

Characterization of morbilliviruses isolated from dolphins and porpoises in Europe

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A previously unidentified morbillivirus was isolated from two harbour porpoises (*Phocoena phocoena*) that had died in the Dutch Waddensea (North Sea) in 1990. This porpoise morbillivirus (PMV) and a dolphin morbillivirus (DMV), which had recently caused a heavy mortality in Mediterranean striped dolphins (*Stenella coeruleoalba*), were compared antigenically with other members of the genus *Morbillivirus*, including the newly recognized phocine distemper virus type 1. DMV and PMV proved to be similar but distinct morbilliviruses, closely related to rinderpest virus and peste-des-petits-ruminants virus. Cell cultures of cetacean, pinniped,

ruminant and canine origin showed a different pattern of susceptibility to DMV and PMV infection. Ruminants and dogs proved to be susceptible to experimental infection with DMV and PMV, which both caused a transient leukopenia most pronounced in the ruminants. Pre-exposure of dogs to DMV and PMV protected them from developing CDV viraemia and clinical signs upon challenge infection with virulent CDV. A serological survey among stranded animals of different cetacean species in Europe indicated that infections with DMV- and PMV-like morbilliviruses are not uncommon among these aquatic mammals.

Introduction

Within the genus *Morbillivirus* of the Paramyxovirus family, five major pathogens have been recognized: measles virus in humans, canine distemper virus (CDV) in dogs and other carnivores, rinderpest virus (RPV) and peste-des-petits-ruminants virus (PPRV) in ruminants and, recently, phocine distemper virus type 1 (PDV-1) in seals (for reviews, see Pringle, 1991; de Vries & Osterhaus, 1993). Under natural conditions the host range of each of these viruses is restricted to members of one mammalian order (Black, 1991). The members of the genus are closely related antigenically but may be differentiated in virus neutralization (VN) assays and in ELISA with specific monoclonal antibodies (MAbs) and polyclonal antisera (Taylor, 1979; Örvell *et al.*, 1990; Visser *et al.*, 1990; Bostock *et al.*, 1991; Harder *et al.*, 1991; Ross *et al.*, 1992).

During the last 5 years, we and others have reported several cases of heavy mortalities in aquatic mammals,

which were caused by infection with morbilliviruses. In 1987 thousands of Baikal seals (*Phoca sibirica*) died from infection with a morbillivirus closely related or identical to CDV (Grachev *et al.*, 1989; Osterhaus *et al.*, 1989). From 1988 onward, more than half of the population of harbour seals (*Phoca vitulina*) in the North and Baltic Seas died from an infection with a newly identified PDV-1 (Mahy *et al.*, 1988; Osterhaus & Vedder, 1988), that proved to be closely related but not identical to CDV (Visser *et al.*, 1989; Haas *et al.*, 1991; Kōvamees *et al.*, 1991).

The first indication for the presence of morbillivirus infections in cetacean species came from the demonstration of morbillivirus antigen in organs of harbour porpoises (*Phocoena phocoena*) that died along the Irish coasts during the PDV-1 outbreak among harbour seals (Kennedy *et al.*, 1988; McCullough *et al.*, 1991). In 1990 a dolphin morbillivirus (DMV) was isolated from Mediterranean striped dolphins (*Stenella coeruleoalba*) during an epidemic that started in the western part of the

Mediterranean Sea (Domingo *et al.*, 1990, 1992; Van Bresseem *et al.*, 1991). So far DMV has not been characterized and questions concerning its origin and relationships with other morbilliviruses remain to be addressed.

Here we describe the isolation and characterization of a morbillivirus from harbour porpoises (porpoise morbillivirus; PMV) that died in the Dutch Waddensea (North Sea) in 1990 and the comparison of antigenic and other properties of this virus with those of DMV. These two viruses isolated from cetaceans proved to be similar but distinct morbilliviruses, most closely related to RPV and PPRV.

Methods

Virus isolation. Organs were collected from two harbour porpoises (29-90 and 53-90) that had become stranded at the Dutch Waddensea coast in February and December of 1990. A 10% lung homogenate of harbour porpoise 29-90 was prepared in Iscove's modified Eagle's medium (IMEM) for virus isolation in African green monkey (Vero) cell cultures, using standard procedures (Mohanty & Dutta, 1981*b*). Animal 53-90 stranded in a fresh state, which allowed the preparation of primary kidney cell cultures, as previously described (Osterhaus *et al.*, 1985). Inoculated Vero cell cultures and primary kidney cell cultures were passaged every 10 days and checked for the development of cytopathic changes at regular intervals. Culture supernatants were tested for the presence of morbillivirus antigen by antigen capture ELISA (see below).

Viruses. For ELISAs, VN assays and *in vitro* infection studies, clarified culture supernatants of Vero cells were used after infection with the Convac or Bussel strain of CDV, PDV-1 1 (Visser *et al.*, 1990), the RBOK attenuated strain of RPV, the Nigeria 75-1 strain of PPRV, a previously described DMV isolate (16A) (Van Bresseem *et al.*, 1993) and PMV 29-90 or PMV 53-90, which were isolated in the course of these studies.

For *in vivo* infection of ruminants and dogs, culture supernatants of the third passages of PMV 53-90 or DMV 16A in Vero cell cultures were used. All virus isolates were propagated in Vero cells and infectivity titres were calculated according to Reed and Muench (Mohanty & Dutta, 1981*a*).

Physical and morphological characterization of viruses. Viruses were purified from clarified supernatant of DMV 16A-, PMV 29-90- or PMV 53-90-infected Vero cell cultures by 10% to 60% sucrose gradient centrifugation as previously described (Visser *et al.*, 1990). Gradient fractions were titrated for infectivity in Vero cell cultures (Visser *et al.*, 1990). The buoyant density of the virus was calculated from the refractive indices of the fractions showing highest infectivity as previously described (Visser *et al.*, 1990).

Lysates of PMV 53-90- and 29-90-infected Vero cell cultures prepared by osmotic shock were placed on formvar/carbon-coated grids, negatively contrasted with 2% phosphotungstic acid (BDH, pH 6.0 adjusted with 1 M-KOH) and examined for the presence of viral structures using standard electron microscopic procedures.

Cell substrates. Vero cell cultures were used for virus isolation, propagation, titration and neutralization procedures as previously described (Appel, 1969; Osterhaus *et al.*, 1985). Primary harbour seal kidney cell cultures (SeKC), primary harbour porpoise kidney cell cultures (HPKC), spotted dolphin (*S. plagiodon*) kidney cells (SpIK, ATCC CCL 78), Madin-Darby canine kidney cells (MDCK) and

marmoset B59 cells were cultivated in 25 cm² bottles (Corning) in IMEM supplemented with penicillin (100 units/ml), streptomycin (100 µg/ml), glutamine and 2% fetal calf serum (IMEM-S) and used for virus susceptibility studies. Peripheral blood mononuclear cells (PBMCs) were isolated by Lymphoprep (Nycomed Pharma) gradient centrifugation of heparinized blood sampled from a goat (G.PBMC), a sheep (S.PBMC), a cow (C.PBMC), a specific pathogen-free (SPF) beagle dog (D.PBMC), a horse (H.PBMC), a harbour porpoise (*P. phocoena*) (PP.PBMC), a bottlenose dolphin (*Tursiops truncatus*) (TT.PBMC), a harbour seal (*P. vitulina*) (PV.PBMC), a Mediterranean monk seal (*Monachus monachus*) (MM.PBMC) and a Hawaiian monk seal (*M. schauinslandi*) (MS.PBMC) using standard procedures (Siebelink *et al.*, 1992). After Lymphoprep centrifugation the cells were washed and cultured at a concentration of 5×10^4 cells per well in 96-well round-bottomed plates (Greiner) using IMEM-S. The PBMCs were stimulated with the mitogen concanavalin A (Con A; Flow Laboratories) at a concentration of 5 µg/ml and maintained in the presence of recombinant human interleukin 2 (IL-2) (Boehringer Mannheim) (100 units/ml) as previously described (Siebelink *et al.*, 1992).

Antibody preparations. Previously raised panels of MAbs against PDV-1 (Örvell *et al.*, 1990), CDV (Örvell *et al.*, 1985) and a panel of newly generated MAbs raised against PPRV and RPV were used to study serological cross-reactivities between morbilliviruses by indirect ELISA (see below).

Serum or plasma samples from 37 harbour porpoises, from 22 common dolphins (*Delphinus delphis*), from five white-beaked dolphins (*Lagenorhynchus albirostris*) beached in northwest Europe between September 1989 and July 1992 and from 14 striped dolphins beached on the Mediterranean coasts of Spain, Italy and Greece from April 1991 to January 1992 were tested for the presence of virus-neutralizing antibodies.

Serological assays. For detection of morbillivirus-specific antibodies present in serum or plasma samples from stranded animals a previously described micro VN assay was used (Visser *et al.*, 1990). In short, serial twofold dilutions of the samples were incubated in 96-well flat-bottomed plates (Greiner) for 1 h at 37 °C with suspensions containing 50 to 100 TCID₅₀ of virus. After addition of 10⁴ Vero cells to each well, cultures were screened for the development of cytopathic changes during a 14 day incubation period. Virus-neutralizing serum antibody titres were expressed as the reciprocals of the highest dilution that prevented the cultures from developing cytopathic changes.

For comparison of the reactivities of MAbs with different morbilliviruses, a previously described indirect ELISA (Örvell *et al.*, 1985) was used. Briefly, 50-fold dilutions of MAb preparations were incubated for 1 h at 37 °C on morbillivirus-coated ELISA plates. Bound MAbs were detected with a polyclonal horseradish peroxidase (HRP)-labelled rabbit anti-mouse antibody preparation (Dakopatts). Absorbance (at 450 nm; A_{450}) values exceeding twice the mean of the reaction obtained with unrelated MAbs were considered positive.

Susceptibility of cell substrates to infection with DMV and PMV. The *in vitro* host range of DMV and PMV was determined by inoculating different cell lines, primary cell cultures and PBMCs from different mammalian species with 10³ to 10⁴ TCID₅₀ of the respective viruses. PBMC cultures were inoculated in the same way with 10³ to 10⁴ TCID₅₀ of CDV (Bussel strain) or PDV-1. After incubation for 1 h at 37 °C, all cells were washed and subsequently cultivated at 37 °C using standard procedures (Siebelink *et al.*, 1992). During a period of 2 weeks after inoculation the cell lines and primary cell cultures were monitored for the development of cytopathic changes at regular intervals. When cytopathic changes were observed or at the end of the incubation period, a cell-free supernatant of these cultures was tested in the antigen capture ELISA for the presence of morbillivirus antigen. The

replication of DMV, PMV, PDV-1 or CDV in PBMCs was detected by immunofluorescence analysis (IFA) or by fluorescence-activated cell sorter (FACS) measured fluorescence (FMF) analysis demonstrating morbillivirus F protein expression on the cell membrane (see below) on days 5, 7 and 11. If F protein expression was observed by FMF analysis, the expression at day 7 proved clearest in all cases.

Infection of ruminants and dogs with DMV and PMV

(i) *Ruminants.* To study the susceptibility of ruminants to infection with DMV or PMV one young adult goat, sheep and cow were inoculated intranasally and intraperitoneally with 1 ml clarified culture medium of the third passage in Vero cells containing 10^3 TCID₅₀ of DMV 16A (goat 1, sheep 2 and cow 3) or 10^3 TCID₅₀ PMV 53-90 (goat 4, sheep 5 and cow 6). The animals inoculated with DMV and those inoculated with PMV were housed in two separate isolated rooms. After inoculation the animals were observed for the development of clinical signs, particularly those concerning the respiratory tract, and body temperatures were measured. Heparinized blood was collected at regular intervals after inoculation and white blood cell (WBC) counts were performed. Plasma samples were tested for DMV, PMV, PPRV or RPV virus-neutralizing antibodies and the presence of viraemia was demonstrated by FMF analysis of PBMCs isolated from the heparinized blood samples. Therefore 10^6 isolated PBMCs were stimulated with Con A, cultured for 3 days and cocultivated with 10^4 TT.PBMC for 7 days and with separate cultures with Vero cells which were subsequently passaged three times. After cocultivation with TT.PBMC the presence of virus infection was tested by the detection of membrane expression of morbillivirus F protein in an IFA. After cocultivation of PBMCs with Vero cells morbillivirus infection was demonstrated by the development of cytopathic changes or the formation of syncytia.

(ii) *Dogs.* SPF beagle dogs were also tested for their susceptibility to infection with DMV or PMV. Two dogs were inoculated with clarified culture supernatant containing 10^3 TCID₅₀ of the third passage on Vero cells of DMV 16A (dogs 1 and 2) or PMV 53-90 (dogs 3 and 4). Dogs 1 and 2 and dogs 3 and 4 were kept in two separate pressurized glove boxes during the experiments. After an observation period of 4 weeks after the inoculation, the four dogs and two control dogs (dogs 5 and 6), housed separately from the other four dogs, were challenged intranasally with a dog brain suspension containing 5×10^3 TCID₅₀ of the virulent CDV Snyder-Hill strain (de Vries *et al.*, 1988). During the 4 week observation period following inoculation and during another 2 week period after challenge, the animals were checked for the development of clinical signs and elevation of body temperature. Heparinized blood samples were collected at regular intervals, WBCs were counted and the plasma samples were used for the detection of DMV-, PMV- and CDV-specific virus-neutralizing antibodies. For the detection of PBMC-associated viraemia, serial 10-fold dilutions of PBMCs isolated from the heparinized blood samples (starting at a concentration of 10^6 PBMCs per culture), were mixed with autologous pre-infection PBMCs to a final concentration of 10^6 cells per culture. These cells were Con A-stimulated and cocultivated with 10^4 TT.PBMC. After 7 days morbillivirus F protein expression was demonstrated by screening these cultures by FMF analysis (see below). The numbers of infected PBMCs identified in the blood by this analysis were expressed as the reciprocal \log_{10} of the dilution of the tested PBMC still giving positive FMF results. For the detection of PBMC-associated viraemia by ELISA or PCR analysis, PBMCs taken from the experimental animals were Con A-stimulated and cocultivated with Vero cells. Cultures were regularly checked for virus replication by the formation of syncytia and the detection of morbillivirus antigen by antigen-capture ELISA (see below). When syncytium formation was observed, Southern blot analysis of PCR products amplified with morbillivirus- and CDV-specific oligonucleotide primers was carried out (see below).

Antigen capture ELISA. The broadly reactive MAb F3-5 raised against the MV F protein (de Vries *et al.*, 1990) was used to coat 96-well ELISA strip plates. These plates were used to detect the presence of morbillivirus antigen in clarified culture supernatant as previously described (Van Bressem *et al.*, 1993). Briefly, supernatants were incubated overnight at 4 °C on F3-5-coated ELISA strip plates, followed by a 1 h (37 °C) incubation with a biotinylated MAb F3-5 preparation and a subsequent 1 h (37 °C) incubation with HRP-labelled streptavidin. A_{450} values exceeding twice the background value measured after incubation with supernatant of uninfected cultures were considered positive.

IFA and FMF analyses. Infected PBMCs after *in vitro* or *in vivo* inoculation were detected by demonstrating the presence of membrane expression of the morbillivirus F protein by IFA or by FMF analysis. PBMCs were washed three times with PBS supplemented with 0.4% BSA and 0.02% sodium azide, incubated on ice with MAb F3-5 for 1 h, washed three times, incubated for 1 h on ice with fluorescein isothiocyanate (FITC)-labelled goat-anti-mouse conjugate (Becton-Dickinson), washed again and fixed with 1% paraformaldehyde in PBS. Fluorescence was determined either by inverted fluorescent microscopy (Visser *et al.*, 1990) or by FMF analysis using a FACSscan and methods recommended by the manufacturer (Becton-Dickinson). For PBMCs of each species the viable cell population was gated in the FITC channel yielding a FACS profile confining 97.5% of the PBMCs to the non-fluorescent fraction. Fluorescent PBMCs were expressed as percentages of total numbers of viable PBMCs with fluorescence exceeding this threshold value.

PCR analysis. Total RNA was extracted from Vero cells with or without cytopathic changes after cocultivation with PBMCs from dogs 1 to 6, using RNazol (Campro Scientific), according to the procedure recommended by the manufacturer. cDNA was prepared with oligo(dT) (Boehringer Mannheim) and reverse transcriptase H⁻ Superscript (Gibco BRL). Morbillivirus-specific sequences were amplified using broadly reactive morbillivirus P gene-specific primers, which amplify a fragment of approximately 450 bp (T. Barrett, I. K. G. Visser, L. V. Mamaev, L. Goatly, M. F. van Bressem & A. D. M. E. Osterhaus, unpublished results) and CDV F gene-specific primers, which amplify a fragment of approximately 800 bp (I. K. G. Visser, R. W. J. van der Heijden, M. W. G. van de Bildt, M. Kenter, C. Örvell & A. D. M. E. Osterhaus, unpublished results). Southern blot analysis was performed on PCR products separated on 1% agarose gels and transferred to Duralose-UV 0.45 µm membranes (Stratagene) and hybridized with ³²P-labelled DMV-P, PMV-P, CDV-P or CDV-F probes. These probes were prepared by PCR amplification using the morbillivirus-P and CDV-F primer pairs on cDNA derived from Vero cell cultures infected with the respective viruses. Radioactivity bound to PCR products transferred to the membranes was quantified with a 400A Phosphor Imager (Molecular Dynamics).

Results

Isolation of a morbillivirus from harbour porpoises

Five days after passaging Vero cell cultures inoculated with the lung homogenate of harbour porpoise 29-90, for the third time, cytopathic changes including the development of small syncytia and foci of rounded cells were observed. Similar changes were observed in the primary kidney cells of harbour porpoise 53-90 about 20 days after establishment of the culture. In cell lysates of both cultures morbillivirus antigen was demonstrated by ELISA and characteristic paramyxovirus particles or

nucleocapsids were shown by negative contrast electron microscopy (data not shown). Infectivity titres of culture supernatants of Vero cell cultures peaked at a buoyant density of 1.18 to 1.20 g/ml in sucrose gradients (not shown). Western blot analysis (Towbin *et al.*, 1979) of the H, F, NP and P proteins of the PMV isolate showed that the M_r s of these structural proteins (not shown) were within the range found for the respective proteins in other morbilliviruses (Rima, 1983; Diallo *et al.*, 1987; Grubman *et al.*, 1988; Anderson *et al.*, 1990; Norrby & Oxmann, 1990; Visser *et al.*, 1990; Barrett *et al.*, 1991). A comparison of the M_r s of these proteins from the two PMV isolates with those of the two DMV isolates from Mediterranean striped dolphins showed that the M_r s of the NP protein of the PMV isolates were slightly higher. No differences in the M_r s of the other three proteins of PMV and DMV studied by Western blot analysis could be distinguished (not shown). Collectively these data confirmed that PMV, like DMV, should be considered a member of the genus *Morbillivirus*. The PMV 53-90 and the DMV 16A isolates were further passaged in Vero cells and used for subsequent studies.

Antigenic cross-reactivities between DMV, PMV and other morbilliviruses

A panel of MAbs raised against the NP ($n = 3$), H ($n = 11$) and F ($n = 5$) proteins of PDV-1 (Örvell *et al.*, 1990) was used to define antigenic cross-reactivities between PDV-1, DMV, PMV and CDV in the indirect ELISA (Table 1). Of these MAbs, two H-specific and all the F-specific MAbs cross-reacted with CDV and three of the F-specific MAbs cross-reacted with DMV and PMV. This indicated that DMV and PMV are closely related morbilliviruses, distinct from PDV-1 and CDV. Subsequently a panel of MAbs raised against the NP ($n = 9$), P ($n = 9$), H ($n = 8$) and F ($n = 8$) proteins of CDV (Örvell *et al.*, 1985) was used to define antigenic cross-reactions between CDV, PDV-1, DMV, PMV, PPRV and RPV in the indirect ELISA (Table 2). The reaction pattern obtained with these MAbs was identical for DMV and PMV, and it was quite similar to the pattern found with PPRV; only one of the 34 reactions was found to be different. As previously shown (Visser *et al.*, 1990) the cross-reaction between CDV and PDV-1 indicated that these were similar but distinct morbilliviruses. They both proved to be antigenically distinct from DMV and PMV. The reaction pattern with RPV was quite different from the other patterns found and most similar to those found with DMV, PMV and PPRV: 26, 26 and 25, respectively, of the 33 MAb reactions tested were identical.

A newly generated panel of MAbs raised against the NP ($n = 2$) and H ($n = 6$) proteins of PPRV and one

Table 1. *Reactions in indirect ELISA of PDV-1-specific MAbs with different morbilliviruses*

Clone designation	Specificity	Virus			
		PDV-1	CDV*	DMV*	PMV*
1.064C5	PDV NP1	+	+	—	—
1.071E2	PDV NP1	+	—	—	—
1.069G2	PDV NP2	+	—	—	—
		3/3†	0/3	0/3	0/3
1.062G5	PDV H1	+	—	—	—
1.063C3	PDV H1	+	—	—	—
1.063E9	PDV H1	+	—	—	—
1.069D9	PDV H1	+	+	—	—
1.071E5	PDV H1	+	—	—	—
1.067E5	PDV H2	+	—	—	—
1.068F2	PDV H3	+	—	—	—
1.070B5	PDV H4	+	—	—	—
1.072C4	PDV H4	+	—	—	—
1.085C4	PDV H5	+	—	—	—
1.122D11	PDV H6	+	+	—	—
		11/11	2/11	0/11	0/11
1.062E2	PDV F1	+	+	—	—
1.068B2	PDV F1	+	+	—	—
1.067D2	PDV F2	+	+	+	+
1.073G10	PDV F3	+	+	+	+
1.092C3	PDV F3	+	+	+	+
		5/5	5/5	3/5	3/5

* CDV, Convac strain; DMV (16A); PMV, 53-90 strain.

† Symbols + and —: reaction and no reaction respectively.

‡ No. of positive reactions per number of protein-specific MAbs tested.

MAb against the NP protein of RPV were used to further define antigenic cross-reactions between PPRV, RPV, DMV, PMV, PDV-1 and CDV in the indirect ELISA (Table 3). The patterns of cross-reactions of these MAbs with these viruses were all different indicating that DMV and PMV, although closely related, are distinct viruses.

Susceptibility of different cell substrates to DMV and PMV infection

Four continuous cell lines of primate (Vero, B59), dolphin (Sp1K) and canine (MDCK) origin and primary kidney cell cultures from harbour seals and porpoises were tested for their susceptibilities to infection with DMV and PMV. After inoculation with DMV or PMV, all these cell substrates produced morbillivirus antigen in the culture supernatant as shown by antigen capture ELISA. All except the PMV-infected primary kidney cell cultures also developed cytopathic changes (not shown). Similarly, freshly isolated PBMCs from different cetacean, pinniped and ruminant species and from dogs and horses were tested for their susceptibilities to infection with DMV, PMV, CDV and PDV-1. To this end, PBMCs were tested 7 days after inoculation for the expression of morbillivirus F protein by FMF analysis. As shown in Fig. 1, all PBMC substrates expressed the F

Table 2. Reactions in indirect ELISA of CDV-specific MAbs with different morbilliviruses

Clone designation	Specificity	Virus					
		CDV*	PDV-1	DMV*	PMV*	PPRV§	RPV§
3.564	CDV NP	+†	-†	-	-	-	-
3.662	CDV NP1	+	-	-	-	-	-
3.740	CDV NP	+	+	+	+	+	-
3.755	CDV NP	+	+	-	-	-	-
3.851	CDV NP	+	+	-	-	-	-
3.991	CDV NP4	+	-	-	-	-	-
4.100	CDV NP5	+	+	-	-	-	-
4.271	CDV NP	+	+	+	+	+	NT‡
5.224	CDV NP	+	NT	-	-	+	-
		9/9	5/8	2/9	2/9	3/9	0/8
3.630	CDV P	+	-	-	-	-	-
3.695	CDV P3	+	-	-	-	-	-
3.698	CDV P1	+	+	-	-	-	+
3.768	CDV P	+	+	+	+	+	-
3.788	CDV P	+	+	-	-	-	-
4.051	CDV P5	+	-	-	-	-	-
4.088	CDV P6	+	+	+	+	+	-
4.415	CDV P1	+	+	-	-	-	-
4.420	CDV P	+	NT	-	-	-	-
		9/9	5/8	2/9	2/9	2/9	1/9
1.347	CDV H1	+	+	-	-	-	-
2.267	CDV H2	+	-	-	-	-	-
3.734	CDV H3	+	-	-	-	-	-
3.775	CDV H4	+	-	-	-	-	-
3.900	CDV H3	+	-	-	-	-	-
4.043	CDV H5	+	+	-	-	-	-
4.074	CDV H5	+	+	-	-	-	-
4.275	CDV H6	+	-	-	-	-	-
		8/8	3/8	0/8	0/8	0/8	0/8
3.551	CDV F2	+	+	-	-	-	+
3.584	CDV F2	+	+	-	-	-	+
3.633	CDV F1	+	+	+	+	+	+
3.697	CDV F2	+	+	-	-	-	+
4.068	CDV F2	+	+	-	-	-	-
4.985	CDV F3	+	-	-	-	-	-
5.086	CDV F1	+	+	+	+	+	+
5.148	CDV F3	+	+	-	-	-	-
		8/8	7/8	2/8	2/8	2/8	5/8

*† See Table 1 footnotes.

‡ NT, Not tested.

§ PPRV (Nigeria 75/1 strain); RPV (RBOK attenuated strain).

protein after infection with PMV. Inoculation with DMV did not result in the expression of the F protein in the PBMCs of pinnipeds, the cow and the horse. CDV inoculation did not result in F protein expression in PBMCs of monk seals, sheep, the cow and the horse, whereas PDV-1 inoculation did not result in F protein expression in PBMCs of the goat, the cow and the horse.

Experimental infection of ruminants and dogs with DMV and PMV

Since DMV and PMV could infect cells of ruminants and dogs *in vitro*, and a close antigenic relationship was observed between these two viruses on the one hand and

the ruminant morbilliviruses on the other, an experimental infection was carried out in a goat, a sheep, a cow and two dogs with DMV. The same numbers of animals were inoculated in the same way with PMV.

(i) Ruminants

No clinical signs were observed in the small ruminants and cows after intraperitoneal and oculonasal inoculation with 10^3 TCID₅₀ DMV or PMV per animal. However, as shown in Table 4, the goats, sheep and cows developed a leukopenia ($< 3 \times 10^6$ WBC/ml) between days 3 and 13 after inoculation with DMV or PMV, and this was most pronounced in the small ruminants. All

Table 3. Reactions in indirect ELISA of PPRV-CDV-specific MAb and one RPV-specific MAb with different morbilliviruses

Clone designation	Specificity	Virus					
		PPRV*	RPV†	PMV*	DMV†	PDV-1	CDV*
B8D10	PPRV NP	+†	+	+	+	+	+
B10D8	PPRV NP	+	+	+	+	-†	-
		2/2	2/2	2/2	2/2	1/2	1/2
B2G6	PPRV H	+	-	-	+	-	-
B4B8	PPRV H	+	-	-	+	-	-
B9G3	PPRV H	+	+	-	-	-	-
B2G3	PPRV H	+	-	+	+	+	+
B2G4	PPRV H	+	-	+	-	-	-
B3F2	PPRV H	+	-	-	-	-	-
		6/6	1/6	2/6	3/6	1/6	1/6
IE6-1	RPV NP	+	+	+	+	+	-

*† See Table 1 footnotes.

† See Table 2 footnotes.

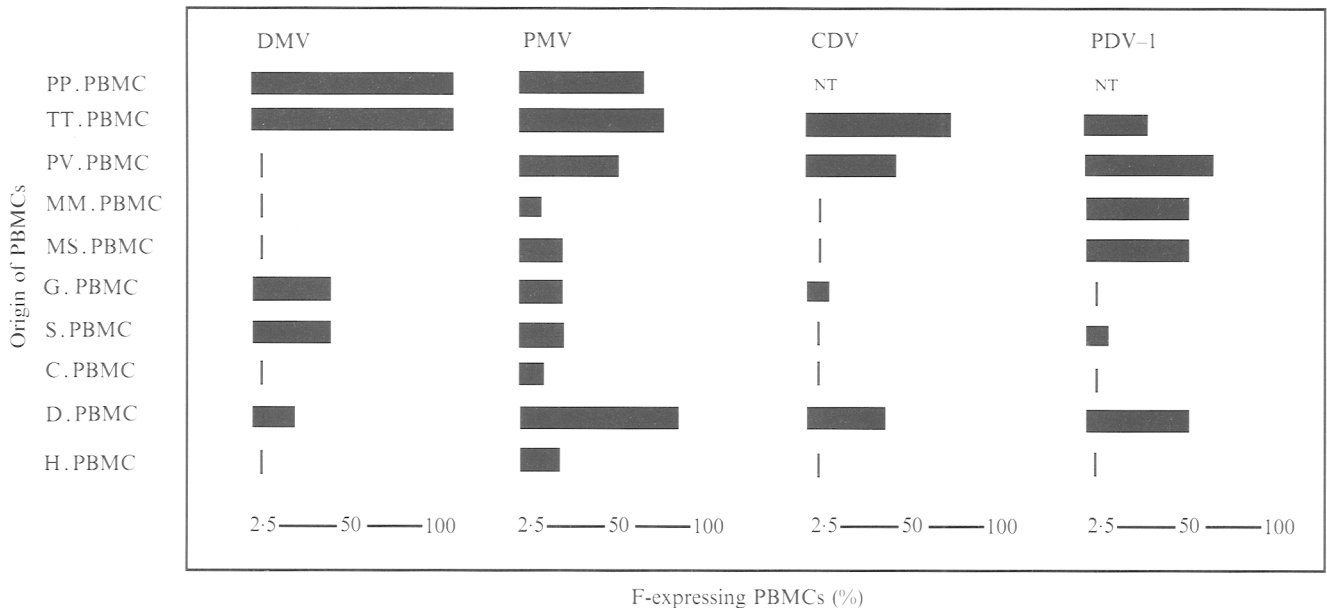


Fig. 1. Horizontal histogram: percentages of Con A-stimulated PBMCs from different animal species with membrane expression of morbillivirus FITC-labelled F protein 7 days after inoculation with DMV (16A), PMV (53-90), CDV (Bussel strain) or PDV-1, as detected by FMF analysis, with allowed background fluorescence of the tested PBMC population set at 2.5% (|); NT, Not tested.

these animals also developed viraemia, which persisted for about 10 days in the cows and for at least 20 days in the small ruminants. An elevated body temperature was observed for only 1 day in the goat inoculated with PMV. All six of these ruminants developed DMV- and PMV-neutralizing serum antibodies within 10 days after inoculation which reached maximum titres of 80 to 640. Titres to the homologous virus developed slightly faster and reached higher levels in most cases. Maximum virus-neutralizing serum antibody titres to PPRV and RPV were at least fourfold lower than maximum

homologous virus-neutralizing antibody titres in these animals. The titres to CDV were consistently lower than those to PPRV or RPV (not shown).

(ii) Dogs

No clinical signs or body temperature rise were observed during the 4 week observation period in the two dogs inoculated via the oculonasal route with 10^3 TCID₅₀ DMV (dogs 1 and 2) or in the two dogs inoculated via the same route with 10^3 TCID₅₀ PMV (dogs 3 and 4). All

Table 4. Development of clinical signs, viraemia and VN serum antibody titres in a sheep, goat and cow at different times after infection with DMV or PMV*

DMV-inoculated animals	Time after inoculation (days)							PMV-inoculated animals	Time after inoculation (days)						
	1	3	6	8	10	13	20		1	3	6	8	10	13	20
Goat 1 T	—	—	—	—	—	—	—	Goat 4 T	—	—	—	—	—	—	—
L	—	—	+	+	+	—	—	L	—	—	+	+	—	—	—
V	—	+	+	+	—	—	+	V	—	+	+	+	+	+	+
PMV VN	—	—	—	—	40	160	160	PMV VN	—	—	—	80	160	160	320
DMV VN	—	—	—	—	80	320	640	DMV VN	—	—	—	80	80	160	320
PPRV VN	—	—	—	—	40	40	160	PPRV VN	—	—	—	20	40	80	80
RPV VN	—	—	—	20	20	40	40	RPV VN	—	—	—	20	40	80	40
Sheep 2 T	—	—	—	—	—	—	—	Sheep 5 T	—	—	—	+	—	—	—
L	—	—	+	+	+	—	—	L	—	—	+	+	—	—	—
V	+	+	+	+	+	+	+	V	+	+	+	+	+	—	+
PMV VN	—	—	—	—	20	40	40	PMV VN	—	—	—	20	20	80	320
DMV VN	—	—	20	20	40	160	320	DMV VN	—	—	—	—	40	80	320
PPRV VN	—	—	—	20	20	80	40	PPRV VN	—	—	—	—	—	20	40
RPV VN	—	—	—	—	—	40	40	RPV VN	—	—	—	—	—	40	40
Cow 3 T	—	—	—	—	—	—	—	Cow 6 T	—	—	—	—	—	—	—
L	—	—	+	—	—	—	—	L	—	—	+	—	—	—	—
V	+	+	+	+	+	—	—	V	+	+	+	+	+	—	—
PMV VN	—	—	—	40	80	80	40	PMV VN	—	—	20	20	160	320	80
DMV VN	—	—	20	320	320	320	40	DMV VN	—	—	—	20	40	80	40
PPRV VN	—	—	—	40	160	80	40	PPRV VN	—	—	—	—	—	20	20
RPV VN	—	—	—	—	—	20	20	RPV VN	—	—	—	—	—	20	20

* T, Body temperature rise; L, drop in WBC counts; V, viraemia. PMV VN, DMV VN, PPRV VN and RPV VN: specific virus-neutralizing antibody titres in serum samples taken at several intervals after inoculation, as indicated. +, positive titre; —, negative titre (≤ 10).

these dogs showed a drop in WBC counts of 20 to 40% on day 4 after infection (not shown). As shown in Fig. 2 a biphasic PBMC-associated viraemia was demonstrated in all four dogs between days 4 and 28 by showing expression of the F protein in FMF analysis. From the PBMCs isolated on these, but not on the other days indicated, DMV or PMV were isolated by cocultivation with Vero cells. In these cells cytopathic changes developed and antigen was demonstrated by morbillivirus antigen capture ELISA. Serum antibodies neutralizing DMV, PMV and CDV developed within 1 week after infection and reached maximum titres of 60 to > 2560 . Titres to the homologous virus were consistently higher than to the heterologous viruses. On day 28 all dogs and two control dogs were challenged with 5×10^3 TCID₅₀ virulent CDV. The control dogs developed clinical signs from day 11 after challenge onward, similar to those previously observed (Appel, 1987; de Vries *et al.*, 1988). Dogs 1 to 4, pre-exposed to DMV or PMV, developed no sign of disease upon challenge. Also the CDV-specific virus-neutralizing serum antibody titres did not reach the levels found in the two animals which had not been pre-exposed to morbillivirus infection (dogs 5 and 6). A PBMC-associated viraemia was demonstrated in dogs 5 and 6 from day 7 post-infection onward, using the same procedure described above for the demonstration of DMV and PMV viraemia. Also in

dogs 1 to 4 PBMC-associated viraemia was demonstrated by showing expression of the F protein in FMF analysis in some of the PBMC samples collected from day 7 post-CDV challenge onward. It was shown that the virus isolated after CDV challenge of dogs pre-exposed to DMV or PMV was probably not CDV but DMV or PMV.

This was concluded since after CDV challenge no genomic sequences could be amplified from the infected PBMCs of dogs 1 and 2 and dogs 3 and 4 with a primer pair based on the F gene sequence of CDV that did amplify genomic sequences from the infected PBMCs of dogs 5 and 6 (Fig. 3). When these PCR products were selected according to size on a 1% agarose gel, blotted on Duralose-UV filters and hybridized with the CDV-F probe, no hybridization with the products derived from PBMCs of dogs 1 to 4 was detected, whereas products derived from dogs 5 and 6 after challenge strongly hybridized with the CDV-F probe (data not shown). From infected PBMCs of dogs 1 to 4, collected before and after CDV challenge, genomic sequences could be amplified with a primer pair based on a P gene sequence conserved among morbilliviruses (Fig. 3). When these PCR products were transferred to filters and hybridized with CDV-P, DMV-P and PMV-P probes, ssDNA derived from DMV-infected PBMCs of dogs 1 and 2 and PMV-infected PBMCs of dogs 3 and 4 before challenge

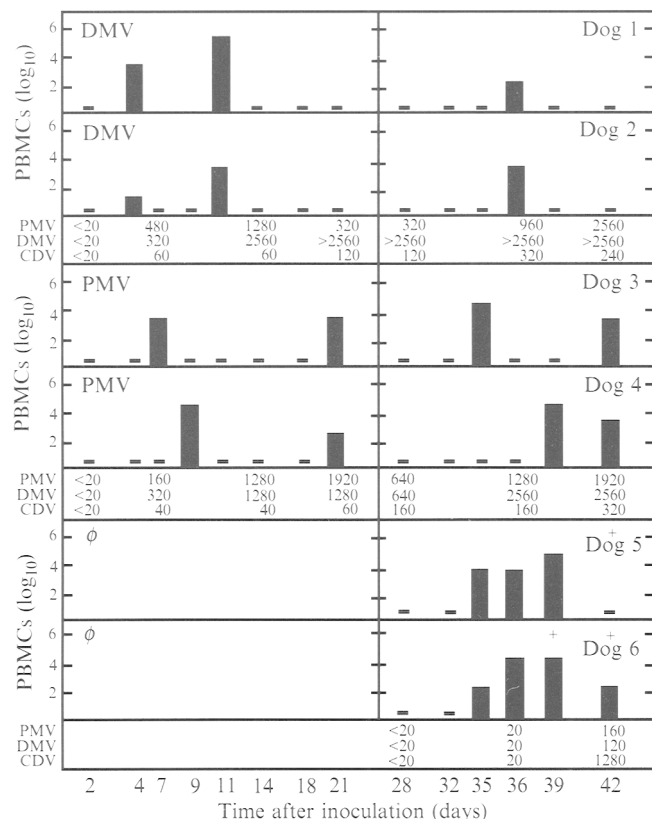


Fig. 2. Development of viraemia, respiratory signs and virus-neutralizing antibody titres after *in vivo* inoculation of SPF beagle dogs with PMV (53-90) or DMV (16A) and subsequent challenge with CDV. Viraemia expressed as the reciprocal (\log_{10}) of the number of infected PBMCs/ 10^6 PBMCs as indicated. —, no viraemia; +, respiratory signs; PMV-, DMV- and CDV-specific virus-neutralizing serum antibody titres at different times after inoculation are expressed as the mean value detected in serum samples taken from two dogs inoculated with the same virus.

did not hybridize with the CDV-P probe, but hybridized strongly with the respective homologous probes. A weak cross-hybridization between these DMV and PMV amplified sequences and probes was also observed. After CDV challenge, ssDNA amplified with the P-specific primer pairs from dogs 1 and 2 and dogs 3 and 4 showed a very low degree of hybridization with the homologous P probes and not with the heterologous probes including the CDV-P probe. This suggests that after CDV challenge only DMV and PMV were present in the PBMCs isolated from dogs 1 to 4.

Morbillivirus-specific antibodies in cetacean species

Serum samples collected from dead or moribund dolphins and harbour porpoises beached on the coasts of the North and the Mediterranean Seas during the last 4 years were tested for the presence of virus-neutralizing

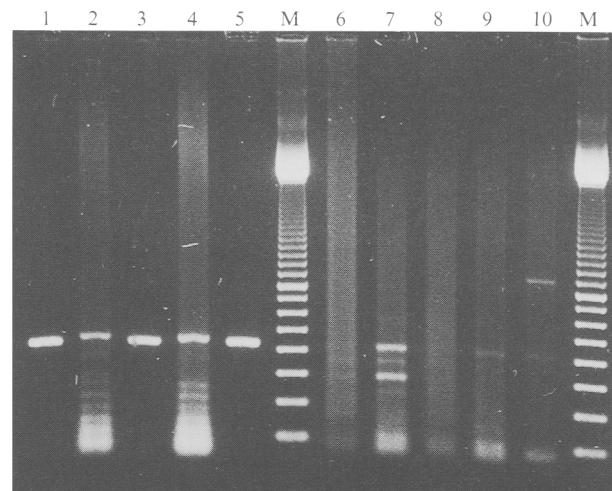


Fig. 3. Analysis by agarose gel (1%) electrophoresis of PCR products amplified with morbillivirus P-specific (lanes 1 to 5) and CDV-F-specific (lanes 6 to 10) primers on cDNA derived from PBMCs isolated from DMV-infected (lanes 1 and 6), DMV-infected and CDV-challenged (lanes 2 and 7), PMV-infected (lanes 3 and 8), PMV-infected and CDV-challenged (lanes 4 and 9) and CDV-challenged (lanes 5 and 10) dogs. Marker (M) lane represents 100 bp ladder (Pharmacia LKB).

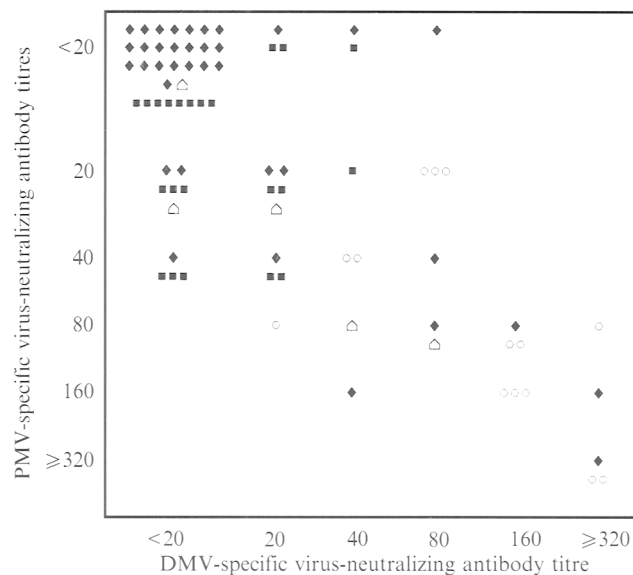


Fig. 4. Virus neutralizing antibody titres to PMV (53-90) and DMV (16A) detected in serum samples from: harbour porpoises (*P. phocoena*), ♦; striped dolphins (*S. coeruleoalba*), ○; common dolphins (*D. delphis*), ■; or white beaked dolphins (*L. albirostris*), ◊.

antibodies to DMV, PMV, CDV and PDV-1. As shown in Fig. 4, all the 14 sera from striped dolphins, 14 of the 22 sera from common dolphins, four of the five sera from white-beaked dolphins and 15 of the 37 sera from harbour porpoises contained virus-neutralizing antibodies to DMV and/or PMV with titres ranging from 20 to > 320 . The titres found to DMV and PMV were not

significantly different in these samples ($r = 0.88$). Titres to PDV-1 and CDV in these sera were generally fourfold lower than those to DMV and PMV (not shown).

Discussion

In the present paper we have described the isolation of a morbillivirus (PMV) from stranded dead harbour porpoises and its comparison with a virus closely related antigenically, DMV, that was recently isolated from Mediterranean striped dolphins during an outbreak causing heavy mortality (Van Bressem *et al.*, 1991).

A difference observed in the M_r s of the NPs of both viruses in Western blot analysis was the first indication that DMV and PMV, although similar, are different morbilliviruses.

Analysis of the reactivities of DMV and PMV with previously described panels of MAbs raised against PDV-1 and CDV and with a newly generated panel of PPRV- and RPV-specific MAbs also indicated that DMV and PMV were distinct viruses closely related to, but different from, the ruminant morbilliviruses PPRV and RPV, and less closely related to the morbilliviruses of carnivores CDV and PDV-1 (Tables 1 to 3). It is interesting to note that a similar analysis of morbillivirus identified in harbour porpoises with a panel of CDV-specific MAbs showed a different pattern of reactions between PMV and this virus (McCullough *et al.*, 1991). This analysis was not carried out by ELISA but by IFA, and with a more limited panel of MAbs. Subsequent analysis of this virus by the same group using a more extended pattern of MAbs in an ELISA, however, revealed that the pattern of reactions with this virus was identical to that obtained with our PMV isolates (Welsh *et al.*, 1992). The name proposed for the porpoise morbillivirus isolated by the Northern Irish group, delphinoid distemper virus, is confusing, since porpoises do not belong to the family Delphinidae and PMV proved to be distinct from DMV.

Differences in the biological properties of DMV and PMV were observed. The pattern of susceptibilities of PBMCs from different animal species to replication of DMV and PMV was not identical. PBMCs were chosen for this purpose since lymphocytes are the natural target cells for morbilliviruses. DMV showed a more restricted pattern of replication than PMV, which replicated in PBMCs of all the species tested. DMV replicated in cells from cetaceans, small ruminant species and dogs, but not in those of pinniped species, cows and horses. Although these results might suggest that the latter species are not susceptible to infection with DMV, it should be realized that only 11 day *in vitro* infection experiments in PBMCs were carried out, without any further passaging. For example it was shown that a cow could be infected with

DMV, although PBMCs from cows proved not to be infected *in vitro* (Fig. 1).

Since cells of ruminants were susceptible *in vitro* to DMV and PMV infection and a close antigenic relationship with PPRV and RPV was observed, *in vivo* infection experiments were carried out in goats, sheep and cows. The ruminants proved to be susceptible to infection with both viruses, as shown by the development of viraemia and virus-neutralizing serum antibodies. However clinical signs were quite mild or absent in these animals. The levels of virus-neutralizing serum antibody titres that developed to DMV, PMV, PPRV and RPV upon infection in these animals also indicated that these are closely related but distinct viruses. Because of the logistic restrictions associated with experiments in ruminants, a similar experiment was carried out in SPF dogs. Since cross-protection may be induced between more or less distantly related morbillivirus infections (de Vries *et al.*, 1988; Visser *et al.*, 1992) we subsequently challenged these dogs with virulent CDV and looked for protection against CDV infection and CDV-induced disease. The two control dogs, not pre-exposed to DMV or PMV, developed clinical signs and PBMC-associated viraemia as expected (Appel, 1969; de Vries *et al.*, 1988). The dogs which had been pre-exposed to DMV or PMV were protected from CDV infection and from developing signs of CDV-associated disease. The comparatively limited rise in CDV-specific virus-neutralizing serum antibodies in dogs 1 to 4 also indicated that these animals were protected from CDV infection. The limited PBMC-associated viraemia observed after CDV challenge in these dogs was not due to CDV infection, but probably to DMV and PMV infection, as shown by PCR analysis using a discriminative panel of F and P gene primer pairs (Fig. 3). From these data it cannot be concluded whether this viraemia was elicited by the CDV infection or would also have occurred in the absence of this challenge.

Taken together these *in vivo* studies showed that ruminants and dogs are susceptible to experimental infection with DMV and PMV, although these infections do not result in serious disease signs. However, it cannot be ruled out that the viruses had been attenuated owing to the three passages in Vero cells.

Although DMV and PMV proved to be closely related antigenically to the ruminant morbilliviruses, it is not likely that under natural circumstances DMV and PMV would infect ruminants, since morbillivirus infections in general seem to be restricted to one order of mammalian species (Black, 1991). The sudden appearance of DMV and PMV infections in dolphins and porpoises raises questions about the origin of these closely related morbilliviruses in aquatic mammals.

The demonstration of a high incidence of virus-neutralizing antibodies in the sera of different cetacean

species in the North and Mediterranean Seas during the last 4 years (Fig. 4) suggests that infections with DMV and PMV, or closely related morbilliviruses, are widespread among different dolphin species and harbour porpoises. The analysis of the nucleotide sequences of DMV and PMV and the evaluation of sequence homologies with those of other morbilliviruses may provide more insight into the origin and phylogenetic relationships of these newly recognized viruses of aquatic mammals.

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