The Science of the Total Environment, 115 (1992) 117–132
Elsevier Science Publishers B.V., Amsterdam

Molecular and serological studies on the recent seal virus epizootics in Europe and Siberia

T. Barrett[a], J. Crowther[a], A.D.M.E. Osterhaus[b], S.M. Subbarao[c], J. Groen[d], L. Haas[e], L.V. Mamaev[f], A.M. Titenko[g], I.K.G. Visser[b] and C.J. Bostock[a]

[a]*Institute for Animal Health, Pirbright Laboratory, Pirbright, Surrey GU24 0NF, UK*
[b]*National Institute of Public Health and Environmental Protection, P.O. Box 1, 3720 BA Bilthoven, Netherlands*
[c] *Seal Rehabilitation and Research Centre, Pieterburen, Netherlands*
[d]*Dept of Microbiology and Cell Biology, Indian Institute of Science, Bangalore, India*
[e]*Institut für Virologie, Tierärztliche Hochschule Hannover, Büntweg 17, D-3000 Hannover 21, Germany*
[f]*Limnological Institute, Siberian Division of the Russian, Academy of Sciences, 664033 Irkutsk, Lermontova 281, Russia*
[g]*Laboratory of Specific Pathogens, Antiplague Research Institute, Trilisser St. 78, Irkutsk 664047, Russia*

ABSTRACT

The virus epizootics which occurred in seals in both Europe and Siberia during 1987/1988 were caused by two different morbilliviruses, referred to as phocid distemper virus (PDV) 1 and 2, respectively. Molecular and serological studies have shown that the European virus is quite distinct from canine distemper virus (CDV), its closest relative in the morbillivirus group. Analysis of tissues obtained from infected seals from a wide geographical distribution over Northern Europe showed that the infectious agent (PDV 1) was identical in all cases. Nucleotide sequence analysis of one of the virus genes suggested that this virus has evolved away from CDV over a long time period and is most probably an enzootic virus of marine mammals. In contrast, the virus (PDV 2) which caused the deaths of many Siberian seals was indistinguishable, both serologically and at the molecular level, from CDV and must have originated from a land source.

Key words: cDNA hybridisation, seal morbilliviruses, PCR, ELISA, virus neutralisation

INTRODUCTION

The virus epizootic in seals in Northern Europe during the summer and autumn of 1988 was caused by a morbillivirus closely related to canine distemper virus (Osterhaus and Vedder, 1988). A similar disease in seals in

Lake Baikal, Siberia, was seen somewhat earlier than the European epizootic in December 1987 (Grachev et al., 1989; Likhoshway et al., 1989; Osterhaus et al., 1989a,b; Titenko et al., 1990). A morbillivirus was also isolated from porpoises in Northern Ireland (Kennedy et al., 1988) and more recently evidence was obtained of a similar infection in Mediterranean dolphins (Domingo et al., 1990). It appears that now there is one or more distinct morbillivirus(es) in marine mammals whose origin is unknown. The virus isolated from European seals has been called 'phocid distemper virus-1 (PDV-1)'.

The morbilliviruses which form an antigenically related genus within the Paramyxoviridae, are negative strand RNA viruses with an unsegmented single-strand genome of approximately 15 kb encoding six structural proteins and at least two non-structural proteins (Cattaneo et al., 1989; Barrett et al., 1991). In addition to canine distemper, virus (CDV) infecting dogs and Mustelidae, the group also includes measles virus in man (MV), rinderpest virus (RPV) in cattle and other large ruminants and peste-des-petits-ruminants virus (PPRV) in sheep, goats and other small ruminants.

Initial nucleic acid hybridisation studies using a variety of cDNA clones derived from several genes of four morbilliviruses (MV, CDV, RPV and PPRV), indicated that the European seal morbillivirus was genetically distinct from all of these and was probably a new virus (Mahy et al., 1988). Several European laboratories have independently isolated a morbillivirus from infected seal tissue (Cosby et al., 1988; Hofmeister et al., 1988; Osterhaus et al., 1988; Liess et al., 1989; Blixenkrone-Møller et al., 1989; Visser et al., 1990). This enabled more detailed biochemical and immunological analyses of the isolates and comparison of them with other known morbilliviruses. For example, the nucleocapsid protein of the seal isolates migrates more slowly on polyacrylamide gels than does the corresponding protein of CDV (Cosby et al., 1988; Rima et al., 1990). Recently partial sequence data on several genes from a European isolate have confirmed that the seal virus is genetically distinct from CDV (Curran et al., 1990; Haas et al., 1991). Sixteen independent isolates of PDV from the coasts of Northern Germany were found to be identical in reactivity with a panel of PDV-derived monoclonal antibodies but were distinguishable from CDV and other morbillivirus isolates (Harder et al., 1991). On the other hand, monoclonal antibody reactivities have established that a Siberian isolate of PDV is more closely related to Coin than are the European isolates and that the European and Siberian seal isolates are quite different from each other (Osterhaus et al., 1989a; Visser et al., 1990). This paper summarises molecular and serological data associated with the seal epizootic and provides new genetic and immunological evidence that the viruses isolated in Northern Europe are very similar, if not identical viruses, but that they differ

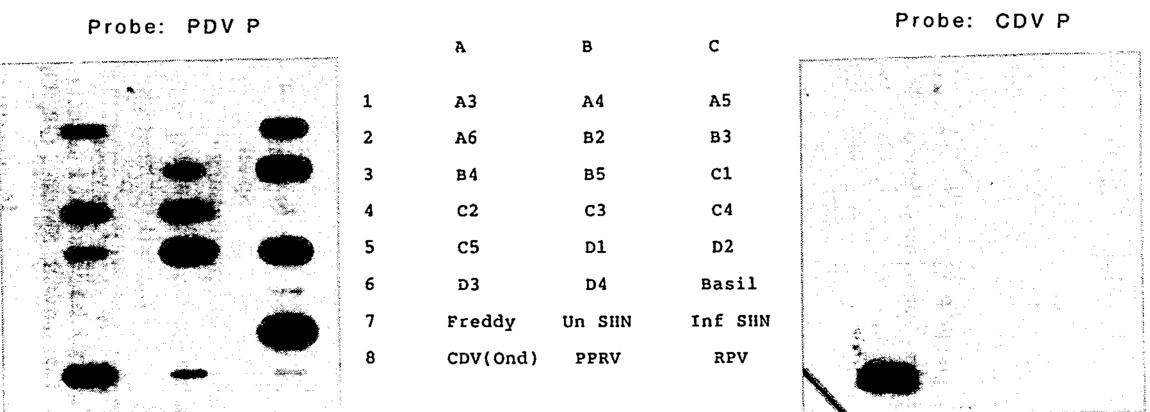


Fig. 1. Comparison of *P* gene-specific cDNA probes for the detection of virus-specific nucleic acid in RNA derived from seal tissues. The cDNA probes were derived from PDV-1 and CDV (Onderstepoort). Identical amounts of RNA (15 μg) were transferred in duplicate to 'Hybond N' nylon membrane (Amersham, UK Ltd) using a Sclheicher and Schuell 'Slot Blot' apparatus. One set of samples (left-hand panel) was probed with the PDV-specific probe and the other with the CDV-specific probe. After hybridisation the membranes were washed four times for 15 min in 2 × SSC, 0.1% SDS at room temperature followed by two washes in 0.2 × SSC, 0.1% SDS at 50°C for 20 min each. The dried membrane was autoradiographed at -70°C with an enhancer screen. The code listing the tissue and geographical origin of each sample is given in the legend to Table 1. Control RNAs from CDV, PPRV and RPV infected tissue culture cells were placed along the bottom row (row 8). Control uninfected (Un SHN) and PDV 1-infected (Inf SHN) seal kidney tissue culture cell RNAs were also blotted (row 7).

from the Siberian virus which closely resembles CDV and recent morbillivirus isolates from dog and ferret.

NUCLEIC ACID HYBRIDISATION ANALYSIS

European tissue samples

We have isolated and sequenced a P-gene specific cDNA clone from the Hannover/2558/Han-88 isolate of PDV (Haas et al., 1991) and confirmed that PDV-1 is distinct from CDV, since they share only about 80% nucleotide sequence identity in their *P* genes. Using these *P* genes as probes, hybridisa-

TABLE 1

Comparison of nucleic acid hybridisation and PCR analyses for the detection of virus-specific RNA in seal tissues collected from various locations during the morbillivirus outbreak. Those spleen tissues which were positive by hybridisation but negative in the PCR assay are marked with an asterisk. The primers used in the PCR assay were homologous to the 5' end of the *P* gene of the Hannover/2558/Han-88 isolate

Sample	Hybridisation	PCR	Tissue	Origin[a]
A1	ND	–	Spleen	NL
A2	ND	–	Lung	NL
A3	–	–	Lung	DK
A4	–	–	Lung	DK
A5	±	–	Lung	D
A6	++	+++	Spleen	NL
B1	ND	–	Spleen	USSR
B2	–	–	Lung	USSR
B3	+++	+++	Lung	S
B4	–	–	Lung	S
B5*	++	±	Spleen	D
C1*	++++	–	Spleen	NL
C2	+++	++	Lung	NL
C3*	+++	–	Spleen	D
C4	±	+	Spleen	GB
C5	+	+	Spleen	NL
D1*	++++	–	Spleen	NL
D2	++++	++++	Lung	NL
D3	±	++	Lung	D
D4	–	–	Lung	D
Basil	±	+++	Spleen	GB
Freddy	–	–	Spleen	GB

[a]NL, Netherlands; DK, Denmark; D, Germany; S, Sweden; GB, Great Britain; USSR, Union of Soviet Socialist Republics.

tion analysis of RNA derived from a range of tissues collected from seals which had died from the disease at sites widely distributed over Northern Europe has confirmed that the European isolates fall into a single group (referred to as PDV-1 by Visser et al., 1990). In Fig. 1 are shown the results of this analysis where it can be seen that the *P* gene from CDV (Onderstepoort strain) does not hybridise significantly with any of the tissues examined, whereas the PDV-specific clone hybridises very strongly with many of them. Positive results were obtained with samples from a wide geographical distribution indicating that the same or a very similar virus was responsible for the infections throughout Northern Europe (see also Table 1).

Siberian tissue samples

Unlike samples from European seals, those from the Baikal seal tissues hybridised at similar, but low levels, with cDNA clones from the *P* genes of both CDV and European PDV. Similar levels of hybridisation were also seen with RNA derived from Vero cells infected with two recent CDV isolates from mink and dog in Germany in 1989 (Fig. 2). As shown before in Fig. 1, the CDV (Onderstepoort) probe did not hybridise significantly with PDV-1. In studies of this kind it is not possible to make precise quantitative assessments of homology since the strength of the hybridisation signal is dependent on the amount of virus-specific RNA on the filter and this remains an unknown quantity. This is especially so in the case of seal tissue-derived samples where the amount of virus-specific RNA is much lower than in tissue culture-derived RNA. Nevertheless, the results indicate that the European and Siberian seal viruses are genetically distinct from each other and that the Siberian isolate is more closely related to CDV isolates confirming the recent immunological data based on monoclonal antibody reactivities (Osterhaus et al., 1989a; Visser et al., 1990).

POLYMERASE CHAIN REACTION (PCR) ANALYSIS

European seal tissue samples

Application of the PCR technique to the tissue samples isolated from seals from many different parts of Northern Europe generated in many of the samples a DNA fragment of the expected size which, on Southern blotting, hybridised with the specific PDV *P* gene probe (Fig. 3). Some of the spleen tissue samples, which had hybridised strongly with the PDV probe, failed to generate a PCR fragment. This inhibition of the reaction has been noted by others (De Franchis et al., 1988; Higushi, 1989) and it appears that

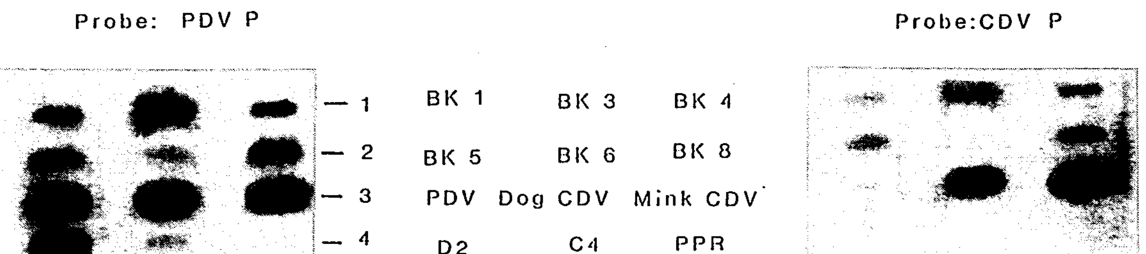


Fig. 2. Comparison of P gene-specific cDNA probes for the detection of virus-specific nucleic acid in RNA derived from Siberian seal tissue samples. The RNA samples were blotted onto nylon membrane and hybridised as described in the legend to Fig. 1. Rows 1 and 2 contained RNA derived from various Baikal seal tissues collected during the epizootic: BK 1, heart; BK 3, spleen; BK 4, liver; BK 5, kidney; BK 6, infected tissue culture cells; BK 8, brain. RNAs derived from Vero cells infected with PDV 1, PPRV and two recent German isolates of CDV from dog and ferret were also blotted in rows 3 and 4. D2 and C4 were from European seal tissues (see Table 1).

haemoglobin breakdown products can interfere with the activity of the Taq polymerase. This effect applies particularly to the analysis of the spleen tissues, since this organ contains a high content of haemoglobin, which would become degraded through autolysis resulting from delays in sample collection and handling. In contrast, some other tissues which were negative or borderline positive in the hybridisation test proved positive by PCR, which is clearly the more sensitive assay if inhibitors are not present. Table 1 lists the results of both hybridisation and PCR assays on the tissue samples.

RNA from CDV isolated from dog and ferret at about the same time as the Hannover 2558/Han-88 isolate of PDV generated a different-sized fragment of approximately 600 base pairs using the PDV-specific amplimers. Although of unpredicted size, this was a morbillivirus-specific fragment since it cross-hybridised at low levels with the PDV *P* gene cDNA clone on Southern blotting of the gel (see tracks 10 and 11, bottom of Fig. 3). It is probable that a sequence, sufficiently similar to the upstream primers to allow annealing, is present in a different part of the gene in the field isolates of CDV which is responsible for the different sized fragment. Examination of the nucleotide sequence of the *P* gene of CDV Onderstepoort failed to show any region of sufficient homology to this primer, but a sequence homologous to the downstream primer was present. Since there is no sequence data for the field isolates of CDV, it is not possible to confirm the presence and location of primer-homologous sequences. A faint band of the size expected for PDV was also present, but in lower amounts, in the Southern blot. Unlike the larger fragment, however, this was not visible on staining with ethidium bromide and may represent vestigial homology at the original site.

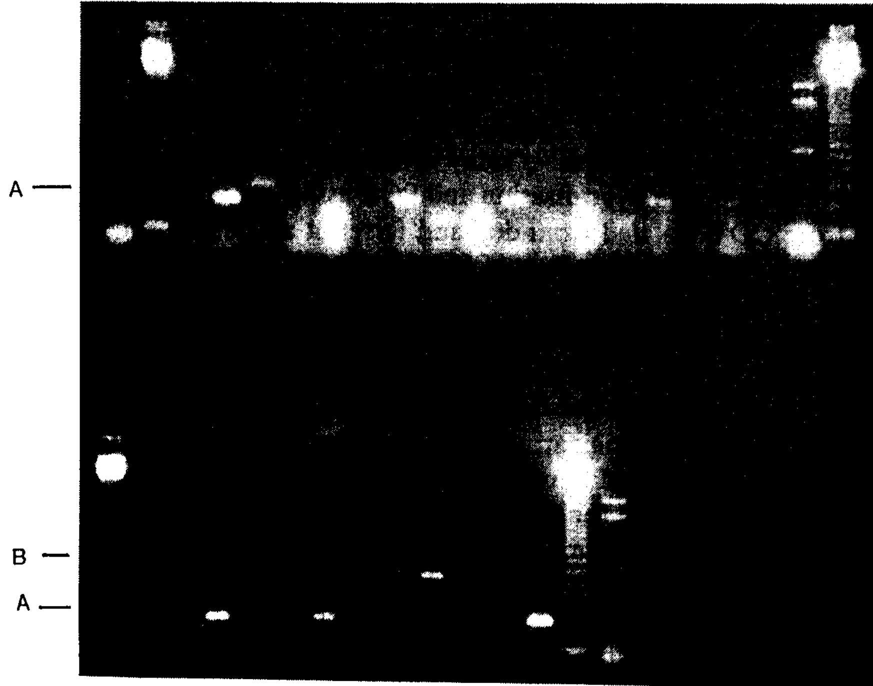
Siberian seal tissue samples

RNA from Siberian tissues generated a larger sized fragment similar to that seen previously with the RNA derived from the recent CDV isolates from Europe (compare track 1 with track 5 in Fig. 4) which also hybridised weakly with the PDV P gene probe. This indicates that the Siberian virus is genetically closely related to CDV isolates.

SEROLOGICAL STUDIES

Detection of PDV-specific antibodies

Sera collected from healthy, ill and dead common and grey seals from around Britain's coast at various times during the epidemic have been examined by a variety of serological tests (Bostock et al., 1990). Sera were assessed for the presence of antibodies with specificities for the related morbillivirus


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
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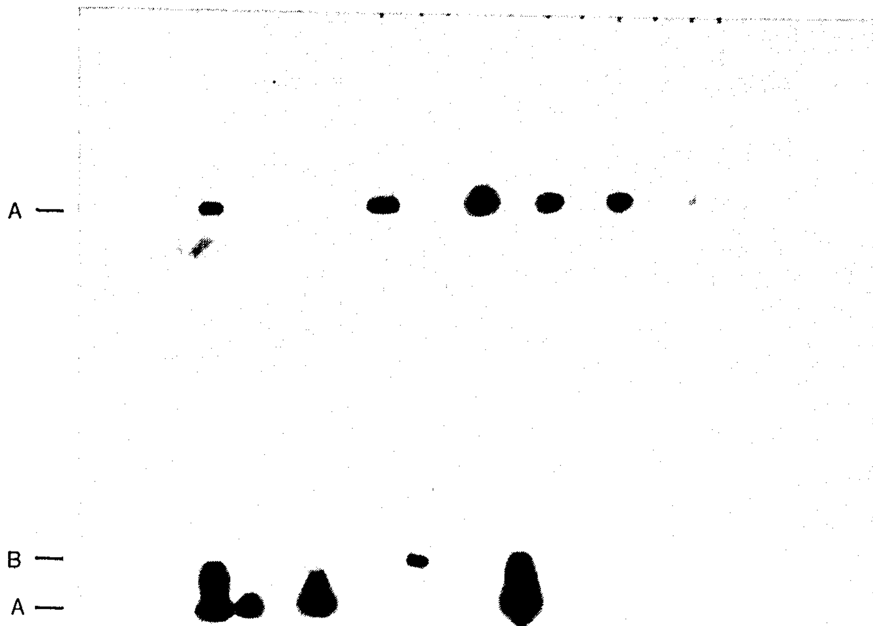
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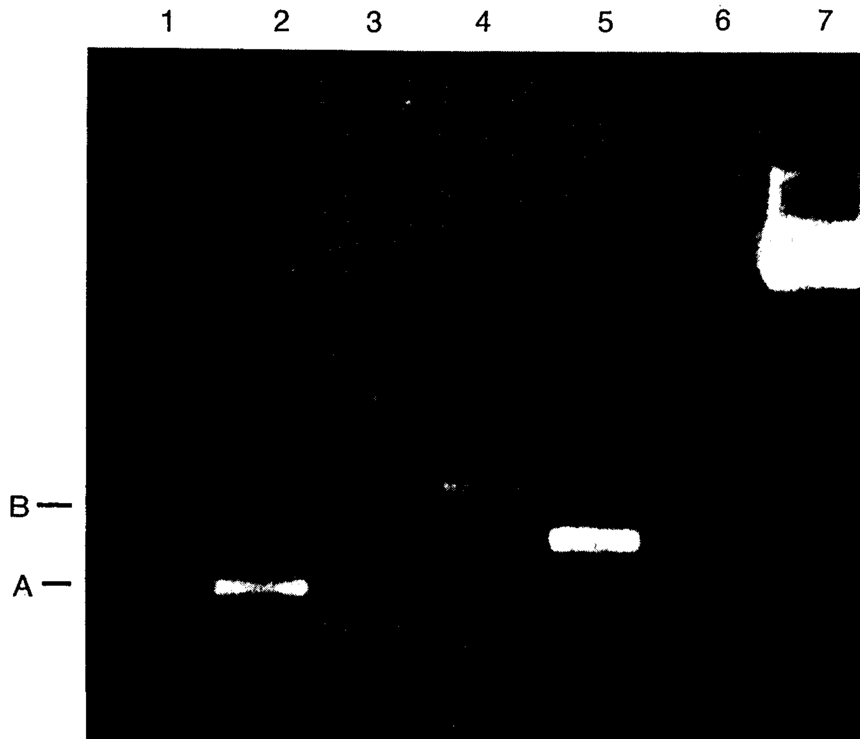
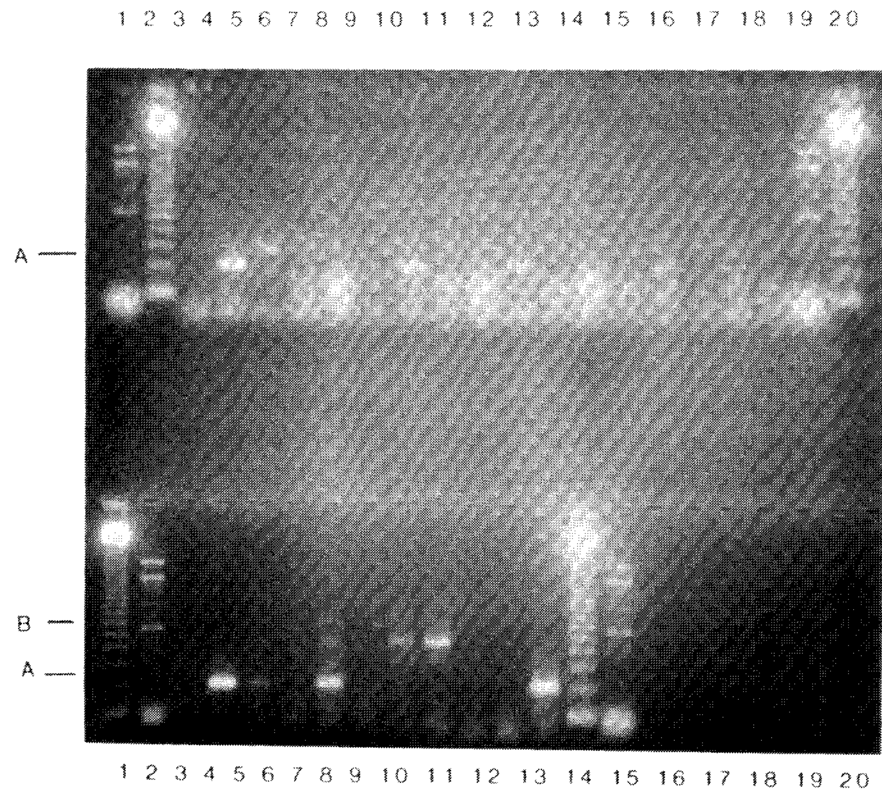


Fig. 4. PCR analyses on RNAs derived from European and Siberian seal tissues. The PCR-derived DNAs were separated on a 2% agarose gel as detailed in Fig. 3. The samples were: 1, recent dog CDV isolate; 2, A6 European seal spleen; 3, Siberian seal spleen (BK 3, Fig. 2); 4, Siberian seal brain (BK 8, Fig. 2); 5, DNA derived from RPV RNA using RPV fusion gene-specific primers; 6 '123' base pair marker DNA. The fragment generated by sample 5 is 376 base pairs and co-migrates with the third band in the '123' marker ladder.

Fig. 3. PCR analyses on RNAs derived from European seal tissues and recent dog and ferret CDV isolates from Germany. The upper panel shows an ethidium bromide-stained agarose gel separation of the PCR-generated DNAs. The lower panel is a Southern blot of the same gel hybridised with the PDV-1-specific P gene probe. The agarose gel consisted of 2% agarose in TBE buffer (TBE = 89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.3). The PCR reactions were carried out as described by Haas et al. (1991) except that the annealing temperature was reduced to 25°C because of the high A/T content of the PDV-specific primers used. The samples were loaded in two rows on the same gel. A size marker DNA consisting if a '123' base pair ladder was placed in lanes 2 and 20 on the upper gel, and lanes 1 and 14 on the lower gel. Another size marker, consisting of a *Rsa* I digest of plasmid pUC 19, was placed in lanes 1 and 19 on the upper gel and lanes 2 and 15 on the lower gel. The assay samples were placed in lanes 3–18 on the upper gel and lanes 3–13 on the lower gel. A marks the position of the DNA fragment of 245 base pairs (i.e. comigrating with the second DNA band of 246 base pairs in the '123' marker). The DNA fragment of an unexpected size which was produced from RNA derived from the recent German ferret and dog isolates is indicated by B at the side of each panel. It migrated at a position between the fourth and fifth DNA band in the '123' marker and so is between 500 and 600 base pairs in length (see lanes 10 and 11).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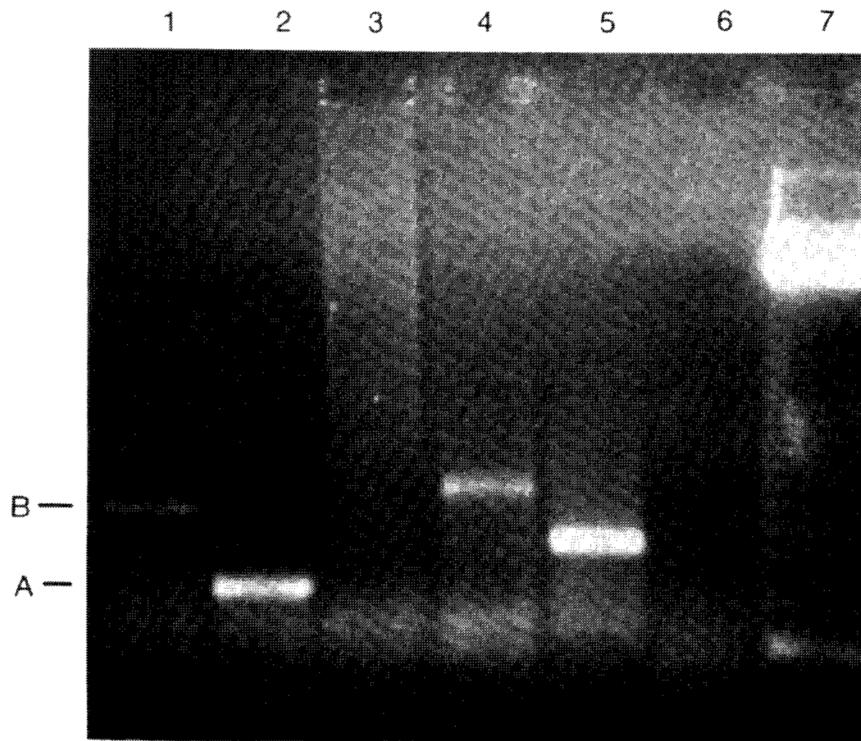
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RPV using virus neutralisation (VN) tests and indirect ELISAs based the assay, developed by Anderson et al. (1983, 1991), for the detection of bovine anti-RPV antibodies. These assays were used since at the time there was no PDV-specific test available but were found to be suitable for analysis of morbillivirus-specific antibodies in sera from the British outbreak. Subsequently sera became available from seals sampled at various times before the outbreak and these were used to assess negative serum values with defined confidence limits. Table 2 details the results of this study and compares the virus neutralisation and ELISA data for these sera. On the basis of these results, and the clinical findings, the samples were placed into six groups with an overall correlation between positive ELISA and disease.

Subsequently 1000 sera, collected from seals from other parts of Northern Europe were examined for the presence of antibodies using both CDV and RPV indirect ELISA with protein A and anti-seal conjugates as well as VN tests for both viruses (Osterhaus et al., 1989c). This study confirmed that the virus which infected European seals induced antibodies that cross-neutralised both CDV and RPV. It also confirmed the specificity of the CDV

TABLE 2

Classification of seals sampled during the British morbillivirus outbreak based on clinical and serological data (Bostock et al. 1990)

Group	RPV ELISA	RPV VN	Clinical Details	Comment
A	–	–	Healthy	No exposure to virus.
B	–	–	Sick/dead	Animals sampled or died early in infection (1–6 days) before antibody response.
C	++	++	Healthy	Recovered animals (typical of Grey seal populations).
D	–	+	Healthy	Low titre VN may indicate problem in determining negative threshold of VN test
E1	+/–	–	Sick/dead	Animals representing early stages at 6–14 days of infection during which production of antibody has begun.
E2	++	–	Dead	Antibodies only detected by ELISA because of greater sensitivity or because tests may detect different antibodies.

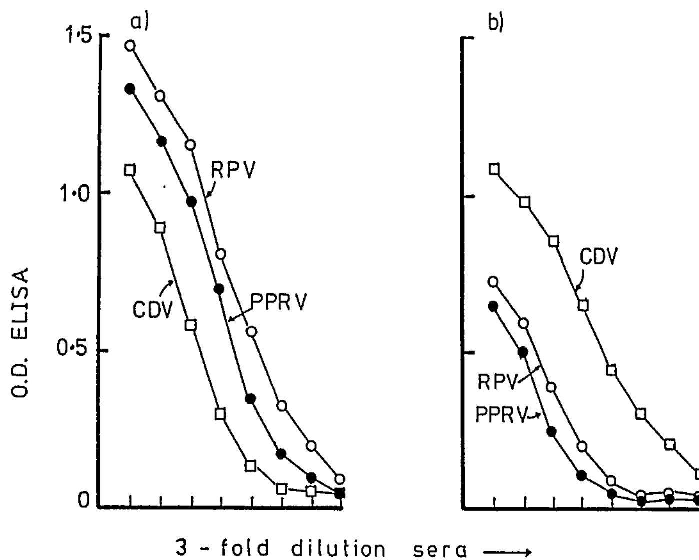

Fig. 5. Cross-reactivity of seal sera in ELISAs using purified RPV, PPRV and CDV as antigens. Purification was carried out by centrifugation through both sucrose and tartrate gradients (see Barrett et al., 1989). The antigens were adsorbed to microtitre plates at saturating concentration as assessed by titration against homologous rabbit sera prepared against the respective purified antigens. The sera diluted (3-fold dilutions) and reacted with the antigens. Bound antigen was detected using Protein A horse radish peroxidase conjugate followed by the addition of OPD/H_2O_2. Panel (a): reactivity of a typical serum from the British outbreak; panel (b): reactivity with a serum sample from an infected Lake Baikal seal.

and RPV ELISAs for detecting the presence of antibodies to the seal virus and, for sera collected after the onset of the epizootic, the correlation between positive ELISA and virus neutralisation tests was high (Osterhaus et al., 1989c).

Cross-reactivity of seal antibodies

The cross reactivity of a serum from a single infected seal from Lake Baikal was examined using indirect ELISA where sucrose and tartrate gradient purified RPV, CDV and PPRV (Barrett et al., 1989) were adsorbed to microtitre plates at a saturating concentration as assessed by titration against homologous rabbit sera prepared against the respective purified antigens. The Lake Baikal serum and several positive seal sera from the British outbreak were then diluted and reacted with the antigen coated plates. The relative binding with the different antigens was compared. Figure 5a shows the titration of a single positive British seal serum, in which the reaction with

RPV and PPRV is six-fold higher than with CDV, and is typical of 15 similar sera examined. On the other hand, with the Lake Baikal serum titrations (Fig. 5b) the reaction with CDV is greater (approx. 10-fold higher) than for PPRV or RPV. These results confirm the serological data of Visser et al. (1990) and are in accordance with the molecular data presented above which indicates that the Baikal outbreak was caused by a virus very closely related or identical to CDV.

DISCUSSION

This paper illustrates the usefulness of molecular biological and immunological methods for the comparison of closely related viruses. Members of the genus Morbillivirus show considerable antigenic cross-reactivity and have been difficult to differentiate solely by serological means using polyclonal antisera. This was highlighted by the fact that an ELISA using RPV as the antigen could be used for the detection of antibodies against the seal morbillivirus and the close agreement between data using CDV and RPV as antigens. Recent research using monoclonal antibodies show that they can be used for rapid discrimination of viruses (Cosby et al., 1988; Osterhaus et al., 1989a; Blixenkrone-Møller et al., 1990; Visser et al., 1990; Harder et al., 1991), however, the most detailed way to characterise and differentiate these viruses is by genome sequence analyses. The development of the PCR technique and its use in sequence analysis of virus isolates has shown the practical feasibility of this approach (Yamada et al., 1989; Taylor et al., 1990a; Haas et al., 1991).

In the past diagnosis of the morbilliviruses was generally based on the species of animal from which the virus was isolated. The recent seal virus epizootic showed that this is not a satisfactory method of classification since more than one virus can infect a particular species. Similar problems are encountered with the closely related viruses of large and small ruminants, RPV and PPRV, which can cross-infect the other species in certain cases (Diallo et al., 1989; Anderson et al., 1990). Molecular hybridisation has been used successfully to classify these viruses from postmortem samples and from isolates grown in tissue culture. For example isolates of PPRV from a recent outbreak in Oman more closely resembled RPV antigenically than did older African isolates and the nucleocapsid protein migrated in a position intermediate between that of RPV and the older PPRV strains (Taylor et al., 1990b). However, hybridisation analyses clearly differentiated the two viruses. Similarly, nucleic acid hybridisation has been used to show that RPV and PPRV viruses co-circulate in small ruminants in India (Shaila et al., 1990).

The hybridisation studies described here clearly showed that the various

European seal virus (PDV-1) isolates are similar, if not identical to each other, and that they differ from the Siberian virus (PDV-2) isolate which more closely resembles recent CDV isolates from dog and ferret in Europe. The PCR analyses also agreed with this conclusion. However, the hybridisation analyses were not clear-cut and the PDV-1 cDNA clone cross-hybridised more strongly with RNA from recent CDV isolates than with RNA from the laboratory strain (CDV Onderstepoort) This may reflect evolution within this group of viruses where PDV-1 is a distinct virus which is more closely related to recent European CDV isolates than to CDV Onderstepoort. In turn, this could reflect the different geographical origins of the CDV strains (Europe vs. Africa). Within the measles virus group it has been shown that geographical differences are reflected in sequence differences in this virus (Taylor et al., 1990a). In contrast it would appear that the Siberian seal virus PDV-2 is indistinguishable from the recent European CDV isolates both by nucleic acid hybridisation and PCR analyses. This conclusion is consistent with all the serological tests carried out on the PDV isolates which showed that PDV-1 is a distinct virus but that PDV-2 is almost indistinguishable from CDV.

CDV infects a wide range of carnivore species (Appel, 1987) but, until the identification of phocid distemper virus as the cause of the seal deaths in Northern Europe in 1988, no morbillivirus had been isolated from a marine or freshwater mammal. It is probable that the virus was present for some considerable time in some unknown seal population in the North Atlantic (Diets et al., 1989) and then spread to infect other seal populations. CDV is known to infect a wide variety of carnivore species and its effect in wildlife populations can be devastating. CDV almost wiped out the last remaining blackfoot ferrets in the United States. There is also a danger in using live attenuated vaccines developed for domestic animals since they are not necessarily attenuated for non-target species (Carpenter et al., 1976). This widely different response of a virus in different but related species was illustrated by the fact that PDV-1 had a devastating effect on harbour seals but was mainly a sub-clinical infection in grey seals. In the case of the Lake Baikal epizootic it is probable that the source of infection was through land animals infected with the virus and an outbreak of CDV in dogs was seen at the same time (Grachev et al., 1989).

Since PDV-1 was antigenically closely related to other morbilliviruses, cross-reactivity in immunological tests makes clear differential diagnosis difficult, particularly when a disease is seen in an unusual species. The ELISA and neutralisation tests available in 1988 for CDV and RPV diagnosis were equally good at detecting the presence of a morbillivirus antibody in seal sera but could not be used to further identify the virus. Since then the availability of specific cDNA probes, PCR primers and new monoclonal antibodies has

enabled us to draw finer distinctions between the viruses in the group and will aid in the identification of new morbillivirus isolates. The degree of evolution within the individual members of the morbillivirus group can only be finally demonstrated by comparative sequence analysis and this work is now underway in several laboratories.

ACKNOWLEDGEMENTS

S.M.S. and T.B. were the recipients of Royal Society short-term Fellowships.

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