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Four years of monitoring for viral haemorrhagic septicaemia virus in marine waters around the United Kingdom

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ABSTRACT: Between 1995 and 1998, marine fish from around the coast of the UK were collected and samples analysed for viral haemorrhagic septicaemia virus (VHSV) using cell culture isolation methods. In 1997 and 1998 the samples were also analysed for VHSV by reverse transcription PCR (RT-PCR). A total of 1867 fish of 11 species were tested, but VHSV was isolated on only 1 occasion, from herring *Clupea harengus*, in 1996 . However, despite VHSV not being isolated in 1997 and 1998, in both years samples of herring from the west and south coasts of England produced positive signals in the RT-PCR, and in 1997 cod from the east coast of England also produced positive signals in the RT-PCR. These results are believed to be true indications of the presence of VHSV nucleic acid in the fish. In 1997, birnaviruses from Serogroup B1 were isolated from herring (a previously unrecorded host for the virus) and cod *Gadus morhua*, and a birnavirus from Serogroup A2 was also isolated from cod. In 1998, an aquareovirus was isolated from haddock *Melanogrammus aeglefinus*, a previously unrecorded host for the virus.

KEY WORDS: Viral haemorrhagic septicaemia virus · VHSV · Birnavirus · Aquareovirus · Marine fish · UK · Survey

INTRODUCTION

Marine-fish disease monitoring around the United Kingdom has been undertaken by staff of the CEFAS Weymouth Laboratory on an annual basis since 1982. The dab *Limanda limanda* is the target species and the incidence and prevalence of external disease conditions (lymphocystis, papillomas, hyperpigmentation and ulcers) and liver pathology are monitored according to international guidelines (Bucke et al. 1996). Diseases in other species, such as *Ichthyophonus* in Atlantic herring *Clupea harengus* and pseudobranch 'tumours' in Atlantic cod *Gadus morhua*, are also monitored. Between 1995 and 1998, fish other than dab were sampled and tested for the presence of viral haemorrhagic septicaemia virus (VHSV), primarily because of an increasing awareness of the potential for transfer of viruses from wild fish to fish in marine aquaculture, but also to broaden the knowledge of the health status of marine fish. The main impetus for this was reports that VHSV had been identified in a number of marine fish species (Meier et al. 1994, Meyers & Winton 1995), and that VHSV from a marine fish reservoir might have been responsible for a VHS outbreak in turbot *Scophthalmus maximus* cultivated on the Scottish island of Gigha (Munro 1996). The results of these annual surveys complement others conducted over a similar time period in different UK waters and in different areas of the North Sea (Smail 1995, 2000, Mortensen et al. 1999, King et al. 2001). We have previously described the isolation of VHSV from herring *C. harengus* during one of the cruises (Dixon et al.

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1997), and we report here the full results of the virus monitoring between 1995 and 1998.

MATERIALS AND METHODS

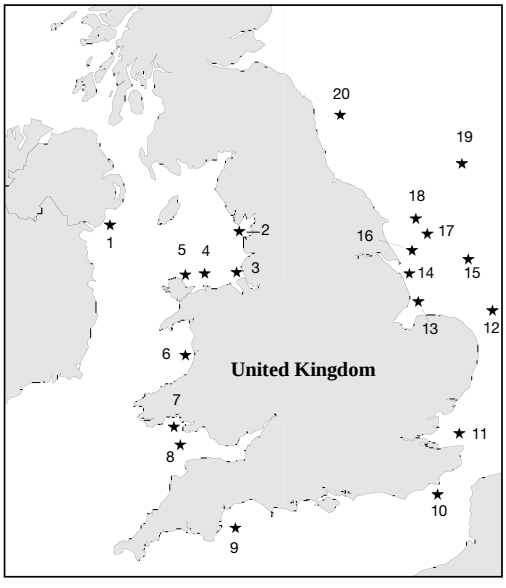
Fish sampling. Fish were caught during January or February using a Granton trawl or a beam trawl. The sampling sites are shown in Fig. 1 and the species tested are shown in Table 1; between 1 and 4 trawl tows were made at each sampling site. In some years certain sites could not be sampled because of adverse weather conditions. Each species from a trawl tow were sampled separately and portions of liver, spleen, heart, kidney and brain were taken from each fish, up to a maximum of 50 fish per species per station. Organs from the first 10 fish of a species were put into 10 separate sample containers, and then organs from the next 10 fish of that species were added sequentially to those sample containers; according to the number of fish of a species available at a station, samples comprised from 1 fish to a maximum of 10 pools each of 5 fish. The tissue samples were covered with virus transport medium then blast frozen; they were stored frozen at 20°C for up to 2 mo until they were processed at the laboratory. In 1997, 5 hypermelanised and 5 normal appearing dab from one site were processed individually, and 5 hypermelanised dab from a second site were also processed individually. In 1996, 1 cod, and in 1998 2 cod, from different sites were seen to have skin ulcers, and were processed individually. Skin with underlying muscle as well as internal organs were taken from those dab and cod. Portions of tissues showing pathologies were fixed in 10% neutral buffered formalin, processed and stained with haematoxylin and eosin (H&E) using standard histological procedures.

Cell cultures. Between 1995 and 1997, *Epithelioma papulosum cyprini* (EPC) (Fijan et al. 1983) and bluegill fibroblast (BF-2) (American Type Culture Collection, ATCC CCL 91) cells were used; however in 1998, fathead minnow (FHM) (ATCC CCL 42) and chinook salmon embryo (CHSE-214) (ATCC CRL 1681) cells were also used. In that year the samples were diluted to 1:100 and 1:1000 and inoculated onto each cell type with and without pre-treatment of the cell monolayers with 7% polyethylene glycol (PEG) 20 000 for 30 min (Batts & Winton 1989). After 7 d the samples were passaged either with or without PEG pre-treatment according to the initial inoculation.

In 1998, samples that were positive for VHSV by reverse transcription PCR (RT-PCR) but negative for the virus in the above cell cultures were also inoculated onto the following cell lines: rainbow trout gonad (RTG-2) (ATCC CCL 55), Atlantic salmon (AS) (Nicholson & Byrne 1973), striped snakehead (SSN-1) (Frerichs et al. 1991), rainbow trout embryo (RTE) and turbot fin (TF) (the latter 2 cell lines, R. F. Williams,

Table 1. Total numbers of marine fish tested for viruses from the 4 research cruises

Fish species	Year				Total
	1995	1996	1997	1998	
Brill *Scophthalmus rhombus*				5	5
Cod *Gadus morhua*	20	168	217	303	708
Dab *Limanda limanda*			15		15
Haddock *Melanogrammus aeglefinus*		1	13	51	65
Herring *Clupea harengus*	10	50	237	236	533
Poor cod *Trisopterus minutus*		3			3
Saithe *Pollachius virens*				1	1
Scad *Trachurus trachurus*				7	7
Sea trout *Salmo trutta*				1	1
Sprat *Sprattus sprattus*				43	43
Turbot *Scophthalmus maximus*		1	2	8	11
Whiting *Merlangius merlangus*	301	120	49	5	475
Total	331	343	533	660	1867



Sampling Sites

1. Dundrum Bay
2. Morecambe bay
3. Liverpool Bay
4. Great Orme
5. Red Wharf Bay
6. Cardigan Bay
7. Carmarthen Bay
8. Bristol Channel
9. Lyme Bay
10. Rye Bay
11. Outer Thames Estuary
12. Smith's Knoll
13. Humber/Wash
14. Humber off Ground
15. Sole Pit
16. Humber Rough
17. Flamborough off Ground
18. Flamborough
19. Dogger
20. Amble

Fig. 1. Sampling sites (★) for marine fish surveys from 1995 to 1998

CEFAS laboratory, unpubl.). Those cell lines had been selected as they were known to support the replication of VHSV (Jensen 1965, P. F. Dixon & L. Parry unpubl. data), and would broaden the spectrum of susceptible cell lines inoculated with samples known to harbour VHSV nucleic acid. Inoculation was as described above but without PEG pre-treatment. After 7 d the samples were blind passaged at 1:10 and 1:100 dilutions, both in the standard manner and by inoculating cells in suspension. To effect this the passage, inoculum was diluted to 1:5 and 1:50 and dispensed into wells of a cell culture multi-well plate. An equal volume of seed cells were added to each well, and the multi-well plates were gently shaken for 1 to 2 min before being transferred to an incubator, where the cells attached themselves to bottom of the plastic culture vessel as usual.

Sample processing. Tissues were processed by the method stipulated by the Commission of the European Communities (CEC) for detecting VHSV (CEC 1992, 1996) as described by Dixon et al. (1997). On blind passage, the cells were observed for up to 4 wk unless a cytopathic effect (CPE) was seen before then. The processing was done in a microbiological safety cabinet to reduce the risk of both cross-contamination or laboratory contamination of the samples.

The tissue extracts were also tested for the presence of VHSV nucleic acid sequences by RT-PCR.

Virus identification. Cell cultures showing CPE suggestive of rhabdoviruses or birnaviruses were tested in ELISAs for VHSV, infectious haematopoietic necrosis virus (IHNV) (Way & Dixon 1988, Way 1996) and birnavirus Serotype A2 (Dixon & Hill 1983) to ascertain the presence of those viruses. The latter ELISA was modified by changing the enzyme from alkaline phosphatase to horseradish peroxidase and then performed as described by Way & Dixon (1988) and Way (1996). Identification of VHSV in infected cell cultures was also done by RT-PCR. Viruses not identified by the ELISAs were then tested by neutralisation with birnavirus antisera, using the constant antisera, variable virus method (Rovozzo & Burke 1973), as birnaviruses are widespread in the marine environment. Antisera against the viruses, with homologous 50% plaque neutralisation titres of at least 10^6, were diluted to 5×10^{-2} and mixed with equal volumes of virus diluted from 5×10^{-1} to 5×10^{-9}. After incubation at room temperature for 1 h, aliquots of the mixture were inoculated onto cell monolayers according to the cell type in which the CPE was observed. The cells were incubated at 15°C for 7 d then observed for the presence of CPE. This preliminary identification was confirmed by plaque neutralisation as described by Dixon & de Groot (1996).

The CPE produced by one sample was suggestive of an aquareovirus. In order to short-cut the identification process, the tests initially used to identify the cause of the CPE (electron microscopy and electrophoresis of RNA) were directed at supporting that suggestion.

RT-PCR. Total RNA was extracted from 200 µl cell culture supernatant, or from 100 µl of the initial 1:10 dilution of homogenised tissue using the Trizol™ reagent; the reverse transcription step utilised one-tenth of the total RNA extracted from the tissue (i.e. 1 mg). The semi-nested RT-PCR for VHSV was carried out as previously described (Dixon et al. 1997, Strømmen & Stone 1998) using F3 (5′-GAT-CAG-GTC-CCC-CAR-RTC-NGT-3′) in place of F2 in the first round of RT-PCR. In 1996 only those fish samples from which virus was isolated were tested by RT-PCR, but all the 1997 and 1998 samples were screened for the presence of VHSV nucleic acid.

Nucleotide sequencing. RT-PCR products were purified using the GLEANCLEAN® (Anachem) and inserted into the pGEM-T vector (Promega) using the standard protocol. Both DNA strands were sequenced using the M13 universal sequencing primers and the ABI PRISM™ dye terminator cycle sequencing system (Applied Biosystems). Sequencing reactions were analysed on an ABI 310 genetic analyser and multiple alignments performed using Clustal V (Higgins & Sharp 1989).

Reference viruses. The aquareovirus $13p_2$, originally isolated from the American oyster *Crassostrea virginica* (Meyers 1979), was a gift from Dr. T. Meyers, Juneau Fish Pathology Laboratory, Alaska. Brown trout reovirus (BTR) was isolated at the CEFAS laboratory from brown trout *Salmo trutta* (Taylor et al. 1998). Birnavirus reference Serotype A2 (Jørgensen & Bregnballe 1969) was a gift from the late Dr. P. E. V. Jørgensen, and reference Serotype B1 had been isolated at the CEFAS laboratory (Hill 1976, Hill & Way 1995). VHSV isolates H17/5 and H19/1 (Smail 2000) were gifts from Dr. D. Smail, Fisheries Research Services, Marine Laboratory, Aberdeen, and VHSV isolate cod ulcus (= cod rhabdo 79) (Jørgensen & Olesen 1987) was a gift from Dr. N. Olesen, Danish Veterinary Laboratory, Aarhus, Denmark. VHSV isolate 96-43 was isolated at the CEFAS laboratory (Dixon et al. 1997).

Virus concentration. For electron microscopy, 2 × 175 cm^2 flasks of CHSE-214 cells showing gross CPE were frozen at –20°C, thawed and cell debris removed by centrifugation at 1500 × *g* for 10 min at 4°C. The viruses were concentrated by adding sodium chloride to 2.2% and PEG 6000 to 7% to the supernatant. The mixture was stirred overnight at 4°C then centrifuged at 2000 × *g* for 1 h at 4°C. The pellets were resuspended in distilled water (0.5 ml total) and an equal volume of 1,1,2-trichloro-1,1,2-trifluoroethane was

added and mixed thoroughly. The mixture was separated into phases by centrifugation at 1000 × *g* for 5 min at 4°C. The upper aqueous phase was removed and examined by electron microscopy.

For RNA extraction, a single 175 cm^2 flask of CHSE-214 cells showing gross CPE was frozen at –20°C, thawed, and concentrated by centrifugation at 90 000 × *g* for 1 h at 4°C. The pellet was resuspended in 300 µl molecular-biology grade distilled water (Merck) and processed as described below.

Electron microscopy. Concentrated virus was applied to carbon-coated Formvar grids, stained with 2% methylamine tungstate and observed with a JEOL 1210 transmission electron microscope.

RNA extraction and electrophoresis. A mixture of 4 µl of RNAsin® (Promega), 15 µl 10% sodium lauryl sulphate (SDS), and 15 µl Proteinase K was added to 300 µl of concentrated virus, mixed and incubated at 37°C for 1 h. A further 17.5 µl 10% SDS and 1.2 µl 3 M sodium acetate, 10.3 µl molecular-biology grade distilled water and 6 µl lithium chloride were then added. After mixing, 300 µl phenol-chloroform-iso amyl alcohol (PCIA) (24:24:1) (Gibco BRL) were added, mixed, and a further 40 µl 3 M sodium acetate were added. The preparation was mixed then centrifuged at 13 000 × *g* for 15 min at room temperature. The upper aqueous layer was transferred to another tube and 300 µl PCIA was added, mixed and centrifuged as before. The aqueous layer was transferred to a further tube, 1 ml absolute ethanol was added and the tube was kept at –20°C overnight to precipitate the RNA. The RNA was centrifuged to a pellet at 13 000 × *g* for 30 min at room temperature after which the ethanol was aspirated from the tube, and residual ethanol was allowed to evaporate. The pellet was resuspended in 20 µl diethyl pyrocarbonate-treated water.

The RNA was analysed on 6% polyacrylamide gels (approximately 10 × 7 cm) prepared from a stock solution of 30% acrylamide and bis-acrylamide (29:1) in tris-borate buffer. Electrophoresis was carried out for 10 min at 50 V then 1.5 h at 200 V. The gels were fixed and stained using a silver stain kit (Silver Stain Plus, BioRad) according to the manufacturer's instructions.

Table 2. Locations of fish sampled for virus isolation in 1995. Sampling site numbers correspond to those in Fig. 1

Sampling site	Location	Fish sampled	Number tested
12 Morecambe Bay	53° 56.51′ N, 03° 23.64′ W	Cod	10
2 Morecambe Bay	53° 56.51′ N, 03° 23.64′ W	Whiting	50
1 Dundrum Bay	54° 08.20′ N, 05° 37.27′ W	Whiting	40
4 Great Orme	53° 23.09′ N, 03° 38.04′ W	Cod	10
4 Great Orme	53° 23.09′ N, 03° 38.04′ W	Whiting	40
4 Great Orme	53° 23.09′ N, 03° 38.04′ W	Herring	10
6 Cardigan Bay	52° 21.42′ N, 04° 54.23′ W	Whiting	42
18 Flamborough	54° 15.59′ N, 00° 27.06′ E	Whiting	79
15 Sole Pit	53° 45.96′ N, 01° 24.04′ E	Whiting	50

Table 3. Locations of fish sampled for virus isolation in 1996. Sampling site numbers correspond to those in Fig. 1

Sampling site	Location	Fish sampled	Number tested
15 Sole Pit	53° 38.81′ N, 01° 32.90′ E	Cod	6
13 Humber/Wash	53° 18.08′ N, 00° 26.71′ E	Cod	49[a]
18 Flamborough	54° 14.03′ N, 00° 31.15′ E	Cod	6
18 Flamborough	54° 12.52′ N, 00° 37.45′ E	Turbot	1
1 Dundrum Bay	54° 08.54′ N, 05° 47.58′ W	Cod	34
2 Morecambe Bay	54° 04.31′ N, 03° 25.94′ W	Cod	9
2 Morecambe Bay	54° 04.31′ N, 03° 25.94′ W	Whiting	20
3 Liverpool Bay	53° 57.96′ N, 03° 23.55′ W	Cod	27
3 Liverpool Bay	53° 28.67′ N, 03° 44.07′ W	Poor-cod	3
5 Red Wharf Bay	53° 21.82′ N, 04° 10.38′ W	Cod	20
6 Cardigan Bay	52° 15.25′ N, 04° 20.66′ W	Whiting	50
8 Bristol Channel	51° 33.00′ N, 04° 42.33′ W	Cod	1
8 Bristol Channel	51° 33.00′ N, 04° 42.33′ W	Haddock	1
8 Bristol Channel	51° 33.00′ N, 04° 42.33′ W	Whiting	50
10 Rye Bay	50° 51.71′ N, 00° 47.50′ E	Cod	16
10 Rye Bay	50° 51.71′ N, 00° 47.50′ E	Herring	50

[a]One fish was ulcerated

RESULTS

1995 samples

The fish species tested are shown in Table 2. No viruses were isolated, and RT-PCR was not done on any samples as the procedure was not available at the laboratory at that time.

1996 samples

The fish species tested are shown in Table 3. The isolation of VHSV from Atlantic herring in this year has already been described (Dixon et al. 1997). The RT-PCR was only used to test the herring samples from the same station at which the VHSV-positive herring were taken. No other viruses were isolated.

1997 samples

The fish species tested are shown in Table 4. Dab with hypermelanisation were specifically targeted at 2 sites, but no virus of any type was isolated. However, 6 other samples exhibited CPE (Table 5), but only after a blind passage. All of the isolates were tested in the VHSV and birnavirus Serotype A2 ELISAs, and all were negative in the VHSV ELISA. However, Isolates 36-153 (Trawl 36, Sample 153) and 36-154 produced strong positive signals in the birnavirus Serotype A2 ELISA (absorbance at 450 nm was 0.72 and 0.70, respectively, compared to the positive control absorbance of 0.95 and negative control absorbance of 0.13). That identification was confirmed by plaque neutralisation. Isolates 23-44, 43-160, 44-164 and 44-167 were negative in the birnavirus Serotype A2 ELISA, and were also negative in the IHNV ELISA. In subsequent neutralisation tests those isolates were identified as belonging to birnavirus Serogroup B. An antiserum against birnavirus B1, with a homologous 50% plaque reduction titre of 3.6×10^{-6}, had 50% plaque reduction titres of 2.6×10^{-5} and 1.75×10^{-5} against Isolates 23-44 and 44-164, respectively.

Table 4. Locations of fish sampled for virus detection in 1997. Sampling site numbers correspond to those in Fig. 1

Sampling site	Location	Fish sampled	Number tested
12 Smiths Knoll	52° 40.19′ N, 02° 19.76′ E	Cod	8
15 Sole Pit	53° 45.96′ N, 01° 24.04′ E	Herring	30
15 Sole Pit	53° 45.96′ N, 01° 24.04′ E	Cod	5
13 Humber/Wash	53° 18.48′ N, 00° 25.84′ E	Herring	50
13 Humber/Wash	53° 18.48′ N, 00° 25.84′ E	Cod	28
18 Flamborough	54° 15.59′ N, 00° 27.06′ E	Herring	14
18 Flamborough	54° 15.59′ N, 00° 27.06′ E	Cod	13
18 Flamborough	54° 15.59′ N, 00° 27.06′ E	Haddock	2
18 Flamborough	54° 15.59′ N, 00° 27.06′ E	Dab	10[a]
19 Dogger	54° 47.24′ N, 01° 17.15′ E	Herring	5
19 Dogger	54° 47.24′ N, 01° 17.15′ E	Cod	12
19 Dogger	54° 47.24′ N, 01° 17.15′ E	Haddock	1
19 Dogger	54° 47.24′ N, 01° 17.15′ E	Whiting	5
20 Amble	55° 19.23′ N, 01° 15.09′ W	Herring	16
20 Amble	55° 19.23′ N, 01° 15.09′ W	Cod	33
20 Amble	55° 19.23′ N, 01° 15.09′ W	Haddock	10
3 Liverpool Bay	53° 28.17′ N, 03° 42.59′ W	Herring	50[b]
3 Liverpool Bay	53° 28.17′ N, 03° 42.59′ W	Cod	15
3 Liverpool Bay	53° 28.17′ N, 03° 42.59′ W	Dab	5
5 Red Wharf Bay	53° 21.42′ N, 04° 07.58′ W	Herring	50
5 Red Wharf Bay	53° 21.42′ N, 04° 07.58′ W	Cod	50
6 Cardigan Bay	52° 21.85′ N, 04° 54.23′ W	Whiting	24
7 Carmarthen Bay	51° 32.49′ N, 04° 46.30′ W	Cod	7
7 Carmarthen Bay	51° 32.49′ N, 04° 46.30′ W	Whiting	10[c]
7 Carmarthen Bay	51° 32.49′ N, 04° 46.30′ W	Turbot	1
9 Lyme Bay	50° 33.34′ N, 03° 12.62′ W	Herring	14[b]
9 Lyme Bay	50° 33.34′ N, 03° 12.62′ W	Cod	1
9 Lyme Bay	50° 33.34′ N, 03° 12.62′ W	Whiting	10
10 Rye Bay	50° 50.79′ N, 00° 45.82′ E	Herring	8[b]
10 Rye Bay	50° 50.79′ N, 00° 45.82′ E	Cod	34[c]
10 Rye Bay	50° 50.79′ N, 00° 45.82′ E	Turbot	1
11 Outer Thames Estuary	52° 02.54′ N, 02° 06.07′ E	Cod	11

[a]Five fish were hypermelanised
[b]One fish had a granular spleen
[c]One fish had a head ulcer

Table 5. 1997 research cruise. Fish from which the tissue extracts produced a cytopathic effect (CPE) following inoculation onto cell cultures. Sampling site numbers correspond to those in Fig. 1

Sampling site	Sample number	Fish	Cell line in which CPE seen
3 Liverpool Bay	23–44	Herring	BF-2
7 Carmarthen Bay	36–153	Cod	EPC
5 Red Wharf Bay	36–154	Cod	EPC
10 Rye Bay	43–160	Cod	BF-2
10 Rye Bay	44–164	Cod	BF-2
10 Rye Bay	44–167	Cod	BF-2

All the initial 1:10 fish extracts were tested by the RT-PCR for VHSV, and 13 samples from 6 sites were positive (Table 6). Where there was sufficient product the RT-PCR results were confirmed as VHSV in origin by sequencing (Fig. 2). There were between 1 and 4 (0.4 to 1.5%) nucleotide differences between these partial sequences and the isolate from herring caught in the English Channel (96-43), and between 23 and 25 (8.7 to 9.4%) nucleotide differences when compared to the isolates from cod (H17-5 and H19-1) caught in the coastal waters east of the Shetland Islands.

The only virus isolated from any of those samples was a birnavirus from Sample 23-44. As the replication of the birnavirus may not have allowed VHSV to replicate, Sample 23-44 was re-inoculated onto BF-2 and EPC cell cultures following neutralisation of the birnavirus. Dilutions of the original homogenate were incubated for 1 h with an equal volume of an antiserum against birnavirus Serogroup B diluted to 5×10^{-2}, before inoculation onto the cells. No CPE was observed in the cells, even after a blind passage.

Spleens with a granular appearance from 2 herring were examined histologically. Prominent granulomas were observed, consisting of a discrete outer

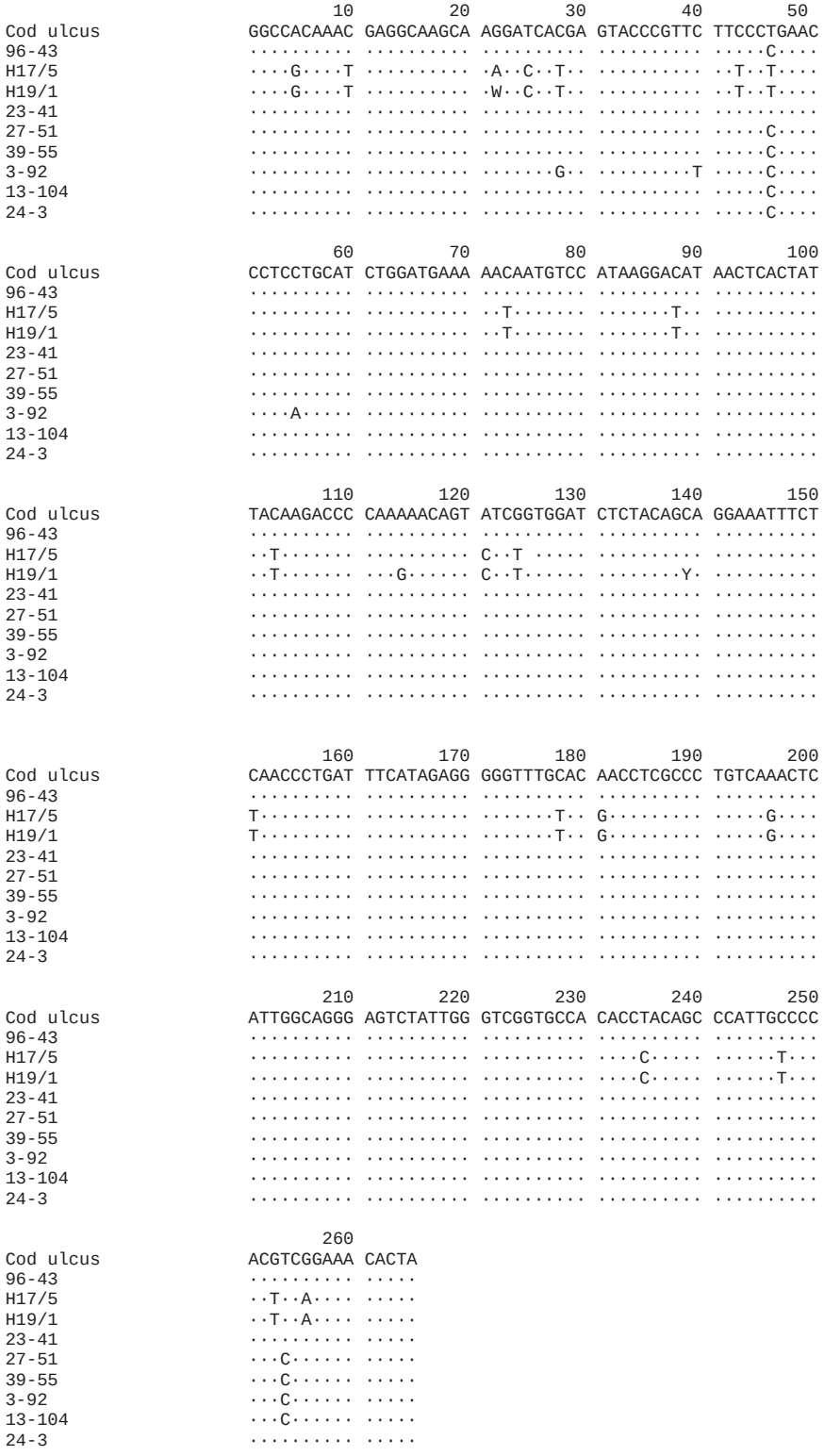


Fig. 2. Alignment of the partial sequences (nucleotides 339-663 of the glycoprotein gene) obtained for the reverse transcription PCR (RT-PCR) products described in Tables 6 and 8. Published sequences for the viral haemorrhagic septicaemia virus (VHSV) isolates from cod caught in the coastal waters east of the Shetland Islands (H17/5 and H19/1) were also included (Stone et al. 1997). (·) indicates the positions of sequence identity compared to the published sequence for the cod ulcus virus (Stone et al. 1997). Multiple alignments were performed using the Clustal V multiple alignment package within MEGALIGN (DNASTAR). International Union of Pure and Applied Chemistry (IUPAC) codes were used where appropriate

Table 6. 1997 research cruise. Fish from which the tissue extracts were positive in the VHSV RT-PCR. Sampling site numbers correspond to those in Fig. 1

Sampling site	Sample number	Fish
3 Liverpool Bay	23-41, 23-42, 23-44	Herring
5 Red Wharf Bay	27-51, 27-52, 27-53	Herring
9 Lyme Bay	39-55, 39-56, 39-58	Herring
15 Sole pit	3-92, 3-93	Cod
18 Flamborough	13-104	Cod
19 Dogger	18-110	Cod

epithelioid layer surrounding necrotic tissue made up of phagocytic cells. Most granulomas contained significant amounts of pigmented material resembling lipofuscin finely distributed throughout the lesions. *Ichthyophonus hoferi* or bacteria were not observed.

1998 samples

The fish species tested are shown in Table 7. One sample, 18-17 from haddock, exhibited CPE. The CPE was only seen in CHSE-214 cells pre-treated with PEG and incubated at 15°C for 25 d following a blind passage. The cells were frozen and thawed and passaged at 10^{-2} and 10^{-3} dilutions onto CHSE-214 cells with and without PEG pre-treatment, and incubated at 15 and 20°C. CPE did not occur at 20°C and progressed slowly at 15°C affecting approximately 25% of the monolayer after 21 d. The CPE occurred in the cells both with and without PEG pre-treatment, although more cells were affected if they had been pre-treated with PEG. After a further passage CPE occurred more rapidly, and affected approximately 50% of PEG pre-treated cells and 25% of non-treated cells after incubation for 9 d. The CPE exhibited as syncytia which gradually increased in size and eventually detached from the rest of the cell monolayer on the plastic substrate; the CPE was typical of that described for aquareoviruses (Lupiani et al. 1995) (Fig. 3). PEG concentrated virus was approximately 70 nm diameter and had a double-layered capsid (Fig. 4). The genome comprised 11 segments of double-stranded RNA (Fig. 5).

All the initial 1:10 fish extracts were tested by the RT-PCR for VHSV, and 6 pools from 2 sites were positive (Table 8). The products generated by RT-PCR (Fig. 6) were confirmed as VHSV by sequencing, and comparison of the partial sequences revealed 0 and 4 (0 to 1.5%) nucleotide differences compared to the isolate made from herring in 1996 and the partial sequences obtained for the 1997 samples (Fig. 2).

The tissue extracts that were VHSV RT-PCR positive were inoculated onto 5 additional VHSV-susceptible cell lines (RTG-2, AS, SSN-1, RTE and TF) and pas-

Table 7. Locations of fish sampled for virus detection in 1998. Sampling site numbers correspond to those in Fig. 1

Sampling site	Location	Fish sampled	Number tested
14 Humber Off Ground	53° 53.21′ N, 00° 57.42′ E	Sprat	23
14 Humber Off Ground	53° 53.21′ N, 00° 57.42′ E	Cod	15
16 Humber Rough	53° 53.95′ N, 00° 15.46′ E	Herring	2
16 Humber Rough	53° 53.95′ N, 00° 15.46′ E	Cod	36[a]
16 Humber Rough	53° 53.95′ N, 00° 15.46′ E	Haddock	20
15 Sole Pit	53° 45.96′ N, 01° 24.04′ E	Cod	6
15 Sole Pit	53° 45.96′ N, 01° 24.04′ E	Whiting	5
15 Sole Pit	53° 45.96′ N, 01° 24.04′ E	Herring	4
17 Flamborough Off Ground	54° 15.03′ N, 00° 03.96′ E	Cod	50
17 Flamborough Off Ground	54° 15.03′ N, 00° 03.96′ E	Herring	50
17 Flamborough Off Ground	54° 15.03′ N, 00° 03.96′ E	Turbot	3
17 Flamborough Off Ground	54° 15.03′ N, 00° 03.96′ E	Scad	5
17 Flamborough Off Ground	54° 15.03′ N, 00° 03.96′ E	Brill	2
19 Dogger	54° 47.24′ N, 01° 17.15′ E	Cod	13
19 Dogger	54° 47.24′ N, 01° 17.15′ E	Herring	3
19 Dogger	54° 47.24′ N, 01° 17.15′ E	Scad	2
20 Amble	55° 19.23′ N, 01° 15.09′ W	Cod	51[a]
20 Amble	55° 19.23′ N, 01° 15.09′ W	Haddock	28
20 Amble	55° 19.23′ N, 01° 15.09′ W	Herring	1
1 Dundrum Bay	54° 08.20′ N, 05° 37.27′ W	Cod	22
1 Dundrum Bay	54° 08.20′ N, 05° 37.27′ W	Herring	50
1 Dundrum Bay	54° 08.20′ N, 05° 37.27′ W	Haddock	2
1 Dundrum Bay	54° 08.20′ N, 05° 37.27′ W	Turbot	1
5 Red Wharf Bay	53° 21.42′ N, 04° 07.58′ W	Herring	50
5 Red Wharf Bay	53° 21.42′ N, 04° 07.58′ W	Cod	20
5 Red Wharf Bay	53° 21.42′ N, 04° 07.58′ W	Turbot	1
5 Red Wharf Bay	53° 21.42′ N, 04° 07.58′ W	Saithe	1
3 Liverpool Bay	53° 28.17′ N, 03° 42.59′ W	Cod	28
3 Liverpool Bay	53° 28.17′ N, 03° 42.59′ W	Herring	20
3 Liverpool Bay	53° 28.17′ N, 03° 42.59′ W	Turbot	1
6 Cardigan Bay	52° 21.85′ N, 04° 54.23′ W	Herring	1
6 Cardigan Bay	52° 21.85′ N, 04° 54.23′ W	Brill	3
7 Carmarthen Bay	51° 32.49′ N, 04° 46.30′ W	Cod	17
7 Carmarthen Bay	51° 32.49′ N, 04° 46.30′ W	Turbot	1
7 Carmarthen Bay	51° 32.49′ N, 04° 46.30′ W	Sea trout	1
7 Carmarthen Bay	51° 32.49′ N, 04° 46.30′ W	Haddock	1
9 Lyme Bay	50° 33.34′ N, 03° 12.62′ W	Cod	5
9 Lyme Bay	50° 33.34′ N, 03° 12.62′ W	Sprat	20
9 Lyme Bay	50° 33.34′ N, 03° 12.62′ W	Herring	5[b]
10 Rye Bay	50° 50.79′ N, 00° 45.82′ E	Turbot	1
10 Rye Bay	50° 50.79′ N, 00° 45.82′ E	Cod	40
10 Rye Bay	50° 50.79′ N, 00° 45.82′ E	Herring	50[b]

[a]One fish was ulcerated and was processed separately
[b]Some fish had granular kidneys and spleens

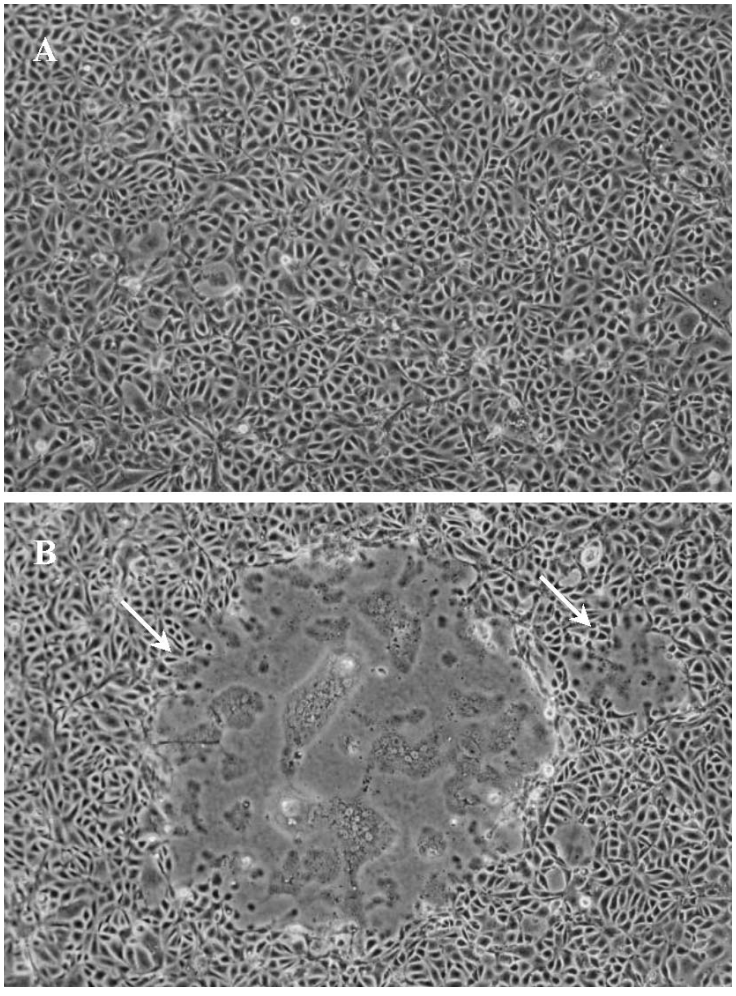


Fig. 3. CHSE-214 cells infected with Isolate 18-17. (A) control cells, (B) syncitia (arrowed) in infected cell culture

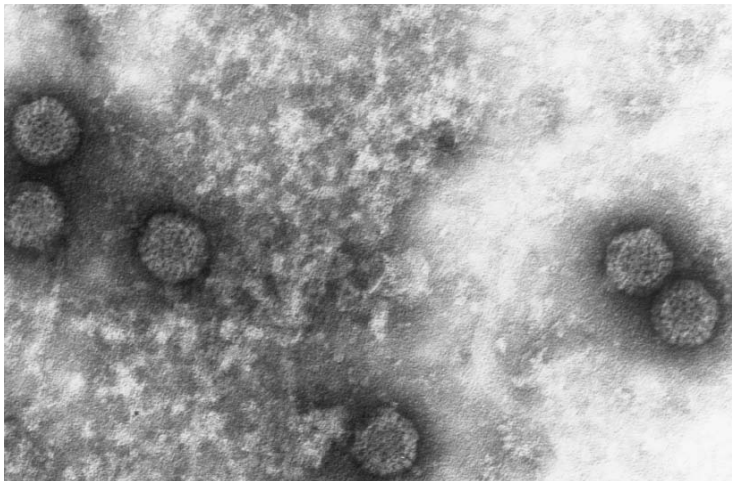


Fig. 4. Electron micrograph of negatively stained Isolate 18-17, concentrated from infected CHSE-214 cells by polyethylene glycol (PEG) precipitation. Virions are approximately 70 nm in diameter

saged after 7 d, but no CPE was observed. The original extract from Sample 24-3 produced a strong signal in the RT-PCR, and the BF-2, CHSE-214 and EPC cells harvested 7 d after inoculation with that material were tested by RT-PCR to determine whether VHSV may have replicated in the cells without producing any CPE; however, the RT-PCR was negative for the virus.

Several herring from Rye Bay had granular kidneys and spleens at post mortem. The histological appearance was similar to that described for herring spleens in 1997.

Data on the virus-positive samples recorded over the 4 yr are summarised in Table 9.

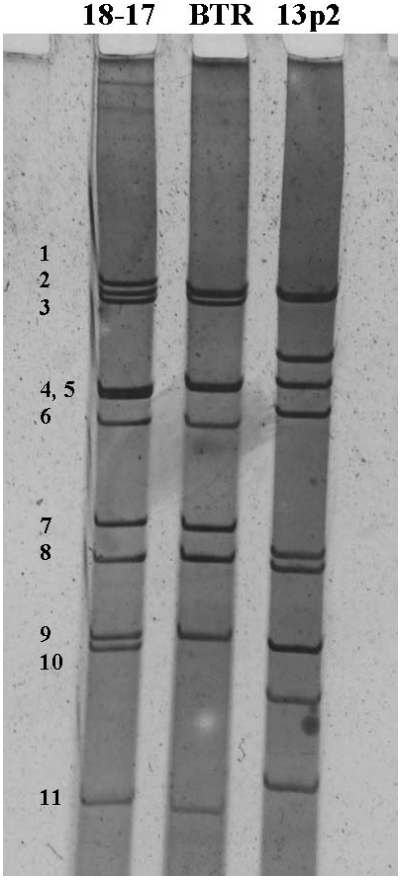


Fig. 5. Comparison of the RNA profiles of Isolate 18-17, BTR and 13p2 aquareoviruses. The viruses were concentrated from infected CHSE-214 cells by centrifugation at 90 000 × *g* for 1 h; the RNA was extracted by the SDS-Proteinase K method and analysed on a 6% polyacrylamide gel. Electrophoresis was for 1.5 h at 200 V, and the gel was silver stained. The RNA segments of Isolate 18-17 are numbered

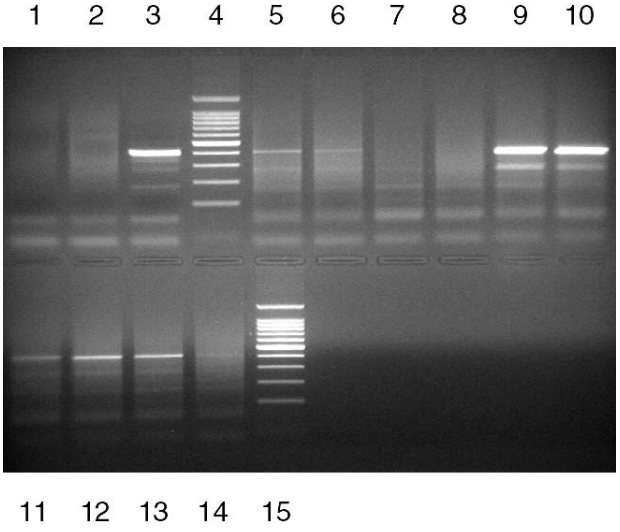


Fig. 6. Detection of the viral haemorrhagic septicaemia virus (VHSV) glycoprotein gene in herring tissue samples by semi-nested reverse transcription PCR (RT-PCR). Lanes 1 and 2: negative controls; lane 3: VHSV-positive control; lanes 4 and 15: 100 bp ladders (Promega); lanes 5 to 14: paired samples corresponding to Isolates 24-1, 24-2, 24-3, 24-4 and 24-5 respectively

Table 8. 1998 research cruise. Fish from which the tissue extracts were positive in the viral haemorrhagic septicaemia virus (VHSV) reverse transcription PCR (RT-PCR). Sampling site numbers correspond to those in Fig. 1

Sampling site	Sample number	Fish
5 Red Wharf Bay	24-1, 24-3, 24-4, 24-5, 24-6	Herring
10 Rye Bay	43-6	Herring

DISCUSSION

The sample sizes of some fish species in these surveys were small, which was a consequence of the virological sampling being an adjunct to the main survey; however, the numbers of fish sampled increased over the years (Table 1). Despite the limitations of sample size, in 2 of the years viruses were isolated. The isolation of VHSV from herring and its identification in herring tissues by RT-PCR has been reported in more detail elsewhere (Dixon et al. 1997), but fish catch data from that cruise have been included here to provide a complete picture of the 4 yr sampling programme. It was interesting to note that a birnavirus, Serotype B1, was isolated from cod from the same location, Rye Bay (Site 10), the following year (1997). However there is nothing to suggest that this is more than a coincidence. Cod from Carmarthen Bay (Site 7) were positive for a serologically distinct birnavirus belonging to Serotype A2. Birnaviruses have been isolated from cod fry in culture in Danish coastal waters and from cod fry in culture on the Faroe islands (Lorenzen et al. 1995); the viruses from those 2 locations also belonged to Serotype A2 (N. J. Olesen pers. comm.). On both those occasions the cod were undergoing mortalities, but there was no indication that the older cod from which the birnaviruses were isolated in this study were diseased.

A birnavirus belonging to Serotype B1 was also isolated from herring (a previously unrecorded host) from Liverpool Bay (Site 3), again in 1997. The significance of these birnavirus isolates for their respective hosts is unknown. Further work is necessary to determine the effect that the birnaviruses and the aquareovirus may have on species in coastal aquaculture or ranching. Birnavirus Serotype B1 has previously been isolated from marine invertebrates, but until recently it had only been isolated from 1 marine fish species, the dab (Olesen et al. 1988). However, reports of similar marine fish surveys have shown that Serotype B1 has been recorded from a wide range of marine fish, predominantly flatfish (Mortensen et al. 1999, Skall et al. 2000).

In 1998 an aquareovirus was isolated from haddock (a previously unrecorded host) which showed no external or internal clinical signs. The pattern of RNA segments of the haddock aquareovirus is similar to that of BTR, which was isolated from a brown trout (sea trout) that had returned to freshwater from the marine environment (Taylor et al. 1998). The haddock were caught off the east coast of the UK, and the brown trout was captured in a river whose mouth is on the west coast of the UK. To what extent these isolates may represent a pool of similar aquareoviruses in fish in the marine environment around the UK is unknown.

Table 9. Summary of fish species sampled during the 4 research cruises from which the tissue extracts were virus-positive by isolation in cell culture or by reverse transcription PCR (RT-PCR). VHSV: viral haemorrhagic septicaemia virus

Sampling site	Year	Fish	Virus	Detection method
10 Rye Bay	1996	Herring	VHSV	Isolation
3 Liverpool Bay	1997	Herring	Birnavirus B1	Isolation
7 Carmarthen Bay	1997	Cod	Birnavirus A2	Isolation
10 Rye Bay	1997	Cod	Birnavirus B1	Isolation
3 Liverpool Bay	1997	Herring	VHSV	RT-PCR
5 Red Wharf Bay	1997	Herring	VHSV	RT-PCR
9 Lyme Bay	1997	Herring	VHSV	RT-PCR
15 Sole pit	1997	Cod	VHSV	RT-PCR
18 Flamborough	1997	Cod	VHSV	RT-PCR
19 Dogger	1997	Cod	VHSV	RT-PCR
20 Amble	1998	Haddock	Aquareovirus	Isolation
5 Red Wharf Bay	1998	Herring	VHSV	RT-PCR
10 Rye Bay	1998	Herring	VHSV	RT-PCR

VHSV was not isolated in cell cultures in 1997, but 13 samples were RT-PCR positive for the virus nucleic acid. The positive samples were from herring from 3 sites on the west and south coasts of England and from cod from 3 sites on the east coast of England. Low numbers of VHSV-positive cod were caught at each site and low numbers of positive herring were caught in Lyme Bay, which might indicate a high prevalence of VHSV in those sites. When VHSV was isolated from the herring in 1996, it was only isolated in BF-2 cells; EPC cells inoculated with the same samples were refractory to the virus. It is therefore possible that both cell lines may have been refractory to the VHSV detected by RT-PCR in 1997. In order to investigate that supposition further, 2 additional cell lines were used in 1998, and PEG pre-treatment of the cells (Batts & Winton 1989) was also used to increase the sensitivity of the isolation method. A greater number of fish were also sampled in 1998. However, VHSV was not isolated in 1998 but again the RT-PCR showed the presence of VHSV nucleic acid in herring from 2 sampling sites. Although there are only data for 2 yr, the RT-PCR data suggest that the prevalence of VHSV varies both spatially and temporally. Longer term studies are required to establish whether VHSV is consistently found in the same sites, and the nature of any temporal trend in prevalence. In 1997, both cod and herring were caught at the same sites where RT-PCR positive fish were identified, but cod caught at the same site as RT-PCR positive herring were RT-PCR negative and vice versa. Likewise in 1998, cod caught at the same sites as the RT-PCR positive herring were RT-PCR negative. That consistent feature may have significance for the epidemiology of VHSV in the marine environment, particularly the transmission of VHSV between the 2 species.

Very few of the fish examined showed gross external signs of disease. Three cod exhibited skin ulcers but were negative for VHSV. The initial isolation of VHSV from Atlantic cod was from ulcerated individuals (Jensen et al. 1979), but VHSV has only been isolated at a low frequency from such fish in later studies, and any relationship between VHSV and the cod ulcers is unresolved (Mortensen et al. 1999, Smail 2000, King et al. 2001). Likewise, Meyers et al. (1992) isolated VHSV from ulcerated skin lesions from Pacific cod *Gadus macrocephalus*. The authors noted the association of virus with the skin lesions, but were unable to determine whether the virus was the primary cause of the lesion, or a secondary pathogen. In one case the lesion appeared to be caused by an inflammatory host response to a possible protozoan parasite. Low numbers of the RT-PCR-positive herring had spleens and/or kidneys containing granulomas, but such fish were also observed in samples that were RT-PCR negative, and fish without such pathology were RT-PCR positive. No specific association of RT-PCR-positive herring and the presence of granulomas can be made because of the low numbers of fish with granulomas. The cause of the granulomas remains unresolved, but similar non-specific granulomatous inflammation was observed in a survey of Pacific herring *Clupea pallasi* in Alaskan waters (Marty et al. 1998).

Comparisons of the nucleotide sequences of the RT-PCR products from the herring and cod samples with those of other VHSV isolates showed that they should be categorised as belonging to Genogroup III (Stone et al. 1997). Their nucleotide sequences were identical to, or differed by up to 1.5% from the nucleotide sequence of the 1996 isolate from herring from the south coast of England (Dixon et al. 1997). Further isolates of VHSV from 5 different fish species, including herring and cod, from around northern UK (Scottish coastal waters and the northern North Sea) were genetically similar to a Genogroup II isolate from turbot (King et al. 2001). This 'north-south divide' in the genetic difference between VHSV from around the UK may be maintained by the geographic separation of different populations of the host species. King et al. (2001) did not isolate VHSV in 1997, but made 21 isolations from fish caught in 1998, of which 17 were from Norway pout *Trisopterus esmarkii* and 1 each were from cod, herring, whiting and poor cod. Smail (1995, 2000) reported the isolation of VHSV from cod (2 in 1993 and 1 in 1995) and from 2 haddock in 1995, from coastal waters off eastern Scotland. In the study reported here, the greater number of RT-PCR-positive sites was recorded in 1997 (3 each for cod and herring), and the greatest prevalence was in herring. No Norway pout were captured in this study; the areas trawled on the east coast of the UK in the North Sea were at or below the southernmost range of significant populations of Norway pout. The Norway pout may be an important reservoir of Genogroup II VHSV in northern UK waters, but the herring, or an as yet unidentified host species, may be the main reservoir of Genogroup III VHSV in southern UK waters.

The reason why VHSV could not be isolated from samples which were RT-PCR positive cannot be specifically determined. The cells used supported the replication of cell-culture adapted VHSV, and the virus was isolated from tissues from VHSV-infected rainbow trout in the BF-2, FHM and EPC cells in laboratory tests (results not shown). This leads to the conclusion that (1) there was no viable virus present in the fish tissues (it could have lost viability during storage or it could have been inactivated by host defence mechanisms), (2) virus was present, but at levels below the sensitivity of the isolation method, or (3) that some marine VHSV does not grow as readily in cell cultures

as the freshwater VHSV. That possible latter phenomenon should be borne in mind when marine fish are screened for VHSV, and samples should also be screened by the RT-PCR; otherwise there may be an underestimation of the prevalence of the virus. The freezing and thawing of the fish viscera prior to processing might have accounted for the loss of viability of VHSV in some of the samples, but it is known that the virus will survive such treatment, as shown by isolation of VHSV from frozen tissues in this study in 1996, and in other reports (Meyers et al. 1994, 1999, Mortensen et al. 1999, Smail 2000, King et al. 2001). Red Wharf Bay and Rye Bay appear to be 'hot spots' of VHSV in herring, and we intend to take more herring samples from those sites to investigate further the inability of the VHSV to grow in cell culture.

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