Short Communication

Identification and real-time PCR quantification of *Phocine distemper virus* from two colonies of Scottish grey seals in 2002

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The North Sea European harbour seal (*Phoca vitulina*) population has endured two phocine distemper virus (PDV) epidemics in 1988 and 2002. The grey seal (*Halichoerus grypus*) is a sympatric seal species that shows little or no mortality from PDV. Two Scottish grey seal breeding colonies were sampled for evidence of PDV infection approximately 2 months after the peak of the 2002 epidemic. In both colonies, a proportion of mothers (13/109) and pups (6/84) tested positive for PDV in their leukocytes. All infected animals were asymptomatic and completed the breeding season successfully. These results illustrate that grey seals come into contact with infectious seals and can become infected themselves without experiencing acute effects. In some seals the virus is able to replicate from the primary site of infection. This study provides evidence that grey seals may have an active role in the spread of PDV during an epidemic.

It was during the outbreak of PDV among European harbour seals (*Phoca vitulina*) in 1988 that the causative virus was first isolated and identified (Mahy *et al.*, 1988; Osterhaus & Vedder, 1988; Curran *et al.*, 1990). The disease spread from the Skagerrak/Kattegat and Wadden Sea in April, reaching the UK by August and eventually killing up to 60 % of North Sea harbour seals (Dietz *et al.*, 1989b). A second PDV outbreak occurred amongst the same populations in 2002 killing around 47 % of the North Sea population (Jensen *et al.*, 2002; CWSS, 2003; Müller *et al.*, 2004). The 2002 virus was shown to be >97 % identical (P gene) to 1988 PDV isolates from harbour seals and distinct from those of *Canine distemper virus* (CDV) and other members of the genus *Morbillivirus* (Jensen *et al.*, 2002).

One putative source of PDV is the harp seal (*Phoca groenlandica*), a species in which the virus appears to be endemic (Dietz *et al.*, 1989a; Markussen & Have, 1992). In 1987, large numbers of harp seals from the Barents Sea invaded northern European waters (Goodhart, 1988), where they probably came into contact with harbour and grey (*Halichoerus grypus*) seals. However, since no similar invasion of harp seals occurred prior to the 2002 epidemic, another vector must have been able to reinfect what was by then a susceptible harbour seal population (Thompson *et al.*, 2002).

Harbour and grey seals in Europe, but particularly in the UK, are sympatric and often share the same haulout sites in coastal waters. Both species are widespread in the UK but show very different susceptibilities to PDV (Harder *et al.*, 1990; Hall *et al.*, 1992; Thompson & Hall, 1993; Lawson & Jepson, 2004). Of the 87 grey seals post-mortemed in 2002 only 10 tested positive for PDV by RT-PCR and none of them had died from the disease (Baker, 1992). Most grey seal deaths reported during both PDV outbreaks were due to other causes (Lawson & Jepson, 2004). Thus, even if a proportion of the grey seal population did acquire PDV infection, the virus did not cause the obvious mortality as it had in harbour seals.

We have recently shown, using serological surveys, that a very large proportion (>85 %) of the breeding females on North Rona (off the NW of Scotland) and on the Isle of May (in the Firth of Forth on the SE coast) had been recently exposed to PDV (Pomeroy *et al.*, 2005). The present study was undertaken to ascertain if the leukocytes of adult female grey seals and a subsample of their pups contained viral RNA, indicative of a state of viraemia, and if maternal transfer was a factor in the spread of the virus. Real-time PCR was chosen over conventional RT-PCR as it allowed for both detection and comparative quantification of the virus particles. Such data would allow comparisons with the levels

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of virus present in different adults and their pups, as well as between the different study sites.

Animals were sampled approximately 2 months after the peak of the epidemic and 1 month after the cumulative number of cases reported in the UK had reached an asymptote (Lawson & Jepson, 2004). This is the time that grey seal breeding colonies form in the UK (between August and December depending on geographical location), and when there are a large number of animals in close proximity. Pups born into these colonies would not have had direct exposure to harbour seals and their antigenic exposure after birth is limited to the colony.

A total of 109 adult female grey seals and 84 of their pups were sampled from the Isle of May, Firth of Forth, Scotland (56° 11′ N, 2° 33′ W) from mid-October to early December 2002; or from North Rona, Outer Hebrides, Scotland (59° 06′ N, 05° 50′ W) from late September to early November 2002. These are two well-studied grey seal breeding colonies that provided some previous knowledge of individual animals' life history (e.g. Pomeroy *et al.*, 2000, 2001). Sampling was undertaken either 1–4 days after pupping ('early' lactation) or $\geqslant$9 days after pupping ('late' lactation). A subpopulation of these mother–pup pairs was sampled at both time periods. All adults were designated either 'old' (breeding during the 1988 epidemic) or 'young' (born after the 1988 epidemic), based on ages derived from tagging or counting cementum and dentine layers in an incisor tooth (Age Dynamics).

All techniques involving live animals were carried out under UK Home Office Licence regulations. Leukocytes were harvested from 10 ml peripheral blood withdrawn from the extradural vein into heparinized Vacutainers (Becton Dickinson) using standard protocols and resuspended in five times the volume with RNAlater (Ambion). In addition, 5–10 ml milk was withdrawn from the mother after intravenous injection of oxytocin (Leo Labs) and a swab of the nasal passage was also taken and stored in liquid nitrogen.

Total RNA was extracted from all samples using Tri Reagent (Sigma) and was DNased before RNA was accurately quantified using RiboGreen (Molecular Probes). cDNA was transcribed from up to 2 μg total RNA with Superscript II (Invitrogen) using random nonamers.

Determination of PDV RNA abundance was performed with a TaqMan probe and a pair of primers for the PDV H gene (Table 1). Initially, gene-specific primers, designed using the PDV H gene sequence from the 1988 epidemic (GenBank accession no. D10371), were used to amplify viral cDNA fragments from harbour seal tissues recovered from carcasses that tested positive for PDV in 2002. A probe and primer set was then designed from a region identical to one in the 1988 isolate and is known to be conserved in this genus of viruses, and all the 2002 carcasses were tested (Table 1) (Curran *et al.*, 1992). A TaqMan probe and primer set for grey seal β-actin was employed as an indicator of the cDNA integrity in each sample and to confirm PCR efficiency (Table 1).

Real-time PCR was conducted on all samples to identify those positive for viral RNA with QuantiTect Probe PCR Mastermix (Qiagen) using an ABI Prism 7000 SDS (Applied Biosystems) and the standard cycling conditions. A standard curve was produced to determine the absolute copy number of the virus in relation to the starting amount of total RNA by cloning a fragment of the H gene into the pCR Topo 2.1 vector (Invitrogen). Plasmid DNA was accurately quantified using PicoGreen double-stranded DNA quantification reagent (Molecular Probes) and used to produce a standard curve. The following equation was used to determine the exact number of H gene fragments in the DNA preparation (taken from Roche Molecular Biochemicals Technical note

Table 1. TaqMan primer and probe sets used for each of the genes

The fluorescent labels used are next to the name of each gene.

Primer/probe parameters	Gene	
	PDV H (FAM)	β-Actin (JOE)
Forward primer	5′-ACCTCGATGGGCAATGTGTT-3′	5′-CCCGGAGGCGCTCTTC-3′
Melting temp	59 °C	59 °C
Optimal conc.	300 nM	300 nM
Reverse primer	5′-GTCTTACCGTAGATCCCTTCTGAGAT-3′	5′-CAACTCCATCATGAAGTGTGACG-3′
Melting temp	59 °C	59 °C
Optimal conc.	300 nM	300 nM
Probe	5′-CATGTCCCTCATATCAAAACCTTCGGAGG-3′	5′-AGCCTTCCTTCCTGGGTATGGA-3′
Melting temp	68 °C	69 °C
Optimal conc.	50 nM	75 nM
Amplicon	115 bp	89 bp

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$$\frac{6\times10^{23}\ (\text{copies mol}^{-1})\times\text{concentration (g }\mu\text{l}^{-1})}{\text{molecular mass (g mol}^{-1})} = \text{amount (copies }\mu\text{l}^{-1})$$

where the average molecular mass of double-stranded DNA is: (no. of bp) × (660 daltons per bp).

Bayesian 95 % confidence intervals (CI), using a flat prior, were used to assess the significance of any differences in the number of PDV-positive females or pups between breeding sites, and between early and late lactation stages. In addition, comparisons were made between the number of PDV adults in young or old age groups wherever possible. For each analysis group, the mean and median positive proportions among the groups were calculated. Statistical analysis was not performed on the virus copy number data as the final sample size for each group was too small.

None of the seals sampled at either site displayed overt signs of PDV infection. However, real-time PCR on the leukocytes revealed that of the 55 adult females sampled on the Isle of May, three tested positive for the presence of PDV [mean 5·5 %, median 6·5 %, 95 % Bayesian confidence limits (CL) 2·0–14·9 %] (Table 2). Of the 54 adult females sampled on North Rona, 10 tested positive for PDV (Table 3), a level significantly higher than on the Isle of May (mean 18·5 %, median 19·3 %, 95 % Bayesian CL 10·4–30·9 %; Table 2). Among the subgroup of females sampled twice, two were found to be positive early in lactation and five positive in late lactation on North Rona. None tested positive on both occasions (Table 3). None of the positive females on the Isle of May were among those tested twice. The small number of PDV-positive samples on the Isle of May precluded comparisons between levels of infection between young and old females or between the lactation stage. There was no relationship on North Rona between the age of the mother and PDV status, or the stage of lactation when testing positive (Table 3).

Among the pups, four of 48 sampled on the Isle of May were found to have PDV RNA in their leukocytes, although none had mothers that also tested positive (Table 3). On North Rona, two of 36 pups tested positive with only one known to have a PDV-positive mother. This positive pair was tested in late lactation. No significant differences were

Table 2. Number and percentage of animals tested positive for PDV on the Isle of May (IOM) and North Rona (NR)

Animals	IOM	NR
Adult females		
Total tested	55	54
Positive	3	10
Positive early lactation	3	5
Positive late lactation	0	5
Mean	5·5 %	18·5 %
Mode	5·6 %	18·6 %
Median	6·5 %	19·3 %
95 % Bayesian CI	20–14·9 %	10·4–30·9 %
Pups		
Total tested	48	36
Positive	4	2
Positive early lactation	0	0
Positive late lactation	4	2
Mean	8·3 %	5·6 %
Mode	8·4 %	5·7 %
Median	9·5 %	7·2 %
95 % Bayesian CI	3·4–19·6 %	1·7–18·2 %

Table 3. PDV copy number in positive animals

Identities of animals tested positive for PDV and the quantity of viral RNA amplified from the leukocyte cDNA by real-time PCR. All the other animals were negative. The PDV mRNA copy number is given as copy number per pg RNA. Animals aged young were born after the 1988 epidemic and animals aged old were alive during the last epidemic. Ages given for the pups are the ages of their mother. An asterisk (*) after the animal ID shows that it was sampled early and late in lactation. Mean copy number for each island and group of animal is given with the standard deviation.

Positive animal ID	Age	Time in lactation	PDV copy number
Isle of May			
IOM female 1	Old	Early	4·38
IOM female 2	Old	Early	1·95
IOM female 3	Young	Early	0·61
IOM pup 1*	Young mother	Late	79·54
IOM pup 2	Old mother	Late	53·49
IOM pup 3	Young mother	Late	4·7
IOM pup 4*	Young mother	Late	1·2
North Rona			
NR female 1	Old	Early	45·56
NR female 2*	Old	Early	26·80
NR female 3*	Old	Late	25·06
NR female 4*	Old	Late	15·02
NR female 5*	Young	Early	11·93
NR female 6	Age unknown	Early	8·27
NR female 7*	Old	Late	3·18
NR female 8	Young	Late	2·62
NR female 9*	Old	Early	1·35
NR female 10*	Old	Late	0·71
NR pup 1*	Old mother	Late	11·2
NR pup 2*	Young mother	Late	6·25
Median copy numbers and interquartile range (per pg RNA)			
Isle of May	4·38 (1·57–29·09)		
North Rona	9·76 (3·04–17·53)		
All mothers	4·38 (1·95–15·02)		
All pups	8·72 (5·09–42·917)		

detected in the amount of viral RNA of mothers and the pups, or between islands.

All RNA obtained from the somatic cells of milk samples of positive mothers tested negative for PDV. RNA could not be obtained from the nasal swabs of positive animals due to the small volume.

This study shows that grey seals were infected with PDV during the 2002 breeding season. As the primary infection site of distemper viruses is the lymph nodes (Appel & Gillespie, 1972), the isolation of viral RNA from leukocytes indicates that some infected animals were likely to have been temporarily viraemic, although the infection appeared to remain subclinical. Whilst it is not clear if infected grey seals are infectious to others, circumstantial evidence is suggested by our finding that pups at each colony became infected. None of the pups that tested positive for PDV did so until a later stage in lactation, implying that these animals were infected post-partum, and not *in utero*. Previously, overtly healthy adult grey seals had not been investigated for their potential to carry and allow the replication of PDV due to their low mortality during the previous epidemics (Harwood, 1990; Hall *et al.*, 1992).

None of the adult animals that tested positive did so at both stages in lactation. Animals that were positive early in lactation (potentially positive when they arrived at the island) were all negative late in lactation, indicating that the virus had been removed from the blood. Adult animals that tested positive only later during lactation were either already infected but PDV was not yet detectable in the blood or they became infected whilst in the breeding colony. Parturition and lactation are potentially immunosuppressive (Lloyd, 1983) and these may be factors involved in inducing viraemia in animals infected during or prior to the breeding season. In harbour seals, the mean generation time for PDV is approximately 15 days (Swinton, 1998). Within this time frame the females that were positive early and late in lactation could have arrived in the colony with PDV.

The six pups that tested positive for PDV were only viraemic later in lactation implying infection occurred in the breeding colony. Only one of these pups had a mother that was also viraemic. As having an infected mother (even early in lactation) did not necessarily cause a pup to become viraemic by late lactation, the infectious period and the factors determining likelihood of pup infection are not clear. None of the milk samples tested from mothers at the time that they were viraemic contained infected somatic cells. There were no positive pups among those tested in early lactation ($n=55$). This indicates that maternal transfer of the virus does not occur before birth. The most likely route for transmission is via nasal secretions but, unfortunately, insufficient nasal mucus was obtained for analysis in this study to test this notion.

Grey seals are unlikely to be in contact with harbour seals in breeding colonies, but depending on location the two species are likely to come in contact prior to the grey seal breeding season. Grey seals mostly forage locally from a particular haulout site and can make large-scale movements between distant haulouts (McConnell *et al.*, 1999), whereas harbour seals do not appear to move regularly between distant sites (Thompson *et al.*, 1996). If infective animals continue to behave normally, grey seals could therefore be a factor for the spread of infection, particularly between distant sites. A simple compartmental model suggested within species contact rates in 2002 were remarkably similar among all populations studied (M. Lonergan, A. J. Hall, H. Thompson, P. M. Thompson, P. P. Pomeroy & J. Harwood, unpublished results).

The finding that the number of PDV-positive mothers was higher at North Rona than the Isle of May was unexpected. In both outbreaks of PDV, cases were first reported from the east coast of the UK, with a complex pattern of spread thereafter. Very few harbour seal deaths from PDV were reported on the west coast of Scotland in 2002 and none of the live harbour seals on the west coast sampled in 2003 had positive morbillivirus antibody titres (M. Lonergan, A. J. Hall, H. Thompson, P. M. Thompson, P. P. Pomeroy & J. Harwood, unpublished results). Indeed, there is little evidence of an epidemic in Scottish harbour seals (Lawson & Jepson, 2004). However, it would seem more likely that an east coast colony would have more extensive and prolonged contact with infective animals than an isolated north-west coast colony.

Comparison of PDV-positive mothers' morbillivirus $\log_{10}$ antibody titres with those from negative animals indicated they were significantly different. In a Welch modified two-sample *t*-test with unequal variances, samples from females that were real-time PDV-positive (mean $1{\cdot}85 \pm$ SEM $0{\cdot}154$, $n=13$) were lower than those that were negative (mean $2{\cdot}24 \pm$ SEM $0{\cdot}039$, $n=176$, $P=0{\cdot}029$). The immunosuppressive nature of morbilliviruses is well known (Lloyd, 1983), but our results confirm those of Cornwell *et al.* (1992) that viraemic animals may have a positive antibody titre.

In conclusion, grey seals are likely to aid in the spread of PDV during an epidemic. However, their potential to act as a reservoir for the virus between outbreaks still needs to be investigated. This study also shows that simple single-host models for the spread of PDV are probably insufficient. Multi-species models and the incorporation of inter- and intra-specific differences in movements and behaviour are needed (Hall, 1995). In particular, more information on the large-scale movements and contact rates between morbillivirus carriers is required.

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