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USE OF A SLAM TRANSFECTED VERO CELL LINE TO ISOLATE AND CHARACTERIZE MARINE MAMMAL MORBILLIVIRUSES USING AN EXPERIMENTAL FERRET MODEL

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ABSTRACT: Two ferrets (*Mustela putorius furo*) were experimentally infected with phocine distemper virus (PDV), from the 1988 seal epizootic in Europe, in order to determine whether the stable transfected Vero cell line (Vero.DogSLAMtag) expressing canine "signaling lymphocyte activation molecules" (SLAM; CD150) receptors, was more suitable for isolating and characterizing PDV when compared with Vero (American Type Culture Collection # C1008) and primary seal kidney (PSK) cells. Both ferrets displayed characteristic clinical signs of distemper, including fever and rash, 10 days postinoculation (dpi) and, due to increased morbidity, they were euthanized 12 dpi. Histologic lesions, suggestive of infection with morbilliviruses, were observed in tissues from both ferrets, and the tissues stained positive using immunohistochemistry. Isolation of PDV from isolated peripheral blood lymphocytes (PBLs), taken at 5 and 10 dpi, was achieved by cocultivation with Vero and PSK cells, following several passages. Cytopathic effects (CPE) were observed in Vero cell cultures at 29 dpi and in PSK cell cultures at 22 dpi. Phocine distemper virus was isolated from frozen infected ferret lung tissue within 48 hr, when isolation was attempted using the Vero.DogSLAMtag cell line. In addition, a reverse transcriptase polymerase chain reaction (RT-PCR) test was developed to detect a 114 base pair (bp) portion of the nucleocapsid gene found only in PDV. This RT-PCR methodology was used to confirm the identity of the virus subsequently isolated from the ferrets. Viral isolates from the infected ferrets, as well as cultures of virus originally isolated from a dolphin and a porpoise and maintained in Vero cells, also replicated faster and produced higher titers of virus when propagated in Vero.DogSLAMtag cells. These results indicate that Vero.DogSLAMtag cells offer a substantial improvement (including faster viral replication resulting in primary viral isolation in a shorter period of time, and higher yield of virus finally obtained) over traditional cell culture methodologies for isolation and characterization of marine mammal morbilliviruses.

Key words: Ferret, marine mammal, morbillivirus, PDV, phocine distemper, RT-PCR, SLAM, virus isolation.

INTRODUCTION

Morbilliviruses infecting marine mammals include canine distemper virus (CDV), phocine distemper virus (PDV), and cetacean morbillivirus (CeMV); this latter group includes both dolphin morbillivirus (DMV) and porpoise morbillivirus (PMV; Rima et al., 2005). All of these genetically related species belong to the family *Paramyxoviridae*, members of which are negative-sense, single-stranded RNA viruses. All are highly contagious and extremely pathogenic in their hosts. Although considered a major threat to marine mammals worldwide, little work has been done to identify and study the morbilliviruses circulating in Canada, in spite of its large and diverse marine mammal populations. Morbilliviruses are known to occur in both phocid and cetacean species in North American waters, and epizootics involving bottlenose dolphins in the Gulf of Mexico and off the US Atlantic coast have been reported (Taubenberger et al., 1996). Morbilliviruses are known to be circulating in pinniped populations in the Canadian Arctic and

the Northwest Atlantic (Henderson et al., 1992; Ross et al., 1992; Duignan et al., 1994, 1995, 1997; Nielsen et al., 2000; Philippa et al., 2004); however, only one harp seal (*Phoca groenlandica*) was reported to have died, due to PDV, in Canadian waters (Prince Edward Island; Daoust et al., 1993). In US waters (Long Island, New York), two harbor seals (*Phoca vitulina*) died due to PDV infection (Duignan et al., 1993). Our understanding of marine mammal morbillivirus epizootiology in North America has been severely limited by inadequate methods by which to obtain viral isolates from infected animals. Most of what is known about morbillivirus infections in marine mammals from North America has come from serologic and reverse transcriptase polymerase chain reaction (RT-PCR) analyses (Taubenberger et al., 1996; Nielsen et al., 2000). Morbilliviruses, especially the wild types, are notoriously difficult to isolate and propagate in cell culture systems, and usually require the cultivation of fresh or frozen infected material in macrophages from susceptible species (Appel and Jones, 1967). Diagnosis of morbillivirus infections has traditionally relied on results obtained from a number of methodologies including serology, histopathology, and less frequently, viral isolation (Appel 1987). Recently, RT-PCR methodology has been used to diagnose and differentiate species of morbilliviruses (Saliki et al., 2002), but isolation of virus is still considered the "gold standard" for a definitive diagnosis. Isolation of the virus responsible for the European seal epizootic in 1988 was delayed because no satisfactory cell line isolation techniques were available (Osterhaus and Vedder, 1988), and PCR technology had not yet been developed. Phocine distemper virus was first isolated by experimentally infecting dogs (Osterhaus et al., 1988), and this result was confirmed by culturing kidney cells from an infected seal (Kennedy et al., 1988). Isolation of the morbillivirus responsible for the mass mortality of Mediterranean monk seals (*Monachus monachus*) in 1997 was accomplished using Vero cells and standard cell culture isolation methods, but first required several "blind passages" (Osterhaus et al., 1997). Although there are now commercial polyclonal and monoclonal antibodies available that can differentiate between CDV and PDV immunohistochemically (Stanton et al., 2004), there is a very limited number of molecular-based methods of species differentiation. Restriction fragment length polymorphism, combined with conventional RT-PCR analysis, can identify species of morbilliviruses (Saliki et al., 2002); however, since some genes are not conserved, and extra manipulations of RT-PCR products are also required, this limits the usefulness of this approach. By using RT-PCR to specifically target an area of the nucleocapsid gene of PDV, it should be possible to distinguish PDV from CDV, PMV, and DMV.

The purpose of this study was to test three cell lines (Vero, primary seal kidney, and Vero.DogSLAMtag), using samples taken from PDV experimentally infected ferrets (*Mustela putorius furo*), in order to develop a cell culture and isolation methodology useful in the study of marine mammal morbilliviruses. Additionally, fresh tissues from experimentally infected ferrets were used to develop and test RT-PCR methodologies in order to follow the clinical course of infection in experimentally infected ferrets. These methods could then be useful in the diagnosis of distemper infections in stranded marine mammals.

MATERIALS AND METHODS

Ferrets

Two 8-week-old unvaccinated, neutered, male, domestic ferrets (*Mustela putorius furo*) were purchased from Marshall Farms (North Rose, New York, USA) and were housed in separate cages in a biocontainment level-2 certified facility.

Viruses

The Lederle strain of CDV, propagated in chicken embryos (VR-128), was purchased from the American Type Culture Collection (ATCC, Manassas, Virginia, USA) and propagated in Vero cells. Phocine distemper virus for the ferret inoculation was obtained from frozen (−80 C) seal tissues from the 1988 PDV epizootic in Europe (Visser et al., 1989) and consisted of a 0.5% (v/v) suspension of a pool of spleen, lung, and intestinal lymph nodes taken from three dead harbor seals (titer 4.8×10^4 plaque-forming units [pfu]/ml in Vero.DogSLAMtag cells). Dolphin morbillivirus and PMV were obtained from Padraig Duignan, Massey University, Palmerston North, New Zealand, and were originally isolated from striped dolphins (*Stenella coeruleoalba*) from the Mediterranean Sea and harbor porpoises (*Phocoena phocoena*) from the North Sea off the coast of Ireland (Barrett et al., 1993), and subsequently propagated in Vero cells.

Experimental infection

After a short period of acclimation, induction of anesthesia of both ferrets at 12 wk of age was carried out in a chamber containing 3% isoflurane delivered with medical grade oxygen at 2 l/min. Once anesthetized, they were maintained, by mask, with 1.5% isoflurane delivered with oxygen at 1 l/min. Baseline samples of blood from the saphenous vein, and swabs from the nose, mouth, penis, and anus, were taken and served as negative controls. Each ferret was inoculated with 0.4 ml of the PDV-containing tissue homogenate via the intranasal route, while under anesthesia, to prevent loss of the inoculum by sneezing. Every five days postinoculation (dpi), the ferrets were anesthetized and sampling was repeated.

Monitoring clinical course of distemper infection

After inoculation, the animals were monitored daily. The following observations were recorded: rectal temperature, respiration rate, heart rate, amount of food and water consumed, and activity level. The presence or absence of any of the following clinical signs of distemper was also noted: conjunctivitis; central nervous system signs (seizures); a chin rash typical of distemper; and generalized erythema. Both animals were weighed pre-inoculation, at 5 dpi and then daily, until euthanasia.

Sampling and determination of end point

Once ferrets were observed to be ill, by display of clinical signs of distemper and with increasing morbidity as assessed by a consulting veterinarian, the ferrets were anesthetized and sampled as indicated above, then euthanized by an intravenous overdose of sodium pentobarbital (100 mg/kg). Organs and tissues were then sampled at necropsy for histopathology, immunohistochemistry, RT-PCR analyses, and virus isolation.

Isolation of PDV

In vitro isolation of PDV was conducted using three cell lines. African green monkey kidney cells (Vero C1008), purchased from the American Type Culture Collection (ATCC) (CRL 1586), were propagated using standard cell culture methodologies. Primary seal kidney cells (PSK), obtained from a presumed healthy weaned 2-wk-old harp seal collected from the Gulf of St. Lawrence in March 2004 with a Fisheries and Oceans Canada scientific permit, were propagated using standard primary cell culture methodology (Chan and Hsuing, 1994). Vero.DogSLAMtag cell line, a stable transfected continuous cell line containing the morbillivirus cell receptor (CD150), as described by Seki et al. (2003), was donated by Yasuke Yanagi, Department of Virology, Kyushu University, Fukuoka, Japan. To ensure that all the Vero.DogSLAMtag cells were still transfected, the cells were grown in media containing 0.5 mg/ml of the antibiotic G418 (Sigma-Aldrich Canada Ltd., Mississauga, Ontario, Canada), prior to use as previously described (Seki et al., 2003).

Blood from the experimentally infected ferrets was collected in heparinized tubes, and peripheral blood lymphocytes (PBLs) were separated by differential centrifugation on a layer of lymphocyte separation medium (Mediatech, Herndon, Virginia, USA). The PBLs were washed twice in Minimal Essential Medium (MEM; HyClone Inc., Logan, Utah, USA) containing antibiotics (penicillin 200 International Units/ml and streptomycin 200 μg/ml), and the pelleted cells were resuspended in 1.0 ml of MEM plus antibiotics. Then, 0.5 ml of the cell suspension was inoculated onto drained, 80% confluent cultures of Vero and PSK cells grown in 25 cm^2 tissue culture flasks (Corning Inc., Corning, New York, USA). Virus was allowed to adsorb for 1 hr at 37 C before 10 mls of fresh medium, containing 2% fetal calf serum (FCS;HyClone), was added. Uninoculated cells and PBLs from the ferrets harvested before inoculation with PDV seeded into separate tissue culture flasks, served as negative controls. Flasks containing the cocultured cells were incubated at 37 C, and the adherent

cells were subcultured at a ratio of 1:2 every week, with replenishment with fresh media for at least 6 wk or until signs of the cytopathic effect (CPE) consistent with morbillivirus infection (rounding up of cells, giant cell formation and syncytia formation), was detected.

Samples of lung from the experimentally infected ferrets, obtained at necropsy, were homogenized to give a 10% w/v cell free suspension in MEM containing antibiotics as indicated above and gentamycin (50 μg/ml) (BioWhittaker Inc., Walkersville, Maryland, USA). An aliquot of 0.5 ml served as inoculum for each 25 cm^2 tissue culture flask containing Vero and PSK cells. The remaining infected ferret lung homogenate was stored in sterile cryovials and held at −80 C. When Vero.DogSLAMtag cells became available, lung homogenate was thawed and again 0.5 ml aliquots were inoculated (undiluted) onto 25 cm^2 tissue culture flasks (in triplicate) of Vero.DogSLAMtag, PSK, and Vero cells. These lung-inoculated flasks were treated in the same way as the PBL inoculated flasks.

Plaque assay

The PSK, Vero, and Vero.DogSLAMtag cells were compared with respect to the time it took for countable plaques to form, and viral titers were determined using four species of morbilliviruses. The inoculum used to infect the ferrets was also assayed by this method. All cell lines were propagated in MEM containing 10% FCS and antibiotics. Cell-adapted strains of CDV, PDV, DMV, and PMV, that had been passaged at least five times in Vero cells, were assayed using a plaque assay method, as described by Wolf and Quimby (1973). Briefly, serial tenfold dilutions of each virus were prepared in MEM containing antibiotics. Aliquots containing 100 μl were placed centrally on duplicate cell monolayers grown in tissue culture dishes (60×15 mm) (Corning Inc., Corning, New York, USA). Virus was allowed to adsorb for 60 min at 37 C before the addition of agarose gel maintenance medium. Plates were then incubated at 37 C and observed daily for plaque development. When visible plaques were observed, plates were fixed with formaldehyde, the gels removed, cell sheets were stained with crystal violet staining solution, and the plaques were counted.

Collection and handling of samples for RT-PCR

Swabs were collected in 0.01 M phosphate buffered saline (PBS) containing double strength antibiotics, and 100 μl samples of whole blood from the ferrets were immediately transferred into tubes containing TriPure (Roche Diagnostics, Laval, Quebec, Canada), according to the manufacturer's directions, and held frozen at −80 C until analysis. Supernatant fluid (100 μl samples) from Vero flasks infected with CDV, DMV, and PMV, that showed extensive CPE, were also transferred to tubes containing TriPure and held frozen at −80 C. A mock-infected flask containing only Vero cells was used as the negative control. Ferret PBL-infected flasks of Vero and PSK cells, incubated for at least 4 wk, were handled in the same way as the CDV, DMV, and PMV-infected flasks. The PDV passaged five times in Vero cells with a titer of 2.7×10^5 pfu/ml, determined by plaque assay in Vero cells, served as the positive control for all subsequent RT-PCR experiments. Tissue samples from the experimentally infected ferrets were homogenized using a bag system homogenizer (Interscience, Topac, Hingham, Massachusetts, USA), whereby each tissue was added to 0.01 M PBS containing penicillin/streptomycin (1 ml/0.1 g of tissue). After 3 min of homogenizing, the samples were clarified by low speed centrifugation at 2060 × G (700 rcf) for 10 min. The following tissue samples were used: spleen, stomach, pancreas, adrenal gland, small intestine, large intestine, lung, eye, liver, footpad, urinary bladder, tracheal and bronchial lymph nodes, spinal cord, conjunctiva, heart, thymus, trachea, and kidney.

RNA extraction

Samples (100 μl), prepared as described above, were combined with 900 μl of TriPure, and the RNA was extracted as per manufacturer's instructions. The RNA pellet from each tube was then resuspended in 20 μl of RNASecure reagent (Ambion, Austin, Texas, USA) and stored at −70 C until further analysis.

RT-PCR assay

The primer set designated N-FOR/N-REV: N-FOR (5′-TCCCATCACCATGAAGTC-3′, position 126–143); N-REV (5′-TGACTCGTC CCATTCAGA-3′, position 223–240), was designed at the National Center for Foreign Animal Disease, Winnipeg, Manitoba, Canada. The RNA was transcribed and amplified using a One-Step RT-PCR kit (Qiagen, Mississauga, Ontario, Canada), with an initial 30-min incubation at 50 C for reverse transcription, followed by PCR cycle; 15 min incubation at 95 C; followed by 40 cycles with denaturation at 95 C for 30 sec; annealing at 58 C for 1 min;

and an elongation step at 72 C for 2 min. A final elongation step of 10 min at 72 C was then performed. The expected product was 114 bp.

A previously tested primer set (Jensen et al., 2002) designated P1/P2, targeting the phosphoprotein gene of morbilliviruses, was also used: P1 sequence (5′-ATGTTTATGATCA CAGCGGT-3′, position 851–870); P2 sequence (5′-ATTGGGTTGCACCACTTGTC 3′, position 1261–1280). The reaction components and conditions of using this primer set were the same as those for the N-FOR/N-REV RT-PCR; however, the annealing temperature in the RT-PCR procedure was 48 C. In addition, RT-PCR, using generic diagnostic beta-actin gene primers and probe, was used to confirm the integrity of the RNA extracted for use in conventional PCR. Specifically, the sequences were as follows: Beta actin 831 Forward (BTCCTTCCTGGGCATGGA); Beta actin 1036 Reverse (GRGGSGCGATGA TCTTGAT); Beta actin Internal Probe 880-908 (TCCATCATGAAGTGYGACGTSGACA TCCG)—5′TET labeled and 3′ BHQ-1 labeled. The resulting product was 205 bp in length. The samples were run on the Smart-Cycler II (Cepheid, Roche Molecular Systems, Inc., USA). The beta-actin RNA was amplified using the Qiagen Quantitect Probe Real-time PCR kit (Qiagen, Mississauga, Ontario, Canada), under the following amplification conditions: 50 C for 30 min; 95 C for 15 min; 45 cycles of 95 C for 10 sec; followed by 60 C for 1 min. Amplification was monitored and analyzed using SmartCycler II software (Cepheid, Sunnydale, California, USA). Five positive RT-PCR products were sequenced using the N-FOR/N-REV primer set and were confirmed to contain amplified PDV sequence, thus confirming the specificity of the RT-PCR reaction for phocine distemper virus (data not shown).

Electrophoresis of RT-PCR products

All RT-PCR samples, derived from conventional PCR, were analyzed by 1% agarose gel electrophoresis (stained with 1 μg/ml of ethidium bromide) using 10 μl samples per lane and a running condition of 100 volts for 2 hr. A 100 bp standard (BioRad, Mississauga, Ontario, Canada) was used to track the mobility of RT-PCR products.

Evaluation of the sensitivity of the N-FOR/N-REV primer set in standard RT-PCR

The sensitivity of the N-FOR/N-REV primer set was done using 10-fold dilutions (from 13,500 50% tissue culture infectious doses [$TCID_{50}$] to 135 $TCID_{50}$) of Vero cell passaged CDV, PDV, PMV, or DMV stock virus.

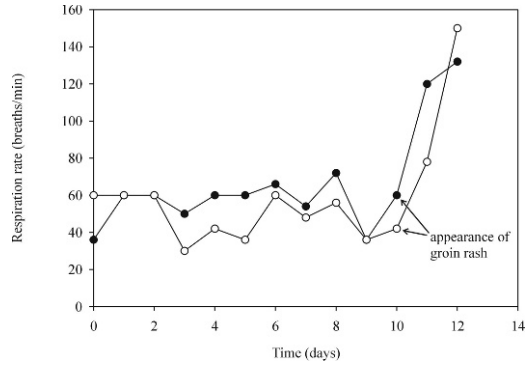

FIGURE 1. Daily respiration rates of two ferrets experimentally infected with PDV.

Histopathology and immunohistochemistry

Organ samples were fixed in 10% neutral buffered formalin for 72 hr, before being embedded in paraffin using standard histologic methodology. Sections were cut at 5–6 μm and stained with hematoxylin, phloxine, and saffron for histopathologic analyses. Using an immunoperoxidase test for PDV, additional sections from the paraffin blocks were stained with a 1:10 and 1:20 dilution of PDV 1.3 monoclonal antibody obtained from Seamus Kennedy, Department of Agriculture for Northern Ireland, Belfast, Ireland, using methodology published by Kennedy et al. (1991). Brain tissue from a dog with distemper served as a positive control. Staining was positive, but weak, likely due to inadequate freezing of the monoclonal antibody during shipment; therefore, additional sections were stained with polyclonal antisera (developed against measles nucleoprotein at the University of Saskatchewan, Western College of Veterinary Medicine).

RESULTS

There were no signs of conjunctivitis or seizure, but a red rash in the groin and near the anus was noted in both animals 10 dpi. On 11 dpi, the ferrets became lethargic, developed fever (maximum rectal temperature for both animals on day 11 was 40 C), and their respiration rates increased dramatically (Fig. 1). Heart rates for both animals were within the

TABLE 1. Results of RT-PCR analyses of samples from PDV experimentally infected ferrets.

Type of infected sample	Primers analyzed	
	Morbillivirus P1/P2 detects P protein (429 bp amplicon)	PDV N protein specific primers (N-FOR/N-REV) (114 bp amplicon)
Vero cell pellets and supernatants	9/12 positive (11/12)[a]	10/12 positive (11/12)
PSK cell pellets and supernatants	11/11 positive (11/11)	11/11 positive (11/11)
Tissues	19/37 positive (33/37)	17/37 positive (33/37)
Swabs	29/40 positive (39/40)	28/40 positive (39/40)

[a] The ratios in brackets reflect the agreement between the two sets of primers.

normal limits throughout the experiment, appetite appeared to decrease starting at 10 dpi, noticeable weight loss was evident by 11 dpi, and activity level was noted as being low starting at 12 dpi. These signs worsened throughout the day, and noticeable facial swelling was also apparent in both ferrets. The ferrets were euthanized 12 dpi.

Phocine distemper virus was isolated from ferret PBLs, collected from both ferrets from the first bleeding 5 dpi, and cocultured with the Vero cell line. Virus isolation was also successful using the PSK cell line cocultured with PBL cells collected on the second bleeding 10 dpi. No virus was isolated from either Vero or PSK cells cocultured with PBLs collected prior to infection (0 dpi). Cytopathic effects consistent with PDV infection, including syncytia formation and rounding-up of cells, was first observed in PSK cells at 22 days and in Vero cells at 29 days post coculture. PDV was subsequently isolated from both ferrets, using PBLs collected from ferrets at 10 and 12 dpi, cocultured in both Vero and PSK cell lines. No cytotoxicity or CPE was noted in any of the cell control flasks, nor in flasks inoculated with PBLs prior to infection with PDV. Viral identity was confirmed by RT-PCR in flasks showing CPE (Table 1). PDV was also recovered in PSK and Vero cells from fresh ferret lung homogenates 4 wk post-incubation. Extensive CPE was evident in the frozen stored ferret lung infected Vero.DogSLAMtag cells, after only 48 hr of incubation; but no CPE was observed in Vero or PSK cells, even after 40 dpi.

RT-PCR testing of swabs indicated that PDV nucleic acid was present in secretions from the eye, mouth, penis, nose, and anus of both ferrets as early as 5 dpi. The RT-PCR compared two sets of primers, using cell culture (pellet and supernatant) samples in comparison with blood, swab, and ferret tissue isolates. Using either the PDV nucleocapsid specific primer set (designated N-FOR/N-REV) or the morbillivirus general phosphoprotein primer set (designated P1/P2), 93% agreement was obtained, with the P1/P2 set being more sensitive. The most accurate results were obtained for the swab samples, where 39/40 RT-PCR results were obtained using either primer set. The four most closely related species of morbilliviruses (DMV, PDV, CDV, and PMV; Jensen, et al., 2002) were titrated using Vero cells. Dilutions of 10^{-1} to 10^{-8} for each virus were used to calculate a $TCID_{50}$ value at 14 dpi. Three logarithmic units of $TCID_{50}$ value were used as the sensitivity of detection for these four viruses. As shown in Figure 2, an initial RT-PCR amplification, using a $TCID_{50}$ 1,000 of each virus, indicated that a substantial amount of RT-PCR product (as highlighted in a 1% agarose gel) could be detected when using the general morbillivirus phosphoprotein primers (Fig. 2, lanes 7–11). However, when RT-PCR was conducted using the nucleocapsid specific PDV primer set, only PDV was detected (Fig. 2, lanes 2–6), outlining the

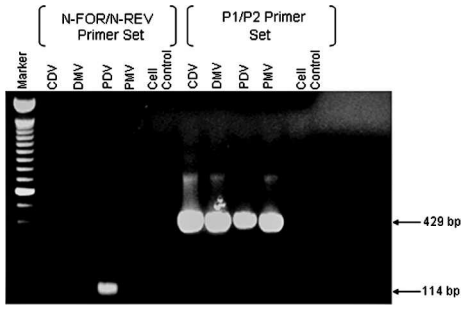

FIGURE 2. Specificity of N-FOR/N-REV primers. Standard RT-PCR: Lane 1, 100 bp marker; Lanes 2–5, N-FOR/REV primer set: CDV, DMV, PDV, PMV ($TCID_{50}$ 1000 stock), respectively; Lane 6, cells only supernatant: Lanes 7–10, P1/P2 primer RT-PCR: CDV, DMV, PDV, PMV($TCID_{50}$ 1000 stock), respectively; Lane 11, cells only supernatant.

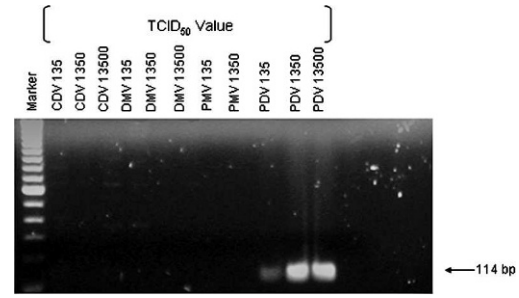

FIGURE 3. Titration of morbilliviruses. Standard RT-PCR using N-FOR/N-REV primers: Lane 1, 100 bp marker; Lanes 2–4, CDV virus at $TCID_{50}$ values of 135, 1,350, and 13,500, respectively; Lanes 5–7, DMV virus at $TCID_{50}$ values of 135, 1,350, and 13,500, respectively; Lanes 8–9, PMV at $TCID_{50}$ values of 135 and 1,350, respectively; Lanes 10–12, PDV at $TCID_{50}$ values of 135, 1,350, and 13,500, respectively.

specificity of the N-FOR/N-REV primer set. It should be noted that the annealing temperature of the N-specific PCR reactions was optimized to reach the high level of specificity shown in Figure 2 for the N-FOR/N-REV primer set. The level of sensitivity of the N-FOR/N-REV primer set, however, seems to be lower than for the P1/P2 primer set (Fig. 2, comparing the intensities of lanes 4 and 9), possibly due to the higher annealing temperature used in the reactions involving the N-FOR/N-REV primer set.

An additional RT-PCR analysis was performed to determine the level of sensitivity of the N-FOR/N-REV primers in relation to the starting $TCID_{50}$ of stock viruses (Fig. 3); using 10–fold serial dilutions of PDV stock virus suggested that the limit of detection by agarose gel analysis was 135 $TCID_{50}$ (lane 10). Again, no level of detection of product was obtained upon titrating PMV, DMV, and CDV (lanes 2–9) using the N-FOR/N-REV primer set, which again could be attributed to the stringency imposed by the primer annealing temperatures for the two sets of primers used. It should be noted that due to the low stock $TCID_{50}$ of PMV, only a $TCID_{50}$ of 135 and 1,350 were used in this analysis.

The RT-PCR performed, using generic designed beta-actin primers, gave unreliable results, probably due to the nature of the samples analysed (data not shown). However, all of the RNA extractions and RT-PCR reactions were done in duplicates or triplicates to confirm the integrity of the RNA. The results from these replicate samples provided confidence that the integrity of the RNA used in these experiments was good.

Histologic lesions, highly suggestive of infection with morbilliviruses, were observed in tissues from both ferrets 12 dpi. The most severe lesions were observed in the lungs, where multifocal to coalescing interstitial pneumonia with marked type II pneumocyte hyperplasia (Fig. 4A), and a diffuse suppurative bronchiolitis, were noted. A marked and diffuse lymphoid depletion was also observed in the lymph nodes, thymus, and spleen of both ferrets. Other lesions were of low magnitude and included a mild ulcerative suppurative conjunctivitis (both ferrets), a mild multifocal neuronal degeneration with mild focal cerebral gliosis (both ferrets), and a mild multifocal suppurative tracheitis (in one of the two ferrets). Acidophilic intracytoplasmic inclusions were easily observed in the epithelial lining of the bronchioles, the urothelial cells of the

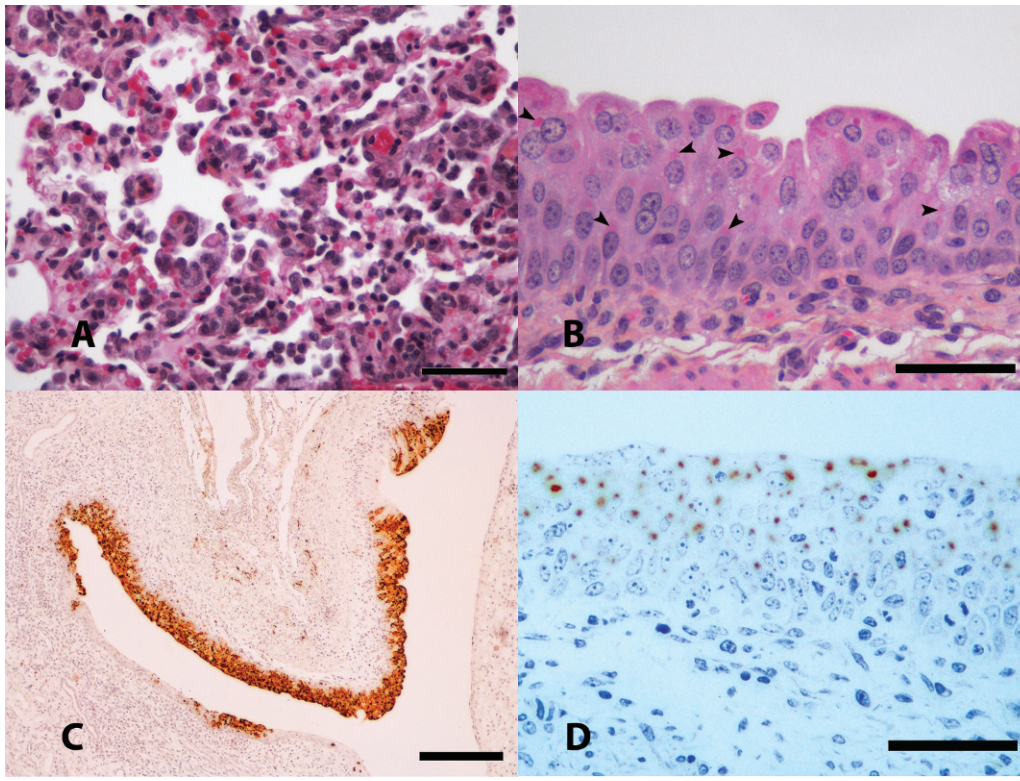

FIGURE 4. (A) Lung of a ferret 12 dpi with PDV. Interstitial pneumonia, characterized by a diffuse thickening of the alveolar walls associated with type II pneumocytes hyperplasia and inflammatory cells infiltrates. Macrophages are often present in the alveolar lumens. HE. Bar=50 μm. (B) Urinary bladder of a ferret 12 dpi with PDV. Numerous acidophilic inclusion bodies (arrowhead) are present in the cytoplasm of the urothelial epithelium. HE. Bar=50 μm. (C) Renal pelvis of a ferret 12 dpi with PDV with intense staining in the cytoplasm of the urothelial cells using morbillivirus polyclonal antisera. Bar=100 μm. (D) Renal pelvis of a ferret 12 dpi with PDV with mild staining in the cytoplasm of the urothelial cells, with PDV monoclonal antisera. Bar=50 μm. Avidin-biotin-peroxidase complex method with hematoxylin counterstain.

renal pelvis and urinary bladder (Fig. 4B), the bile ducts, and the thymus. Immunohistochemical staining of fixed tissues indicated a fulminant infection, in both ferrets, with quite extensive morbillivirus-specific antigen staining in many tissues, primarily epithelium. Morbillivirus antigen was expressed extensively in airway and alveolar epithelium as well as alveolar macrophages; in basal epithelium of hair follicles of the eyelid; in focal areas of the epithelium of the footpad; in epithelium throughout the stomach and all strata of the villi (extensively in the colon and focally in the duodenum and jejunum); and in epithelium of the urinary bladder, pancreatic ducts, kidney, bile ducts in the liver and renal pelvis (Fig. 4C, D). Sampled tissues from all major organs in both ferrets also tested positive for PDV using RT-PCR (Table 1), supporting histologic findings.

When other cell culture propagated species of morbilliviruses were titered in SLAM cells, there was a considerable reduction in time before countable plaques were visible, and a substantial increase in the final titer was noted, compared to that done using Vero and PSK cells (Table 2).

DISCUSSION

This study reports the first experimental infection of PDV in ferrets. Both animals

TABLE 2. Comparison of plaque titration values (plaque forming units/ml) of four species of tissue culture-adapted morbilliviruses assayed in Vero, primary seal kidney cells (PSK), and Vero.DogSLAMtag cells.

Virus[a]	PSK cells (incubation time)	Vero cells (incubation time)	Vero.DogSLAMtag cells (incubation time)
CDV	No plaques visible after 14 days.	1.2×10^2 (6 days)	5.3×10^3 (3 days)
PDV	3.2×10^2 (14 days)	1.1×10^4 (11 days)	3.6×10^4 (6 days)
DMV	1.7×10^3 (9 days)	5.8×10^4 (9 days)	1.1×10^5 (4 days)
PMV	2.7×10^2 (9 days)	4.5×10^4 (9 days)	6.8×10^4 (4 days)

[a] All strains have been passaged at least five times in Vero cells.

used in this study became ill with clinical signs of distemper 10 dpi, using virus recovered from seals during the 1988 seal epizootic in Europe. The clinical signs seen in the ferrets in the present study were similar to those reported in harbor seals experimentally infected with PDV (Visser et al., 1989), including respiratory distress, weight loss, and elevated body temperature. In that study, infected seals died on 14 and 18 dpi, which suggested that PDV infection in seals and ferrets probably follows a similar clinical course. Respiration rate was identified as the clinical sign most indicative of the onset of morbidity in ferrets (Fig. 1), which positively correlated with the extensive interstitial pneumonia and bronchiolitis seen upon histologic examination. Results from this study, albeit using only two animals, suggest that ferrets may offer an alternative method of PDV isolation and may also provide a more convenient animal model (as opposed to dogs or seals) for live-animal studies.

The lesions observed in the infected ferrets are similar to lesions described in ferrets naturally and experimentally infected with CDV (Fox et al., 1998) and with seals naturally infected with PDV (Kennedy et al., 1989). The most prominent histologic changes were seen in the lung and lymphoid tissues. Suppurative infiltrates observed in the trachea, bronchioles, and conjunctiva were suggestive of secondary bacterial infection. The fact that the animals were euthanized only 12 dpi probably accounts for the absence of overt encephalitis and the relatively low number of inclusion bodies observed.

A significant finding was that PDV replication in ferrets and Vero.DogSLAMtag cells progressed rapidly (days), whereas virus replication in PSK and Vero cells took much longer (weeks; Table 2). Lymphoid tissues are the major sites of morbillivirus replication, and CD150 or "signaling lymphocyte activation molecules" (SLAM) have been identified as the cellular receptor for all morbilliviruses (Tatsuo et al., 2001). The Vero.DogSLAMtag cell line used in this study was stably transfected and expresses the canine SLAM molecule (Seki et al., 2003). Presumably, ferret PBLs and the Vero.DogSLAMtag cells both possess similar cell receptors that allow attachment of PDV and subsequent rapid replication of the virus. Since Vero and PSK cells do not have SLAM receptors, PDV is likely infecting these cells by utilizing less-efficient cell receptors present on those cells. This may also account for the observation that PDV was detected, by RT-PCR, in tissues of nonlymphoid origin (brain and gastrointestinal tract) and in the gastrointestinal tract, using immunohistochemistry on infected ferret tissues. However, a further understanding of the distribution of SLAM receptors in ferrets,

as well as the role that other virus receptors may have in the infection of nonlymphoid cells, is needed before these hypotheses can be proven.

PDV was identified from both ferrets using three techniques; namely, virus isolation in tissue culture, RT-PCR, and immunohistochemistry. While RT-PCR was able to detect PDV nucleic acid from swab samples taken from ferrets in as little as 5 dpi, viral isolation was also possible from blood samples (PBLs) collected at the same time. Viral isolation took considerably longer to accomplish (29 days using Vero cells and 22 days using PSK cells, versus 2 days by RT-PCR detection of nucleic acid). However, when Vero.DogSLAMtag cells were used for virus isolation, analysis times were similar. Virus isolation was possible with frozen lung homogenates using Vero.DogSLAMtag cells, but isolation was only possible in Vero cells from fresh material. One possible explanation for this result is that freezing of the lung homogenate may have reduced the titer of virus below the threshold of detection in Vero cells. Both viral isolation and RT-PCR have advantages and disadvantages for the detection of distemper infection in wildlife. Viral isolation is only possible from samples taken from moribund or freshly dead animals, while RT-PCR can be performed successfully on samples that have been degraded or compromised (Forsyth and Barrett, 1995). Viral isolation remains the "gold standard" for identification of distemper infection, and in vitro preparations of these isolates allow investigators to confirm results further by using both molecular and serologic technologies. With the identification of the CD 150 SLAM as the universal cell receptor molecule for morbillivirus attachment, the use of stably transfected cells containing these receptors can be useful in studies where viral isolation is used as a diagnostic tool on clinical samples. These cell lines require no special media or requirements above those found in most tissue culture laboratories. We have also shown that Vero cell-adapted isolates of CDV, PDV, DMV, and PMV are able to replicate faster, and to a higher final titer, in Vero.DogSLAMtag cells than in Vero cells, indicating that these isolates are able to recognise and bind SLAM cell receptors preferentially (see Table 2). Use of SLAM rather than Vero cells, in serologic surveys of distemper in marine mammals, has also reduced analysis times of the plaque neutralization technique, thereby increasing efficiency and decreasing cost (Duignan et al., 1997). A possible explanation for the lower titers obtained when using Vero and PSK cells may be that morbilliviruses produced limited fusion in these cell types, and as a consequence, the final titers may be lower. Use of a vital stain, coupled with immunofluorescence, would be needed to confirm the actual titers in each cell line, but this is outside the scope of the present study. Although the SLAM cell line used in this study only possesses the cell receptor specific for canines, data reported here suggests that there is cross-reactivity between mustelid, canine, phocine, and cetacean SLAM receptors that allows efficient attachment of marine mammal morbilliviruses from a broad host range of animals.

The RT-PCR results in this study have shown that there are two sets of primers (one specific for the phosphoprotein gene and the other for the nucleocapsid gene) that can be used accurately in a clinical setting when testing swab, tissue, and blood samples. The nucleocapsid-specific primer set has also been shown to be specific only to PDV virus (see Fig. 2), demonstrating a quick and accurate methodology for the detection of morbilliviruses and for differentiating PDV from other related morbilliviruses.

Results from this study indicate that it is possible to isolate and quickly identify morbilliviruses from stranded marine mammals. These advances in cell culture and RT-PCR methodologies will aid morbillivirus researchers in identifying new

strains in terrestrial and marine hosts, as well as in providing a reliable cell culture system for viral propagation.

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