European Union Reference Laboratory for Fish Diseases
National Veterinary Institute, Technical University of Denmark, Copenhagen

DTU

Development of a novel one-step RT-PCR for detection of VHSV

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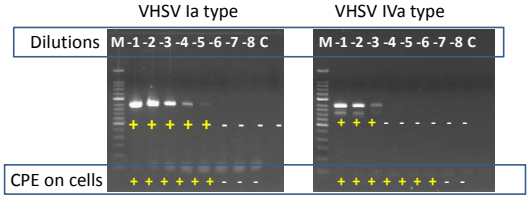
Problems of conventional RT-PCR for VHSV detection

- A conventional RT-PCR for detection of VHS developed by M. Snow et al. is included in the OIE Diagnostic Manual as well as in the EU CD 2015-1554.
- Low sensitivity for detection of VHSV Genotype IVa (Kim 2015, Aquaculture).
- Often non specific reaction close to the VHSV amplicon is seen when testing various non- infected cell cultures leading to false positive reactions. (OIE, 2012)

Current OIE RT PCR problems (1)

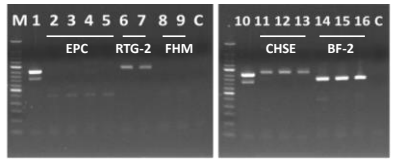
- Low Sensitivity for VHSV IVa



- Results : The sensitivity of OIE primer set was high using VHSV Ia type. But, low sensitivity was showed in VHSV IVa type.

Current OIE RT PCR problems (2)

- Non specific bands appear when normal fish cell lines are used



M : 50 bp DNA size marker (Takara), Lane 1 & 10 : Positive control
Lane 2-5 : EPC, Lane 6 & 7 : RTG-2, Lane 8 & 9 : FHM,
C : Negative control, Lane 11-13 : CHSE, Lane 14-16 : BF-2

→ Non-specific bands can be observed with EPC, RTG-2, CHSE-214 and BF-2.

Therefore:

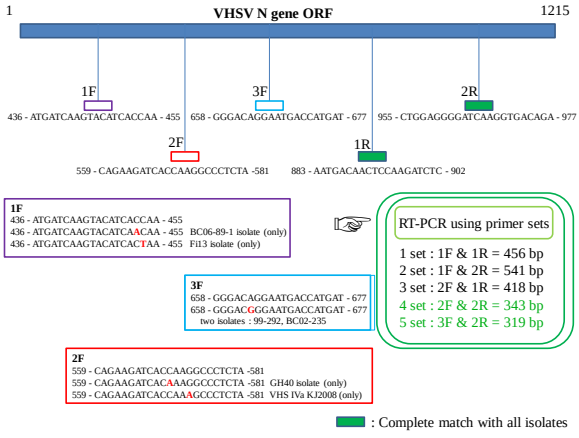
→ We need to improve the specificity of the conventional RT-PCR so that all VHSV genotypes can be detected without the non-specific reaction due to fish cell lines.

New Primer Design

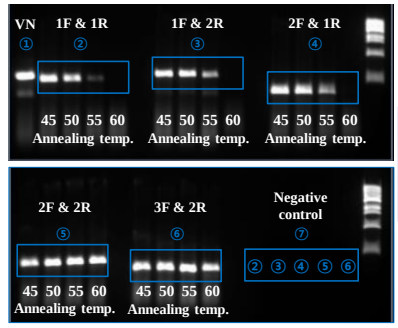
- Primer sets for VHSV gene detection in 37 published articles were investigated against 136 VHSV N-genes - NCBI Genbank (118) + EURL Fishpathogens.eu (18)

Result : No primer set matched all VHSV genotypes therefore

- Candidate primers for 5 regions were designed.



RT-PCR results on VHSV IVa using new primer sets

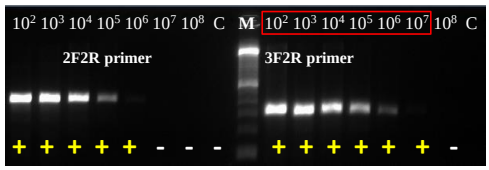


Materials and Methods
- Template : spleen from challenged flounder with VHSV KJ2008 (IVa)
- RNA extraction and RT-PCR
- PCR condition : OIE manual (VN primer set)

→ **2F2R & 3F2R primer sets amplified VHSV IVa at all temperatures.**

RT-PCR titration results using new primer sets

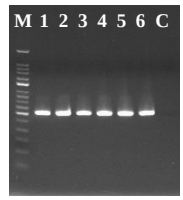
Materials and Methods
- Template : RNA from spleen of challenged flounder with VHSV KJ2008 IVa
- RNA extraction and RT-PCR
- PCR condition : OIE manual (annealing 55 ℃)



→ **3F2R primer set showed higher sensitivity than 2F2R.**
→ **So, the 3F2R primer set was selected.**

Do primer set from various companies influence the 3F2R RT-PCR?

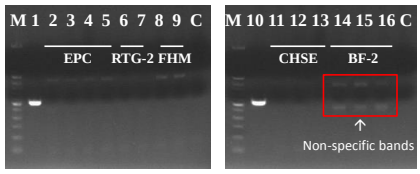
Primer set from 6 companies tested :



M : 50 bp DNA size marker,
Lane 1-5 : 5 Korean companies
Lane 6 : Danish company
C : Negative control

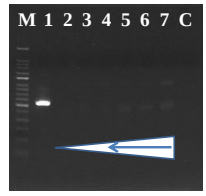
→**No effects of using different companies.**

Do 3F2R cause non-specific reaction with fish cell lines?



→ However, the non-specific bands were detected in all fish cell lines, and specially same bands as target size were observed with BF-2 cell line.

Change of annealing temperatures from 55 to 60°C reduced the non specific bands on BF-2 cell line

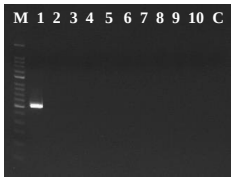


Lane 1 : positive control
Lane 2 : 60 °C
Lane 3 : 59 °C
Lane 4 : 58 °C
Lane 5 : 57 °C
Lane 6 : 56 °C
Lane 7 : 55 °C
Lane C : Negative control

→ **The non-specific bands disappeared when annealing temperatures increased from 55°C to 60°C.**

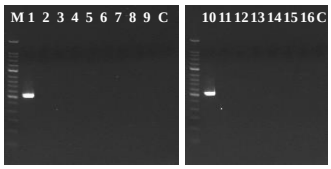
The VHSV RT-PCR using the 3F2R primers at annealing temperature of 60 °C resulted in high specificity with no cross reaction to any heterologous viruses or to any fish cell lines.

Heterologous viruses



Lane 1 : Positive control
Lane 2 : IHNV F-32/87
Lane 3 : IHNV I-4008
Lane 4 : IHNV DW
Lane 5 : IHNV BC
Lane 6 : Birnavirus II
Lane 7 : LGV
Lane 8 : PFR
Lane 9 : SVC 56/70 Fijan
Lane 10 : KHV H361
Lane 11 : Negative control

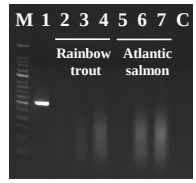
Cell lines



M : 50 bp DNA size marker,
Lane 1 & 10 : Positive control
Lane 2-5 : EPC
Lane 6 & 7 : RTG-2
Lane 8 & 9 : FHM,
Lane 11-13 : CHSE
Lane 14-16 : BF-2
C : Negative control

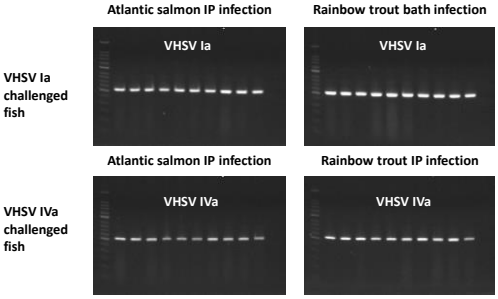
No non-specific reactions were observed in tissue samples from rainbow trout and Atlantic salmon

Non-infected fish samples



Lane 1 : positive control
Lane 2 : Spleen and Kidney in Rainbow trout
Lane 3 : Rainbow trout 2 small fishes
Lane 4 : Rainbow trout 2 small fishes
Lane 5 : Atlantic salmon 1
Lane 6 : Atlantic salmon 2
Lane 7 : Atlantic salmon 3
Lane C : Negative control

RT-PCR using 3F2R primer on samples fromVHSV infected fish



→**It was confirmed that only specific bands were observed using the 3F2R primer set on VHSV fish infected samples.**

Summary

- A highly sensitive primer set was selected among several new candidate primers
- Reaction conditions were established for this conventional RT PCR without non-specific reactions in fish, fish cell lines or with heterologous viruses

Comparison of sensitivities using several methods

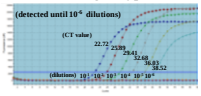
- Real-time RT-PCR (Jonstrup et al. 2013)
- OIE conventional RT-PCR.
- 3F2R conventional RT-PCR
- Cell cultures for virus titration(TCID50)

Testing of 6 VHSV isolates representing all major genotypes (Small panel)

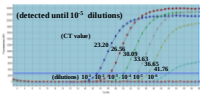
Genotype	Isolate name	Source of isolate	Used cell lines
Ia	DK-3592B	Lorenzen et al. (1993)	BF-2
Ib	DK-1p8	Mortensen et al. (1999)	BF-2
II	DK-1p52	Mortensen et al. (1999)	FHM
III	DK-4p168	Mortensen et al. (1999)	EPC
IVa	KJ2008	Kim & Kim (2011)	EPC
IVb	MIO3, Lakes St. Clair, MI	Elsayed et al. (2006)	EPC

RT-qPCR on titrations from 10^{-1} to 10^{-9} of small panel VHSV

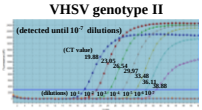
VHSV genotype Ia



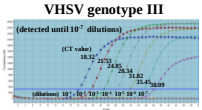
VHSV genotype Ib



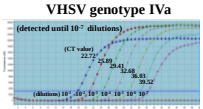
VHSV genotype II



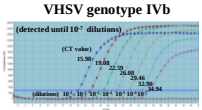
VHSV genotype III



VHSV genotype IVa



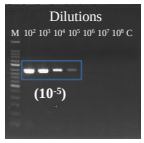
VHSV genotype IVb



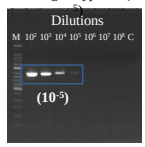
→ **In this small panel, the viral genes were detected at dilutions between 10^{-5} and 10^{-7}**

OIE VN primer RT-PCR on titrations from 10^{-2} to 10^{-8} of small panel VHSV

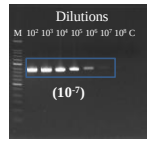
VHSV genotype **Ia** (10^{-5})



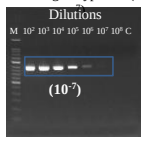
VHSV genotype **Ib** (10^{-



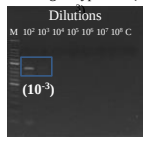
VHSV genotype **II** (10^{-7})



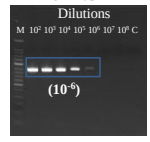
VHSV genotype **III** (10^{-



VHSV genotype **IVa** (10^{-



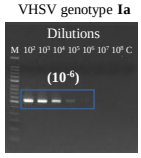
VHSV genotype **IVb** (10^{-6})



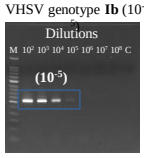
→ **The viral genes were detected at dilutions between 10^{-3} and 10^{-7}**
→ **The OIE VN primer only detected VHSV IVa at a very low level.**

3F2R primer RT-PCR on titrations from 10^{-2} to 10^{-8} of small panel VHSV (annealing at 60 °C)

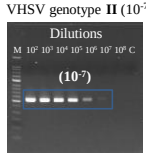
VHSV genotype **Ia**



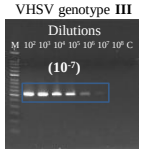
VHSV genotype **Ib** (10^{-



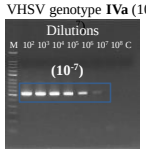
VHSV genotype **II** (10^{-7})



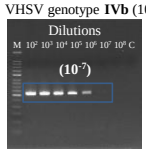
VHSV genotype **III**



VHSV genotype **IVa** (10^{-



VHSV genotype **IVb** (10^{-7})



→ **The viral genes were detected at dilutions between 10^{-5} and 10^{-7}**
→ **The 3F2R primer set detected all VHSV at high level.**

Summary
Sensitivities of 3 RT-PCR and cell culture for detection of VHSV.

Small Panels	Cell culture	Real-time RT-PCR	Conventional RT-PCR using OIE primer	Conventional RT-PCR using 3F2R primer
Ia	-6	-6	-5	-6
Ib	-5	-5	-5	-5
II	-7	-7	-7	-7
III	-7	-7	-7	-7
IVa	-7	-7	-3	-7
IVb	-7	-7	-6	-7

→ **Sensitivity was tested in parallel on cell cultures. Genotype I, II and III on BF-2 and genotype IV on EPC.**

→ **It was concluded that the sensitivity for all genotypes were at the same level when using cell culture, real-time RT-PCR and the conventional 3F2R RT-PCR . While it was lower for the OIE VN RT-PCR.**

Big Panel-1 : Testing 80 VHSV isolates by 3F2R RT-PCR:

Virus number	Isolate	Genotype	Source isolate	Used cell lines
1	DK-F1	I	Jensen (1963, 1965)	BF-2
2	DK-297 (HEDEDAM)	I	Vestergård Jørgensen (1974)	BF-2
3	DK-3592B	Ia	Lorenzen et al. (1993), Jørgensen et al. (1995)	BF-2
4	DK-3971	Ia	Jonstrup et al. (2009)	BF-2
5	DK-6137	Ia	Jørgensen et al. (1995)	EPC
6	DK-7974	Ia	Jonstrup et al. (2009)	BF-2
7	DK-9695377	Ia	Einer-Jensen et al. (2004)	BF-2
8	DK-200051	Ia	Jonstrup et al. (2009)	BF-2
9	DK-200149	Ia	Jonstrup et al. (2009)	BF-2
10	FR-07-71	Ia	Le Berre et al. (1977)	BF-2
11	FR-23-75	Ia	de Kinkelin & Le Berre (1977)	BF-2
12	FR-02-84	Ia	Benmansour et al. (1997)	EPC
13	CZ-7738-R5	Ia	DTU Vet (unpubl.)	BF-2
14	CZ-2077	Ia	Veterinary Research Institute, Brno, Czech Republic (unpubl.)	BF-2
15	DK-5927	Ia	Snow et al. (1999)	BF-2
16	AU-8/95	Ia	University of Veterinary Medicine, Vienna (unpubl.)	BF-2
17	CH-FI 262 BFH	Ia	Cent. for Fish and Wildlife Health, Div. Fish, Univ. of Berne, Switzerland (unpubl.)	BF-2
18	PL-202473	Ia	National Veterinary Research Institute, Pulawy, Poland (unpubl.)	BF-2
19	DK-M. Rhabdo	Ib	Vestergard Jorgensen & Olesen (1987), Jensen, Bloch & Larsen (1979)	BF-2
20	DK-1p8	Ib	Mortensen et al. (1999)	BF-2
21	DK-1p40	Ib	Mortensen et al. (1999)	BF-2
22	DK-1p85	Ib	Mortensen et al. (1999)	BF-2
23	DK-1p86	Ib	Mortensen et al. (1999)	BF-2
24	DK-1p93	Ib	Mortensen et al. (1999)	BF-2
25	DK-1p116	Ib	Mortensen et al. (1999)	BF-2
26	DK-1p120	Ib	Mortensen et al. (1999)	BF-2
27	DK-1p121	Ib	Mortensen et al. (1999)	BF-2
28	DK-5p276	Ib	Skall et al. (2005)	BF-2
29	SE-SVA-14	Ib	Nordblom (1998)	EPC
30	SE-SVA-1033	Ib	Nordblom & Norell (2000)	BF-2

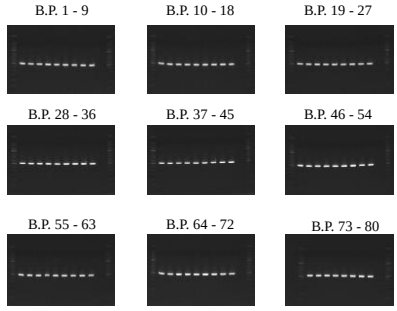
Big Panel-2 : Testing 80 VHSV isolates by 3F2R RT-PCR:

Virus number	Isolate	Genotype	Source isolate	Used cell lines
31	UK-96-43	Ib	Dixon et al.(1997)	BF-2
32	DK-4p37	Ib	Mortensen et al. (1999)	BF-2
33	DK-2835	Ic	Jonstrup et al. (2009)	BF-2
34	DK-5131	Ic	Jonstrup et al. (2009)	BF-2
35	DK-5123	Ic	Jonstrup et al. (2009)	BF-2
36	FIN-ka 422/00	Id	Raja-Halli et al. (2006)	EPC
37	FIN-2ka 66/2000	Id	Einer-Jensen et al. (2004)	EPC
38	NO-A163-68 EG46	Id	Hästein, Holt & Krogsrud (1968)	BF-2
39	GE-1.2	Ie	Lab. of Aq. Animal Health, Russia Res. Inst. for Vet. Viro. and Mic.biol. (unpubl.)	BF-2
40	TR206239-1	Ie	Ito et al. (2012)	BF-2
41	DK-1p49	II	Mortensen et al. (1999)	EPC
42	DK-1p52	II	Mortensen et al. (1999)	FHM
43	DK-1p53	II	Mortensen et al. (1999)	BF-2
44	DK-1p54	II	Mortensen et al. (1999)	BF-2
45	DK-2p51	IIIa	Snow et al. (1999)	BF-2
46	DK-4p101	IIIa	Mortensen et al. (1999)	BF-2
47	DK-4p168	IIIa	Mortensen et al. (1999)	EPC
48	DK-4p51	IIIa	Mortensen et al. (1999)	BF-2
49	UK-H17/5/93	IIIa	Smail (2000)	BF-2
50	UK-860/94	IIIa	Ross et al. (1994)	BF-2
51	UK-H17/2/95	IIIa	Smail (2000)	BF-2
52	FR-L59x	IIIa	Thiery et al. (2002)	EPC
53	NF-GH30	IIIa	Dopazo et al. (2002)	BF-2
54	DK-9795386 (IR-F13.02.97)	IIIa	Snow et al. (1999)	EPC
55	NO-2007-50-385	IIIb	Dale et al. (2009)	BF-2
56	USA-MAKAH	IVa	Brunson et al. (1989)	BF-2
57	USA-KHV	IVa	Hopper (1989)	BF-2
58	USA-Elliott Bay	IVa	USGS (unpubl.)	BF-2
59	Minter Creek, WA	IVa	USGS (unpubl.)	EPC
60	Tokul Creek, WA	IVa	USGS (unpubl.)	EPC

Big Panel-3 : Testing 80 VHSV isolates by 3F2R RT-PCR:

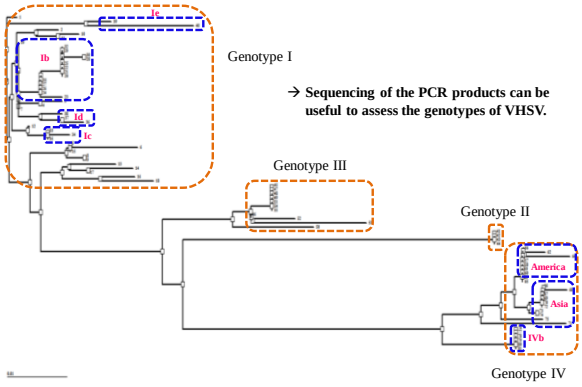
Virus number	Isolate	Genotype	Source isolate	Used cell lines
61	Port Angels, WA	IVa	USGS (unpubl.)	EPC
62	CAN-3624	IVa	Traxler et al. (1995), Pac. Biol. Stat. (unpubl.)	BF-2
63	CAN-99-019	IVa	Ito et al. (2012)	EPC
64	Quatsino, BC	IVa	USGS (unpubl.)	EPC
65	JP-Obama 25	IVa	Takano et al. (2000)	EPC
66	JP-JF00Ehi1	IVa	Nishizawa et al. (2002)	EPC
67	BR01Ehi1	IVa	Ito et al. (2010)	EPC
68	JF01Oit1	IVa	Ito et al. (2010)	EPC
69	JSLOZYam1	IVa	Ito et al. (2010)	EPC
70	PM05Ehi1	IVa	NRIA FRA (unpubl.)	EPC
71	KJ2008	IVa	Kim & Kim (2011)	EPC
72	FYesu05	IVa	Kim et al. (2009)	EPC
73	FWando05	IVa	Kim et al. (2009)	EPC
74	DH2008	IVa	Kim et al. (unpubl.)	EPC
75	MIO3, Lakes St. Clair, MI	IVb	Elsayed et al. (2006)	EPC
76	FPL2006-005, Goby 1-5	IVb	Groocock et al. (2007)	EPC
77	Gizzard shad, Lake Ontario, NY	IVb	USGS (unpubl.)	EPC
78	Blue gill, Budd Lake, MI	IVb	USGS (unpubl.)	EPC
79	Fpl 07-010 Skaneateles Lake, NY	IVb	USGS (unpubl.)	EPC
80	New Brunswick	IVc	Groocock et al. (2007)	EPC

Big Panel of VHSV 80 isolates using 3F2R primer (annealing 60)



→ **Clear and unique amplicons were observed for all 80 VHSV isolates representing a worldwide collection of all known genotypes and subtypes.**

Phylogenetic analysis of Big Panel (80 isolates) using 3F2R primer



→ **Sequencing of the PCR products can be useful to assess the genotypes of VHSV.**

Discussion

- A new conventional RT-PCR have been developed and validated for detection of all genotypes of VHSV.
- The new RT-PCR showed the same sensitivity as cell culture.
- No non-specific responses to heterologous viruses, tissue of several fish species (rainbow trout, atlantic salmon, olive flounder) or fish cell lines were observed.
- We suggest that the new primer set shall replace the current primer set recommended in the OIE manual for detection of VHSV.