose gels and visualised after staining with ethidium bromide. Non-radioactive Southern blotting was used to confirm the specificity of the amplificates (Harder and others 1995). A cloned gD fragment of the PhHV-1 reference isolate was used as a probe in chemoluminiscent assays (ECL; Amersham International).

Measures to minimise the risk of carry-over contaminations consisted of physical separation of pre- and post-PCR procedures, the preparation of templates in a laminar flow cabinet, and the use of master mixes and aerosol-resistant tips. Negative controls (bi-distilled water or tissue culture medium) were routinely run in parallel.

Clinical scoring of PhHV-1-related disease

The serological results obtained from an examination of paired serum samples were used as a 'gold standard' for the diagnosis of active PhHV-1 infection. Seals with a serologically confirmed PhHV-1 infection during the 1993 and 1994 outbreaks were grouped arbitrarily according to the severity of the disease. Group 0 seals showed no clinical signs. Seals of group 1 showed mild clinical signs consisting of anorexia, mucous nasal and/or ocular discharge and rectal temperatures of up to 38·5°C. These seals recovered completely within one week at most. Group 2 animals suffered more severe signs of disease, including mucopurulent nasal and ocular discharge, epistaxis, lower respiratory disease (dyspnoea and coughing) and, in some cases, gastrointestinal symptoms such as vomiting and diarrhoea. Their rectal temperatures frequently exceeded 38·5°C. They recovered completely with palliative antibiotic treatment within two weeks. The seals which succumbed to the infection were classified in group 3.

Results

Virus isolation and serology

Outbreaks of an apparently contagious disease, mainly characterised by upper respiratory signs, were noticed among the harbour seal pups during the summer months of both years. In 1993 an active PhHV-1 infection was confirmed by seroconversion in 24 of 34 seals of all age groups (Table 1). Nineteen of these infected animals developed overt clinical signs. Ten seals were apparently not infected during their stay at the SRRC and did not show clinical

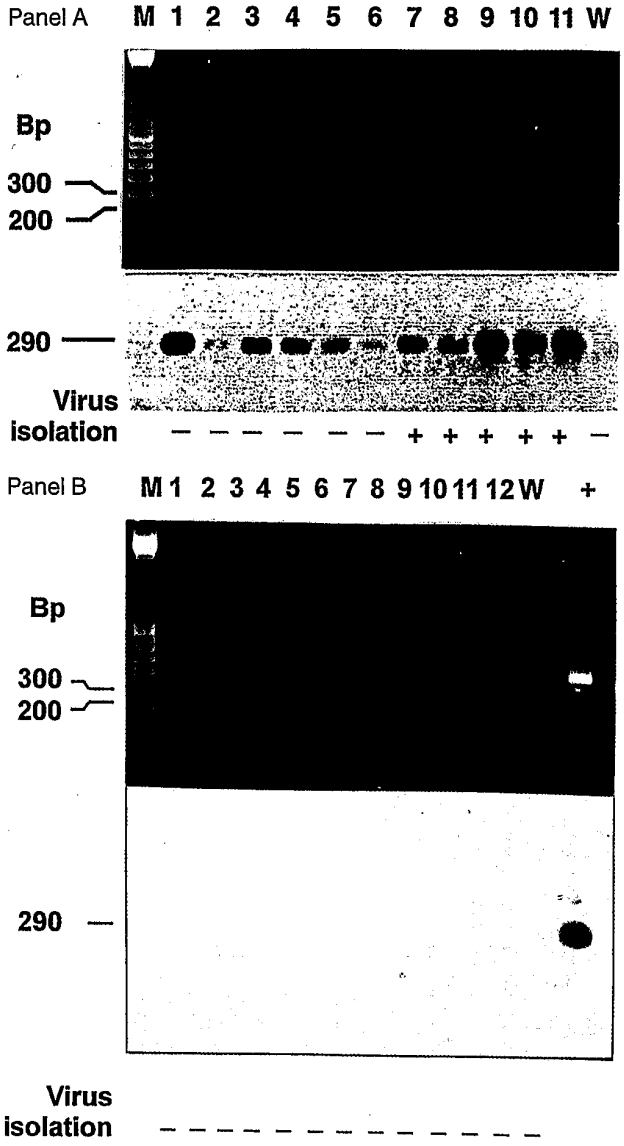

FIG 2: PCR amplification and non-radioactive Southern blot detection of a 290 bp fragment of the gD gene equivalent of PhHV-1 from diagnostic swab samples obtained from harbour seals (*Phoca vitulina*) and a ringed seal (*Pusa hispida*) during outbreaks of respiratory signs in a seal sanctuary in 1993/1994 (panel A). Clinically healthy grey seal pups (*Halichoerus grypus*) were sampled as controls in 1995 when no evidence of PhHV-1-related disease was noticed in the sanctuary's seal population (panel B). DNA was prepared from 100 µl of swab fluid. An equivalent of 20 µl was used for PCR. The results of virus isolation attempts on SeKC cultures using 1 ml of fresh swab fluids is indicated in the bottom line. Before use in PCR, samples of panel A had been stored at –80°C for up to 15 months while other samples were processed within four days after sampling. Panel A: 1,2 – *Pusa hispida* PB94-23 (throat, eye); 3-7 – samples from *Phoca vitulina*: 3 – PB94-22 (nose); 4 – PB94-23 (throat); 5 – PB94/24 (nose); 6 – PB94-40 (nose); 7,8 PB93-Jac. (nose, eye); 9,10,11 – PB93-Cath. (nose, throat, eye); W Swab medium control. Panel B: Swab samples (throat, nose) from *Halichoerus grypus*: 1,2 – PB95-27; 3,4 – PB95-12; 5,6 – PB95-04; 7,8 – PB95-02; 9,10 – PB95-30; 11,12 – PB95-07; W Swab medium control, + DNA sample extracted from CrFK cells infected by PhHV-1 isolate PB84

signs suggestive of the infection. Most of the PhHV-1 isolates were recovered from swab samples. In some animals swabs from the eyes, nose and throat proved virus-isolation positive, but in others only one swab sample was positive, with no clear preference for the location. Leucocyte-associated viraemia could not be detected in any of the animals studied. However, in three newborn seals which succumbed to the disease in 1993, generalised PhHV-1 infection was demonstrated by virus isolation from tissues including lung, kidney, liver and central nervous system.

Within the group of 22 PhHV-1-seropositive adult seals kept separate from other seals, no clinical signs indicative of PhHV-1 infection were observed during the outbreaks. However, in seven adult seals more than four-fold increases in PhHV-1 virus neutralisation titres coincided with PhHV-1-related disease outbreaks in the groups of seal pups.

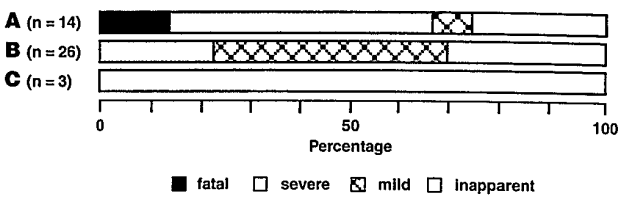

FIG 3: Signs of disease associated with PhHV-1 infection in harbour seals of different age groups. The classification of the signs of disease is described in materials and methods. Three age groups were distinguished: A Pups up to one month, B Juveniles one to 12 months, and C Sub-adults and adults more than 12 months of age

The patterns of reaction of the panel of 32 mAbs with the PhHV-1 isolates obtained in 1993 and 1994 were identical to those obtained with the strains isolated in 1984 (Osterhaus and others 1985) and during the mass mortality of seals in 1988 (Lebich and others 1994).

No morbilliviruses were isolated from the materials used for PhHV-1 isolation.

Diagnosis of PhHV-1 infection by PCR

To determine the sensitivity of the PhHV-1 PCR assay, serial 10-fold dilutions of supernatant from a CrFK cell culture infected with PhHV-1 PB84 were examined. As shown in Fig 1, a positive result was still obtained in the 10^{-5} diluted sample. Titration of the same serial 10-fold dilutions in CrFK cells revealed a titre of $10^{1.9}$ $TCID_{50}$ /20 µl of the undiluted stock. Thus, the detection limit of the PCR assay with Southern blotting (PCR-SB) was $10^{-3.0}$ $TCID_{50}$.

The suitability of this PCR assay for the detection of PhHV-1 DNA in clinical specimens was investigated with swab samples from the eye, nose or throat of six harbour seals and one ringed seal which had all seroconverted to PhHV-1 (Fig 2, panel A). The gD fragment was detected in all the samples from which PhHV-1 had been isolated. Furthermore, six samples which were virus isolation-negative were positive in the PCR-SB. All the seals, except the adult harbour seal PB93-Cath and the subadult ringed seal of this group showed signs of respiratory disease at the time of sampling, indicating that they were asymptomatic virus shedders.

PhHV-1 DNA was not detected by PCR-SB in swabs taken from six clinically healthy grey seal pups sampled as controls when no evidence for a PhHV-1 infection was found (Fig 2, panel B). Swab samples from 10 further clinically healthy harbour seals of the 1993 season, which remained negative in virus isolation assays and did not seroconvert to PhHV-1, also scored negative in the PCR-SB (data not shown).

Correlation of PhHV-1 disease severity with age

In Fig 3 the seals with serologically confirmed active PhHV-1 infection are grouped according to their age and the severity of their clinical signs. Pups up to one month old were distinguished from juveniles aged one to 12 months, and subadults or adults more than 12 months old. The severity of PhHV-1-related disease appeared to be inversely correlated with age. The fatal cases were restricted to three newborn animals.

Discussion

Three different diagnostic methods for the laboratory diagnosis of PhHV-1 infections in pinnipeds have been compared in animals in which the clinical signs were largely dependent on age. During the two pupping seasons studied PhHV-1 was by far the most frequently detected viral agent in this population, which is routinely

vaccinated against PDV (Visser and others 1989). The inverse correlation between PhHV-1-associated disease and age is reminiscent of α-herpesvirus infections of terrestrial carnivores, for example canid herpesvirus (Appel 1987). The results are also in line with earlier studies in which the experimental inoculation of susceptible juvenile harbour seals with PhHV-1 resulted in no apparent infection (Horvat and others 1989). The asymptomatic PhHV-1 infections observed in the adult seals were most probably due to the reactivation of latent virus. During the 1993 outbreak three newborn pups (12·5 per cent of the actively PhHV-1-infected seals) died in the course of infection. This mortality rate is low when compared to the first recognised PhHV-1 enzootic in 1984, when up to 50 per cent of the diseased neonates died (Osterhaus and others 1985). Although it cannot be excluded that PhHV-1 strains with different pathogenic potentials may have been circulating together and that the recent outbreaks may have been caused by milder strains, no significant differences in the antigenic make-up of the isolates obtained in 1984 and in 1993/94 were detected. Since 1984, however, knowledge of and experience with quarantine measures, veterinary care and nursing of seal pups have greatly improved, and these changes may also have contributed to the reduction in losses. They may also explain why, in 1993, 10 of 34 seals did not become infected during their stay at the SRRC.

The origin of the PhHV-1 strains responsible for the outbreaks could not be identified. As in herpesvirus infections of terrestrial carnivores (Appel 1987), pups may be infected directly after birth and thus may have carried the virus when they were admitted to the SRRC. Although experimental evidence for latency of PhHV-1 in seals is lacking, the epidemiological importance of seals which are seropositive to PhHV-1 after the loss of maternal antibodies should not be underestimated, because these animals may be latent virus carriers and shed the virus after reactivation.

The accurate aetiological diagnosis of herpesvirus infections in marine mammals by virus isolation procedures is often hampered by the unsuitability of many diagnostic samples as a result of advanced decay and bacterial contamination. The PhHV-1 gD-specific PCR-SB was approximately 1000 times more sensitive than virus isolation. This factor may be even higher when clinical specimens are analysed after storage at suboptimal conditions, which would be expected to affect herpesviral infectivity more than the integrity of template DNA. Indeed, the PCR-SB was more sensitive than virus isolation procedures in SeKC cultures for the detection of PhHV-1 infection in archived clinical specimens from seals. The limited accessibility of suitable cell cultures is another reason why PCR-SB may be favoured for the rapid diagnosis of active PhHV-1 infection in individual seals. The reported inability to diagnose PhHV-2 infections in this way (Harder and others 1995) is of less clinical relevance, because infection with this virus has so far not been associated with disease in seals. For studies of PhHV-1 infections in seal populations, the testing of paired serum samples for specific antibodies will probably remain the method of choice.

Newborn seals were especially at risk of fatal PhHV-1 infection. Harbour seal pups orphaned before the uptake of colostrum may be particularly threatened because the maternal antibodies conferring temporary protection are mainly transmitted through the colostrum (Ross and others 1994). As a result, passive immunisation with convalescent seal sera can be useful to prevent serious disease during the first weeks of life in a rehabilitation setting. Active immunisation with a non-replicating vaccine could also be considered at an early age, because harbour seals have been shown to be immunocompetent from birth (Ross and others 1994). In the light of their close antigenic relationship with herpesviruses of terrestrial carnivores (Lebich and others 1994), inactivated or subunit vaccines derived from canid or felid herpesviruses might be of value until a homologous PhHV-1 vaccine becomes available.

Acknowledgements. – The study was funded by grants from the Seal Rehabilitation and Research Centre (SRRC), Pieterburen, The Netherlands, and the European Community (Human Capital and Mobility Project ERBCHBGCT920106). The authors gratefully acknowledge the assistance and contributions of all the staff at the centre without which the study would not have been possible.

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Abstract

Trace elements vitamin boluses for calves

CYLINDRICAL boluses 55 mm in length and 18 mm in diameter, suitable for administration to calves weighing more than 75 kg, were made by compressing a mixture of copper oxide powder, sodium selenite, cobalt sulphate, potassium iodide, manganese sulphate, zinc oxide and zinc sulphate, and vitamins A, D, and E, weighing together 30 g. All but one end of the bolus was coated in an inert polymer. When two boluses were administered to cows with a rumen cannula the mean daily rates of release of the elements were 62 mg copper. 0·56 mg selenium, 1·08 mg colbalt, 1·14 mg iodine, 38 mg manganese and 55 mg zinc. When administered to housed dairy calves fed diets containing inadequate levels of copper and selenium, the same dose produced significant increases in plasma copper concentration and in the activity of glutathione peroxidase in comparison with unsupplemented control calves. There were similarly significant increases in suckled beef-cross calves kept at grass for 143 days.

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