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Survey of viral haemorrhagic septicaemia virus in wild fishes in the southeastern Black Sea

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ABSTRACT: Species diversity in the Black Sea ecosystem has been declining rapidly over the last 2 decades. To assess the occurrence and distribution of viral haemorrhagic septicaemia virus (VHSV) in various wild fish species, a wild marine fish survey was carried out in 2009, 2010, and 2011. The pooled or individual samples of kidney, liver, and spleen of 5025 specimens, belonging to 17 fish species, were examined virologically using cell culture. The cells showing cytopathic effects (CPE) were subjected to ELISA and multiplex reverse transcriptase polymerase chain reaction (RT-mPCR), for VHSV and infectious pancreatic necrosis virus (IPNV), after blind passaging to determine the virus species causing CPE. The virus species and possibility of co-infection with IPNV were verified by the RT-mPCR developed in this study. Twelve species of fish (pontic shad *Alosa immaculata*, red mullet *Mullus barbatus*, three-bearded rockling *Gaidropsarus vulgaris*, black scorpionfish *Scorpaena porcus*, Mediterranean horse mackerel *Trachurus mediterraneus*, whiting *Merlangius merlangus* euxinus, stargazer *Uranoscopus scaber*, pilchard *Sardina pilchardus*, garfish *Belone belone*, round goby *Neogobius melanostomus*, thornback ray *Raja clavata*, and anchovy *Engraulis encrasicolus*) tested positive for VHSV Genotype Ie (VHSV-Ie). Except whiting, pilchard, and round goby, the rest are new host records for VHSV. The extent and spread of VHSV-Ie was significantly higher among bottom fish than among pelagic fish. Sensitivity and specificity of the RT-mPCR developed was sufficiently high, suggesting that this assay may be used for both diagnostic and surveillance testing. According to the RT-mPCR results, IPNV was not present in wild fish. These results support the hypothesis that the VHSV-Ie genotype, highly prevalent among fish species in the Black Sea, may have a serious impact on the population dynamics of wild fish stocks.

KEY WORDS: VHSV · Genotype Ie · Marine species · Prevalence · Turkey · Black Sea

INTRODUCTION

Viral haemorrhagic septicaemia virus (VHSV), capable of inducing high mortality in cultured rainbow trout *Oncorhynchus mykiss* (deKinkelin et al. 1979, Wolf 1988) and wild marine fish (Meyers et al. 1999, Traxler et al. 1999) and belongs to the genus *Novirhabdovirus* of the *Rhabdoviridae* family (Walker et al. 2000). Since its first isolation from Atlantic cod *Gadus morhua* in the marine environment (Jensen et al. 1979), accumulating evidence indicates that it is enzootic in Europe, including the Baltic Sea (Mortensen et al. 1999, King et al. 2001, Skall et al. 2005), North America (Hedrick et al. 2003), and Japan (Takano et al. 2001).

Four genotypes of VHSV have been identified around the world (Einer-Jensen et al. 2004): Genotype I (freshwater and marine isolates from continental Europe), Genotype II (marine isolates from the Baltic Sea), Genotype III (marine isolates from the North Sea and North Atlantic), and Genotype IV (North American isolates). Genotype I, representing related freshwater and marine isolates, was further divided into 5 subtypes; Ia, Ib, Ic, Id, and Ie (Einer-Jensen et al. 2004). The isolates obtained to date from rainbow trout *Oncorhynchus mykiss*, turbot *Psetta*

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maxima, and whiting *Merlangius merlangus* in the Black Sea belong to Genotype Ie (Einer-Jensen et al. 2006, Nishizawa et al. 2006, Altuntas & Ogut 2010).

The VSHV belonging to Genotype Ie was first isolated from rainbow trout in Georgia, in the Black Sea (Einer-Jensen et al. 2004), when it was reported from cultured and wild turbot *Psetta maxima* (Nishizawa et al. 2006). Altuntas & Ogut (2010) recently isolated the same genotype from whiting *Merlangius merlangus*, a keystone prey item in the Black Sea ecosystem. The latter report strongly indicated that VHSV was enzootic in the ecosystem. Therefore, the purpose of this study was to assess the extent and distribution of VHSV among marine fish species in the ecosystem. This study has strong implications for managing VHSV in such operations as fish transfers, seed production, and seed release for stock enhancement of marine fish species.

MATERIALS AND METHODS

Sampling of marine fish

Fish were captured from December to April using gill nets, purse-seining, and hook and line off the coast of the southeastern Black Sea (Tables 1 & 2) in 2009, 2010, and 2011. This period for sampling was selected because VHSV was primarily present in January and February in whiting *Merlangius merlangus*, the keystone prey species in the Black Sea ecosystem (Altuntas & Ogut 2010). Samples were collected as pools (4 to 7 fish), or individually, if sample size was small, transported to the laboratory on ice, and processed the same day. Tissue portions from liver, kidney, and spleen were combined as pools of each species, unless otherwise stated. Each fish was additionally inspected for external gross pathological signs, and weight and length of each sample fish were recorded.

Cell culture and viral isolation

Tissue portions from kidney, liver, and spleen were pooled from each fish in minimal essential medium (MEM) at a 1:10 dilution, containing antibiotics (100 IU penicillin ml^{-1}, 100 µg streptomycin ml^{-1}), and a fungicide (0.25 µg amphotericin-B ml^{-1}). The samples were then homogenized and centrifuged for 20 min at 4000 ×*g* at 4°C. After incubating for 12 to 14 h at 4°C, they were inoculated onto BF-2 and epithelioma papillosum cyprini cells (EPC) cell lines at dilutions of 1:10 and 1:100 in duplicate. After pooling duplicate sample dilutions of cell culture fluid for multiplex reverse transcriptase reaction (RT-mPCR) and ELISA (Test Line, Clinical Diagnostics), cells showing a cytopathic effect (CPE) were passaged onto new cells to confirm that the CPE was not the result of toxicity. If CPE was observed in the second passage, the cell culture fluids taken from the first passage were centrifuged at 4000 × *g* for 15 min to pellet cells. Antigen detection ELISA (Test Line) was performed as described by the manufacturer with slight modifications (Altuntas & Ogut 2010).

Multiplex RT-mPCR and sequencing

To determine the possibility of simultaneous detection of VHSV and infectious pancreatic necrotic virus (IPNV), stock solutions of VHSV-Ie (MM12, $10^{6.75}$ $TCID_{50}$ [median tissue culture infection dose] virus ml^{-1}) and IPNV Genogroup III (ilker, $10^{7.00}$ $TCID_{50}$ virus ml^{-1}) were diluted 10-fold, mixed, and inoculated onto BF-2 cells in triplicate (Table 3). At 2 and 3 d post-exposure, CPE types and levels were checked and recorded. On Day 4, 200 µl of clarified cell culture supernatant, from each replicate well, was pooled, and RNA was extracted from 50 µl using TRI© Reagent LS (Sigma). We also added 25 µl Trypsin (0.25%), EDTA (0.05%) solution per 50 µl filtered cell culture fluid before subjecting to TRI© Reagent LS.

The quality and quantity of harvested RNA was determined by measuring the absorbance at 260 nm in a spectrophotometer (Boeco, S30). The amount of RNA template added was 110 ng per 50 µl reaction. The primer set, VP2/NS junction coding region (1403 to 1761 bp), for Jasper-IPNV (forward primer: 5′-AGA GAT CAC TGA CTT CAC AAG TGA C-3′; reverse primer: 5′-TGT GCA CCA CAG GAA AGA TGA CTC-3′) designed by Heppell et al. (1992) and the primer set for VHSV (forward primer: 5′-CCA GCT CAA CTC AGG TGT CC-3′; reverse primer: 5′-GTC ACY GTG CAT GCC ATT GT-3′), targeting a 587 base region of the VHSV G gene (nt 175 to 761), designed by Nishizawa et al. (2002) were used for amplification. The same primer set was also used for detection of VHSV-Ie in an earlier study in the Black Sea (Nishizawa et al. 2006).

We modified the One Tube RT-PCR System (Roche Molecular Biochemicals) for mRT-PCR, mostly following the manufacturer's recommendations. The following program on a thermocycler (MJMini, Bio Rad) was used: an initial denaturation of RNA template (110 ng per 50 µl) with primers (50 pmol of each primer set) was

Table 1. Sampling scheme of marine fish for viral haemorrhagic septicaemia virus Genotype Ie (VHSV-Ie) in 2009, 2010, and 2011. P: number of positive pools; G: number of pools; n: number of fish in all pools

Species	Dec		Jan			Feb			Mar			Total
	2010	2011	2009	2010	2011	2009	2010	2011	2009	2010	2011	
	P/G/n	P/G/n	P/G/n	P/G/n	P/G/n	P/G/n	P/G/n	P/G/n	P/G/n	P/G/n	P/G/n	P/G/n
Akçaabat (41° 01′ N, 39° 33′ E)												
Red mullet *Mullus barbatus*				0/9/43		0/3/13	0/5/24		0/2/8	1/9/43		1/28/131
Bluefish *Pomatomus saltatrix*				0/1/1	0/1/2	0/3/5				0/1/5		0/6/13
Thornback ray *Raja clavata*[a]												1/8/8[a]
Common sole *Solea* sp.				0/2/8	0/1/2	0/1/5	0/1/3					0/5/18
Three-bearded rockling *Gaidropsarus vulgaris*				2/7/32		0/1/5	0/1/3					2/9/40
Anchovy *Engraulis encrasicolus*				0/1/13								0/1/13
Black scorpionfish *Scorpaena porcus*				0/4/17						0/1/4		0/5/21
Mediterranean horse mackerel *Trachurus mediterraneus*				0/20/78	0/10/50	2/16/80	0/10/50	1/18/90	0/4/19		0/10/50	3/88/417
Picarel *Spicara smaris*				0/13/55		0/4/17	0/21/111		0/1/6	0/10/53		0/49/242
Snake blenny *Ophidion barbatum*				0/1/1	0/1/4	0/1/2	0/1/2					0/4/9
Stargazer *Uranoscopus scaber*				0/2/11								0/2/11
Whiting (pooled) *Merlangius merlangus*			0/13/90			0/35/199			1/16/79			1/64/368
Pontic shad *Alosa immaculata*			0/1/1	1/4/16	0/1/2	0/6/17	0/9/23	1/7/21	0/1/4	0/1/1		2/30/85
Black goby *Gobius niger*				0/3/4	0/1/5	0/1/6	0/2/3			0/3/5		0/10/23
Çamburnu (40° 55′ N, 40° 11′ E)												
Red mullet *Mullus barbatus*	1/31/138			0/22/105				0/6/27			1/13/60	2/72/330
Sprat *Sprattus sprattus*	0/5/40				0/16/112							0/21/152
Bluefish *Pomatomus saltatrix*					0/1/1						0/5/15	0/6/16
Thornback ray *Raja clavata*	0/19/20								0/8/8[a]			0/27/28
Common sole *Solea* sp.	0/2/2				0/2/5			0/1/2			0/1/1	0/6/10
Three-bearded rockling *Gaidropsarus vulgaris*	1/8/10				0/11/11			1/2/2			1/4/4	3/25/27
Anchovy *Engraulis encrasicolus*	0/15/105				0/12/84						0/17/114	0/44/303
Black scorpionfish *Scorpaena porcus*	0/9/30				1/13/51			0/5/21			0/11/41	1/38/143
Mediterranean horse mackerel *Trachurus mediterraneus*				3/8/46	0/5/17	0/3/16	1/10/10			0/10/10	0/17/81	4/55/186
Picarel *Spicara smaris*	0/3/8				0/9/23			0/1/3			0/7/30	0/20/64
Snake blenny *Ophidion barbatum*	0/2/3				0/2/2							0/4/5
Stargazer *Uranoscopus scaber*	5/12/51				5/7/26			0/3/4			0/6/20	10/28/101
Whiting (pooled) *Merlangius merlangus*			0/12/88	0/21/106			2/16/81	1/4/20	0/13/65	6/23/114	0/1/7	9/90/481
Whiting (individual) *M. merlangus*					1/97/97			0/105/105			1/137/137	2/339/339
Pilchard *Sardina pilchardus*	0/2/6				0/5/8		0/1/1	0/4/12		0/1/2	2/15/71	2/28/100
Pontic shad *Alosa immaculata*	0/2/2				0/7/15			0/1/2			0/2/5	0/12/24
Garfish *Belone belone*	1/10/49											1/10/49
Round goby *Neogobius melanostomus*	0/4/11				2/11/49		0/2/6	0/4/21			0/7/36	2/28/123
Çarşibaşı (41° 04′ N, 39° 20′ E)												
Thornback ray *Raja clavata*[a]												1/34/34
Anchovy *Engraulis encrasicolus*					1/10/70							1/10/70
Stargazer *Uranoscopus scaber*					0/3/12							0/3/12
Hopa (41° 24′ N, 41° 25′ E)												
Sprat *Sprattus sprattus*							0/10/100					0/10/100
Anchovy *Engraulis encrasicolus*				2/10/100			2/10/100					2/20/200
Mediterranean horse mackerel *Trachurus mediterraneus*							0/15/76					0/15/76
Ordu (41° 04′ N, 37° 46′ E)												
Mediterranean horse mackerel *Trachurus mediterraneus*			4/10/70									4/10/70
Yomra (40° 58′ N, 39° 53′ E)												
Whiting (individual) *Merlangius merlangus euxinus*			4/43/43	2/150/150		4/80/80	8/199/199		1/88/88	1/93/93		20/653/653

[a]All fish were sampled individually

Table 2. Fish species infected with viral haemorrhagic septicaemia virus Genotype Ie (VHSV-Ie) and estimated pooled prevalence. P: number of positive pools; G: number of pools; n: number of fish in all pools; P_{min} and P_{max}: confidence intervals of prevalence estimated from pool prevalence. na: not applicable. For full taxonomic names see Table 1

Species	Sampling date (mm/dd/yyyy)	P/G/n	Prevalence	P_{min}	P_{max}
Akçaabat					
M. barbatus	03/23/2010	1/9/43	2.33	2.29	6.94
G. vulgaris	01/03/2010	2/5/23	9.72	0	23.19
T. mediterraneus	02/07/2009	2/10/50	4.36	0	10.29
T. mediterraneus	02/18/2011	1/18/90	1.14	0	3.52
M. merlangus	03/19/2009	1/16/79	1.28	0	3.80
A. immaculata	01/31/2010	1/3/15	7.79	0	22.55
A. immaculata	02/11/2011	1/7/21	5.01	0	14.58
Çarşibaşı					
R. clavata	04/11/2009	1/8/8	12.5	na	na
E. encrasicolus	01/09/2011	1/10/70	1.49	0	4.40
R. clavata	04/18/2009	1/34/34	2.94	na	na
Çamburnu					
M. barbatus	12/10/2010	1/13/65	1.58	0	4.68
M. barbatus	03/02/2011	1/2/8	15.91	0	45.05
G. vulgaris	12/10/2010	1/1/3	100	na	na
G. vulgaris	02/18/2011	1/2/2	50	na	na
G. vulgaris	03/02/2011	1/2/2	50	na	na
N. melanostomus	01/04/2011	1/2/7	20.63	0	54.58
N. melanostomus	01/19/2011	1/1/2	100	na	na
S. porcus	01/30/2011	1/3/12	9.64	0	27.72
T. mediterraneus	01/06/2010	2/5/30	9.71	0	21.51
T. mediterraneus	01/09/2010	1/3/16	7.79	0	22.08
T. mediterraneus	02/13/2010	1/10/10	10	na	na
M. merlangus	02/03/2010	2/16/81	2.64	0	6.22
M. merlangus	03/30/2010	6/23/114	5.87	1.28	10.46
M. merlangus	02/28/2011	1/4/20	5.59	0	16.27
M. merlangus	01/27/2011	1/43/43	2.33	na	na
M. merlangus	03/28/2011	1/57/57	1.75	na	na
S. pilchardus	03/02/2011	1/10/48	2.09	0	6.21
S. pilchardus	03/03/2011	1/4/20	5.59	0	16.27
U. scaber	12/10/2010	5/9/46	14.97	2.69	27.26
U. scaber	01/17/2011	1/1/6	100	na	na
U. scaber	01/19/2011	1/1/2	100	na	na
U. scaber	01/20/2011	2/2/8	100	na	na
U. scaber	01/23/2011	1/1/2	100	na	na
B. belone	12/15/2010	1/10/49	2.09	0	6.17
Hopa					
E. encrasicolus	02/20/2010	2/10/100	2.21	0	5.24
Ordu					
T. mediterraneus	01/19/2009	4/10/70	7.04	0.32	13.76
Yomra					
M. merlangus	01/20/2009	4/43/43	9.30	na	na
M. merlangus	02/08/2009	4/80/80	5.00	na	na
M. merlangus	03/04/2009	1/88/88	1.14	na	na
M. merlangus	01/10/2010	2/82/82	2.44	na	na
M. merlangus	02/07/2010	6/62/62	9.68	na	na
M. merlangus	02/18/2010	2/73/73	2.74	na	na
M. merlangus	03/26/2010	1/23/23	4.35	na	na

carried out at 68°C for 2 min and immediately placed on ice for at least for 2 min. Master Mix (1 µl dNTP [10 mM] of 2.5 µl of DDT solution, 10 µl RT-PCR reaction buffer, 5× concentrated with 7.5 mM $MgCl_2$ and DMSO, and 1 µl of enzyme mix) was added and subjected to reverse transcription for 30 min at 50°C, followed by 15 min at 70°C, and 7 min at 68°C. The reaction was denatured for 5 min at 94°C, followed by 10 cycles of denaturation at 94°C for 10 s, annealing at 52°C for 30 s, and primer extension at 68°C for 2 min, and another 25 cycles of the same conditions, except that primer annealing was set at 45 s, and 5 s was added in each cycle. Finally, a product extension step was carried out at 72°C for 5 min. The product (10 µl) was stained with ethidium bromide and visualized by UV transillumination following electrophoretic separation (1 h, 110 V) on 1.5% agarose gels.

Using the same protocol for RT-PCR, duplicate sets of viral RNA from VHSV collected from fish species were amplified and purified using a commercial kit (QIAquick PCR Purification Kit, Qiagen), and submitted for sequencing (Macrogen). The sequences were then compared to the GenBank database using the NCBI BLAST server hosting comprehensive sequence-based taxonomic data for species determination of the virus.

Statistical analysis

The evolutionary history from sequences was inferred using the unweighted pair-group method using arithmetic averages (UPGMA) (Sneath & Sokal 1973). The optimal tree with the sum of branch lengths equal to 0.03798513 was used to obtain the dendrogram, and evolutionary distances were computed using the maximum composite likelihood method (Tamura et al. 2004) and are given as the count of base substitutions per site. Evolutionary analyses were conducted in MEGA5 (Tamura et al. 2011). The prevalence of VHSV-Ie in pelagic fish samples was compared with the prevalence of bottom fish using the Mann-Whitney *U*-test for the fact that the data were not normally distributed. Prevalence estimation from pooled samples was carried out as described by Kline et al. (1989) and Altuntas & Ogut (2010).

Table 3. Co-infection assay of infectious pancreatic necrosis virus (IPNV) Genogroup III and viral haemorrhagic septicaemia virus Genotype Ie (VHSV-Ie) on BF-2 cells. $TCID_{50}$: median tissue culture infection dose; NCPE: no cytopathic effect (CPE); SVA: some viral activity (no CPE but rounded cells not detached); NFC: no floating cells; EC: enlarged and floating cells; ICPE: IPNV-like CPE (burst cells); VCPE: VHSV-like CPE (rounded, slightly enlarged grape-like cells); MEM-0: minimum essential medium

Label	VHSV-Ie ($TCID_{50}$)	IPNV-III ($TCID_{50}$)	Day 2 post-challenge	Day 3 post-challenge
A	$10^{5.75}$	10^{0}	NCPE, SVA, NFC, EC	VCPE, 80%
B	$10^{4.75}$	10^{1}	NCPE, SVA, NFC, EC	VCPE, 80%
C	$10^{3.75}$	10^{2}	NCPE, NFC	60% CPE
D	$10^{2.75}$	10^{3}	NFC, <10% CPE	ICPE, 80% CPE
E	$10^{1.75}$	10^{4}	ICPE, 40% CPE	ICPE, 100% CPE
F	$10^{0.75}$	10^{5}	ICPE, 80% CPE	ICPE, 100% CPE
G	0	10^{6}	ICPE, 100% CPE	ICPE, 100% CPE
Control	MEM-0	MEM-0	NCPE, NFC	NCPE, NFC

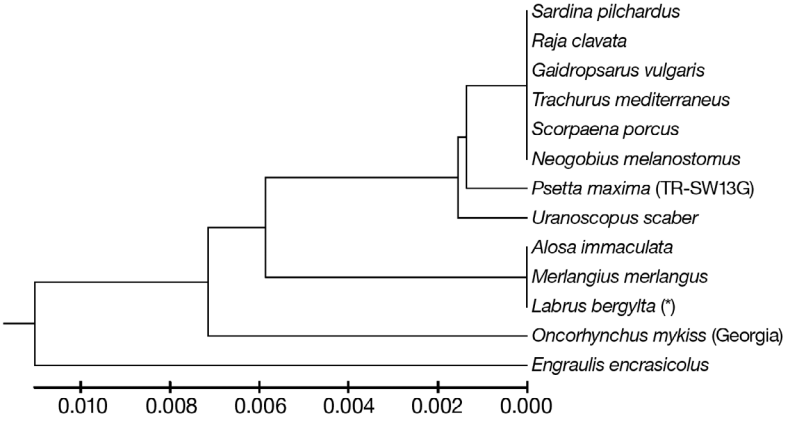

Fig. 1. Evolutionary relationships of viral haemorrhagic septicaemia virus Genotype Ie (VHSV-Ie) in various host species. The evolutionary history was inferred using the unweighted pair-group method using arithmetic averages (Sneath & Sokal 1973). The optimal tree with the sum of branch lengths equal to 0.03798513 is shown. The evolutionary distances were computed using the maximum composite likelihood method (Tamura et al. 2004) and are given as the number of base substitutions per site. (*) the isolate from *Labrus bergylta* was an experimental isolate; the isolate from *Merlangius merlangus* was used to challenge *L. bergylta*

RESULTS

A total of 5025 fish belonging to 17 species of wild marine fish from 6 stations (Akçaabat, Çamburnu, Çarşibaşı, Hopa, Ordu, and Yomra) were sampled during December, January, March, and April of 2009, 2010, and 2011 (Table 1). Twelve species of fish (pontic shad *Alosa immaculata*, red mullet *Mullus barbatus*, three-bearded rockling *Gaidropsarus vulgaris*, black scorpionfish *Scorpaena porcus*, Mediterranean horse mackerel *Trachurus mediterraneus*, whiting *Merlangius merlangus euxinus*, stargazer *Uranoscopus scaber*, pilchard *Sardina pilchardus*, garfish *Belone belone*, round goby *Neogobius melanostomus*, thornback ray *Raja clavata*, and anchovy *Engraulis encrasicolus*) tested positive for VHSV. After analysis of sequenced amplicons, all VHSV isolates belonged to Genotype Ie (Fig. 1). Except *M. merlangus euxinus*, *S. pilchardus*, and *N. melanostomus*, the rest are all new hosts to VHSV. Furthermore, except for *M. merlangus euxinus*, all these species are new host records of VHSV from the area. Except for *T. mediterraneus*, all other fish testing positive for VHSV were asymptomatically infected. *T. mediterraneus* had skin loss (up to 12% in some samples) associated with bacterial diseases (presumptively *Aeromonas* spp.). No parasites were detected, and only some of the fish with skin lesions had VHSV (data not provided).

Bottom fishes had significantly higher rates of VHSV-Ie prevalence than did pelagic fish (Mann-Whitney *U*-test, $p = 0.025$). Stargazer had consistently high prevalence levels. Despite low sample sizes (2 to 8), the majority of fish tested were positive for VHSV-Ie, except in the December sampling, when only 5 out of 9 pools tested positive for VHSV-Ie. Another species showing similar patterns (low sample size but high prevalence) was three-bearded rockling. VHSV-Ie was present in small samples, e.g. 1 of 2 fish, collected in December, January, and February. Similarly, black scorpionfish also had maximum prevalences (P_{max}) up to 28%. Economically the 2 main pelagic fish species, horse mackerel and anchovy, also tested positive for VHSV-Ie. Prevalence of VSHV-Ie in horse mackerel was high (up to P_{max} = 22%) in some instances, whereas it was lower (estimated P_{max} up to 5.2%) in anchovy. Sardine, a new exotic pelagic fish species to the Black Sea were also positive for VHSV-Ie at low prevalence (2.09 to 5.6%).

RT-mPCR for the detection of co-infection in cells having a cytopathic effect attributable to IPNV and/or VHSV was possible (Fig. 2). VHSV-Ie was more efficiently replicated in cell culture than was IPNV. The assay detected 100 $TCID_{50}$ IPNV ml^{-1} or roughly 50 $TCID_{50}$ VHSV-Ie ml^{-1}.

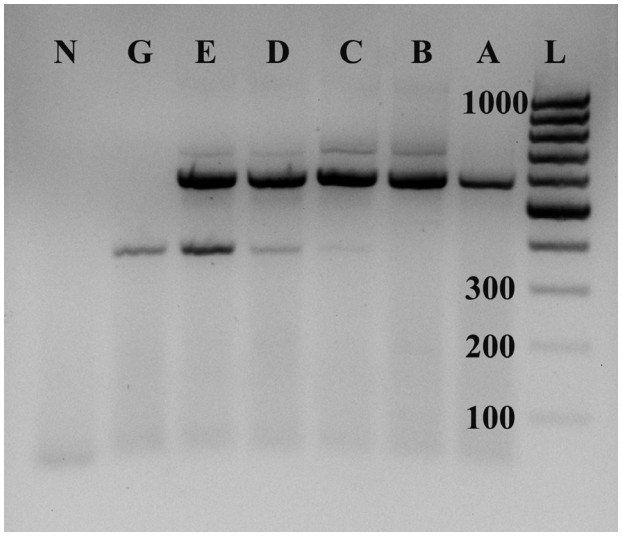

Fig. 2. Multiplex reverse transcriptase PCR of infectious pancreatic necrosis virus (IPNV) and viral haemorrhagic septicaemia virus (VHSV) following co-infection assays on BF-2 cell lines, as described in Table 3. Lane N: negative control, RNA of BF-2 cells (negative controls without RNA template and without primers are not shown). Lanes A–G: Results from co-infection assays A–G (NB assay F was not subjected to multiplex PCR because the CPE results were identical to assays E and G); VHSV concentration increases and IPNV concentration decreases from left to right. Lane L: 100 bp molecular size ladder

DISCUSSION

Turkey is the biggest fisheries producer in the Black Sea. Catches in the last 3 decades have fluctuated greatly; this is attributable to various factors, e.g. introduced species, global warming, and overfishing (Kideys 1994, Richardson et al. 2009). However, the impacts of microbial diseases on economically important schooling pelagic fish have largely been ignored in managing fisheries in the region. We investigated whether VHSV-Ie is endemic among wild fish species in the Black Sea. Our results show that VHSV-Ie is endemic among wild fish species in the region. There were 9 new hosts recorded for VHSV from the area. The results of the survey indicate that VHSV is enzootic among wild fish species in the Black Sea ecosystem. However, our results disagreed with the results of Işıdan & Bolat (2011), who reported that, with the exception of turbot *Psetta maxima*, wild fish in the Black Sea were free of VHSV-Ie. The difference in the results might be due to the sampling design, as sample collection in their study was carried out in the fall and spring. However, Altuntas & Ogut (2010) showed, after extensive monthly sampling, that VHSV-Ie is present in whiting in the Black Sea from January to April, but primarily in January and February. Captured fish by fisherman in fishing ports were accepted as a sample in their study. This sampling strategy could also be problematic since captured fish could be exposed to high temperatures leading to inactivation and degeneration of viral particles due to adverse conditions, including bacteria (Mori et al. 2002). Our results lend further evidence to the suggestion of Altuntas & Ogut (2010) that VHSV-Ie is endemic to the Black Sea.

All VHSV isolations were carried out from asymptomatic fish, except Mediterranean horse mackerel, similar to most VHSV isolations around the world (Dixon 1999, Mortensen et al. 1999, King et al. 2001, Skall et al. 2005). Horse mackerel had some skin lesions presumptively caused by bacteria, *Aeromonas* spp. Low levels of prevalence combined with the lack of any signs of disease suggest that all isolations were from asymptomatic carriers of VHSV-Ie.

Our results provide further evidence that VHSV-Ie is endemic to the Black Sea in that horse mackerel, another keystone prey in the ecosystem, also carry the virus at much higher prevalence levels (P_{max} up to 22%) than do whiting. The high prevalence obtained in mackerel suggests that it could be one of the main sources or hot spots of virus in the Black Sea. Larvae of this species heavily feed on phytoplankton and copepods, while adults prey on larvae of other fish in the summer (Slastenenko 1956, Fischer 1973). Whiting also feed heavily on larvae and juveniles of horse mackerel after anchovy leaves the region after February. More study is needed on feeding habits of horse mackerel in winter to gain insight of the other potential sources of VHSV-Ie. In Ordu province, in 1 pooled sample (5 fish pool^{-1}) collected from mackerel, the prevalence of VHSV in pooled samples was estimated to be 7.04% (0.32 to 13.76%). The data suggest that more rigorous sampling needs to be carried out to conclude that the stock of a given fish species is free of VHSV.

Prevalence rates among pelagic fish were significantly higher than those among bottom-dwelling fish. Skall et al. (2005) suggested that schooling behavior may enhance fish-to-fish transfer of the virus. Anchovy and horse mackerel have the largest stocks in the Black Sea and they form large seasonal schools in the area. Interestingly, during the VHSV season (December to April) in the Black Sea, horse mackerel do not actively forage in the area. All fish live on a narrow continental shelf where the water column depths are a maximum of 250 m. There are no fish living below 250 m due to hydrogen sulfide.

Anchovy *Engraulis encrasicolus*, actively preyed upon by almost all carnivorous fish species, are present in the area and actively forage during the same period. It is likely that bottom-dwelling carnivorous fish are more vulnerable to VHSV-Ie, since infected fish, lethargic and slowly sinking to the bottom, are easy prey for them during winter months when VHSV is readily isolated from wild fish. The same may also be true for infected bottom fish becoming easy prey for other pelagic fish. High prevalence among bottom fish suggests that more research efforts should be spent on the impact of VHSV-Ie on wild fish stocks, as most bottom fish in the region are endangered and their fishery has already been discontinued (GFCM 2012).

The potential source(s) of VHSV are not fully known in the area. Nishizawa et al. (2006) reported that 14 out of 66 turbot *Psetta maxima* captured in our sampling area were positive for VHSV. The high ratio of prevalence observed in wild turbot could be due to feeding on infected whiting, and particularly horse mackerel, both predominant prey items for turbot. Furthermore, before 2005, larval turbot were released into the area without checking for potential viruses during seed production. To our knowledge, brood stock turbot before and during seed production were being fed regularly on whiting as fresh feed. Thus, unintentional release of infected seed into the area could also be one of the main reasons behind the unexpected high prevalence in turbot in the region.

Various protocols for the molecular diagnoses of VHSV and other fish viruses have been developed (Bruchhof et al. 1995, Miller et al. 1998, Guillou et al. 1999). However, due to the unstable nature of RNA and the risk of contamination, Knüsel et al. (2007) suggested that RT-mPCR is more suitable for laboratory samples instead of testing on field samples. Consequently, in this study, we developed an RT-mPCR method with commonly used RT-PCR kits for the detection of VHSV and IPNV. Our approach is also suitable for field samples, as homogenates with IPNV and VHSV can be considered in cell cultures. The results also suggest that co-infections can be detected efficiently. However, more tests with different IPNV and VHSV isolates are needed in order to more accurately determine the level of test sensitivity and specificity. Comparing the propagation of IPNV and VHSV together in cell culture, VHSV-Ie appears to be more competitive than IPNV for binding receptors. De las Heras et al. (2008) showed by flow cytometric assay that both IPNV and VHSV bind to the cells at identical rates. Our RT-mPCR results showed, on the other hand, that the amount of IPNV RNA was probably less than that of VHSV in cell culture fluids of the cells inoculated with equal amounts of IPNV and VHSV-Ie, either due to the early destruction of cells by IPNV restricting production of the virus, or by competition. In brief, VHSV-Ie appears more efficient in co-infections than IPNV according to the RT-mPCR assay. Detection limits were similar to those reported by Williams et al. (1999). Our data show a detection limit of 100 $TCID_{50}$ IPNV ml^{-1}, the same as that reported by Williams et al. (1999) and roughly half that for VHSV-Ie.

In conclusion, VHSV-Ie is endemic in wild fishes living in the southeastern Black Sea. VHSV-Ie was isolated from 12 species of wild fish, of which 9 species represent new host records for VHSV: pontic shad *Alosa immaculata*, red mullet *Mullus barbatus*, three-bearded rockling *Gaidropsarus vulgaris*, black scorpionfish *Scorpaena porcus*, Mediterranean horse mackerel *Trachurus mediterraneus*, stargazer *Uranoscopus scaber*, garfish *Belone belone*, thornback ray *Raja clavata*, and anchovy *Engraulis encrasicolus*. IPNV was not detected during our survey. The high degree of endemicity of VHSV also suggests that the epidemiology of disease need to be considered in managing of fisheries in the Black Sea. The mRT-PCR developed for simultaneous detection of IPNV and VHSV is practical and could efficiently be used for surveillance and diagnostic testing.

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