Validation of Viral Haemorrhagic Septicaemia (VHS) Virus Conventional RT-PCR

Hyoung Jun Kim & Niels Jørgen Olesen

Team of OIE Twinning project on VHS

Background-1

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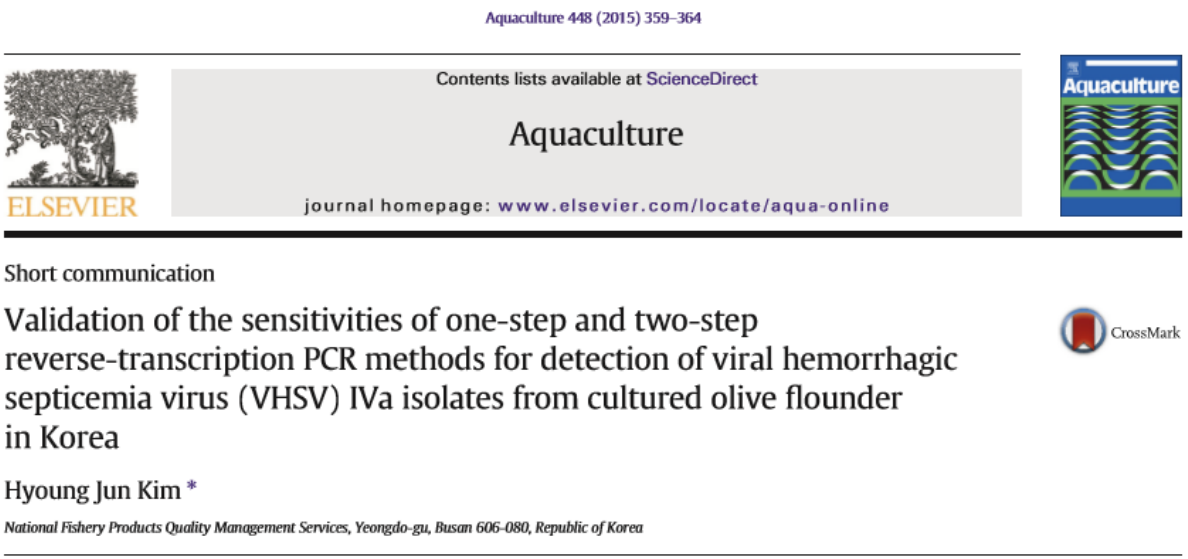
- However, we found a **low sensitivity (10,000 folds)** for detection of **VHSV IVa isolates** using the conventional RT-PCR described in the OIE aquatic manual (VN primer set).



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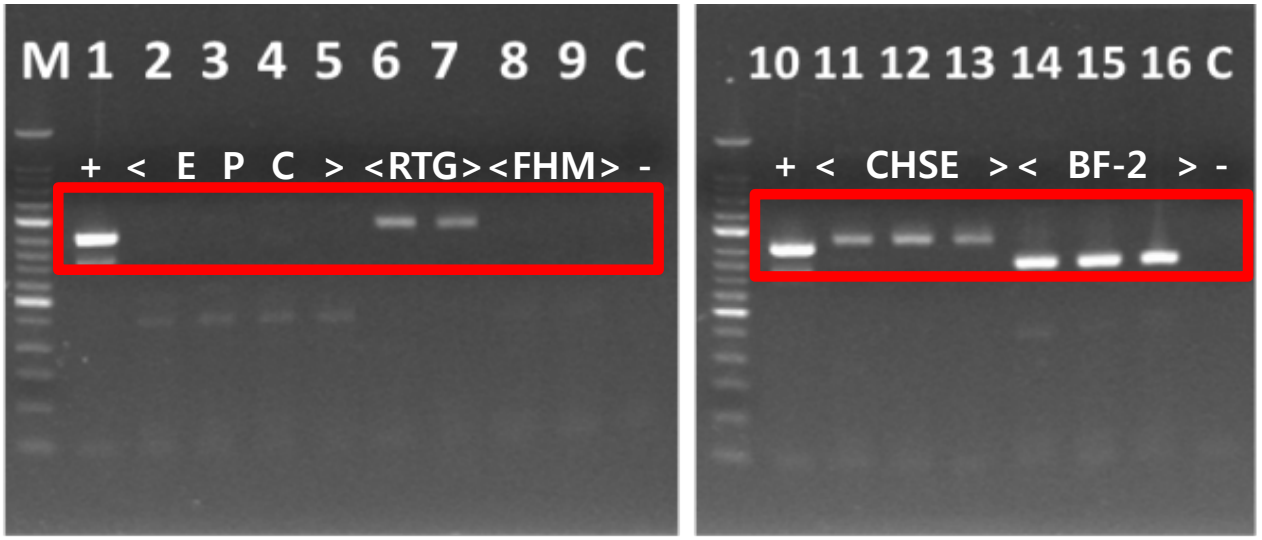
Short communication

Validation of the sensitivities of one-step and two-step reverse-transcription PCR methods for detection of viral hemorrhagic septicemia virus (VHSV) IVa isolates from cultured olive flounder in Korea

Hyoung Jun Kim *

National Fishery Products Quality Management Services, Yeongdo-gu, Busan 606-080, Republic of Korea

Background-1



- And, **non-specific bands with fish cell lines** were often observed when using the OIE RT-PCR.
  - In particular, these non-specific bands showed sizes very close to the positive VHSV control bands.

Background-1

- **Conventional PCR** is regularly used for **detection and genotyping of pathogens.**
- However, we found a **low sensitivity** for detection of **VHSV IVa isolates** using the conventional RT-PCR described in the OIE aquatic manual (VN primer set).
- And, **non-specific bands with fish cell lines** were often observed when using the OIE RT-PCR.
- Thus, a **novel conventional RT-PCR (3F2R)** have been developed and validated for detection of all genotypes of VHSV.

Background-2

- The novel 3F2R method showed the **same sensitivity and specificity** as **cell culture and real-time RT-PCR** using 10 fold diluted viral RNA.

VHSV genotypes (Small Panel)	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

10,000 folds low

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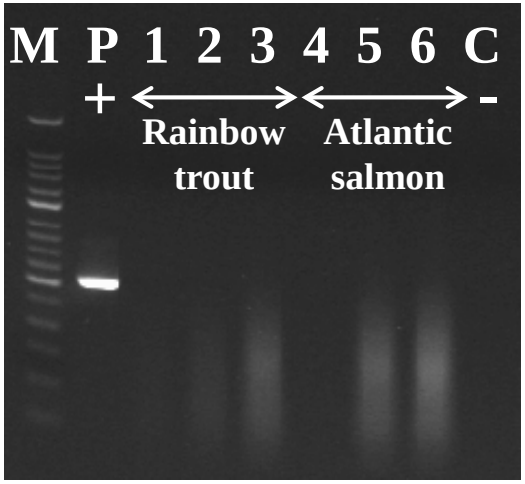
Background-2

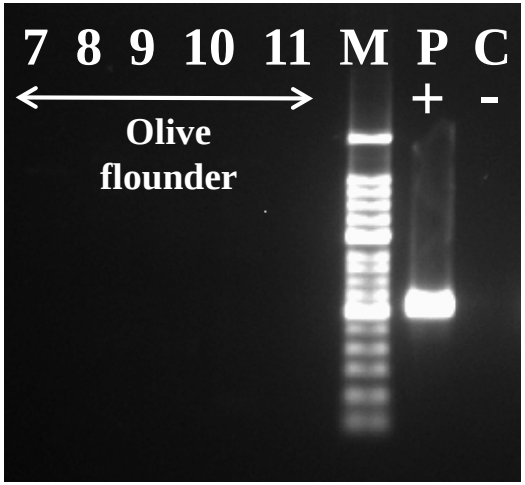
- The novel 3F2R method showed the **same sensitivity and specificity** as **cell culture and real-time RT-PCR** using 10 fold diluted viral RNA.

- **No specific responses** were observed in **heterologous viruses**, **tissue of several fish species** (rainbow trout, Atlantic salmon, olive flounder) and **normal fish cell lines** **using 3F2R method**.

Heterologous viruses

Non-infected fish samles





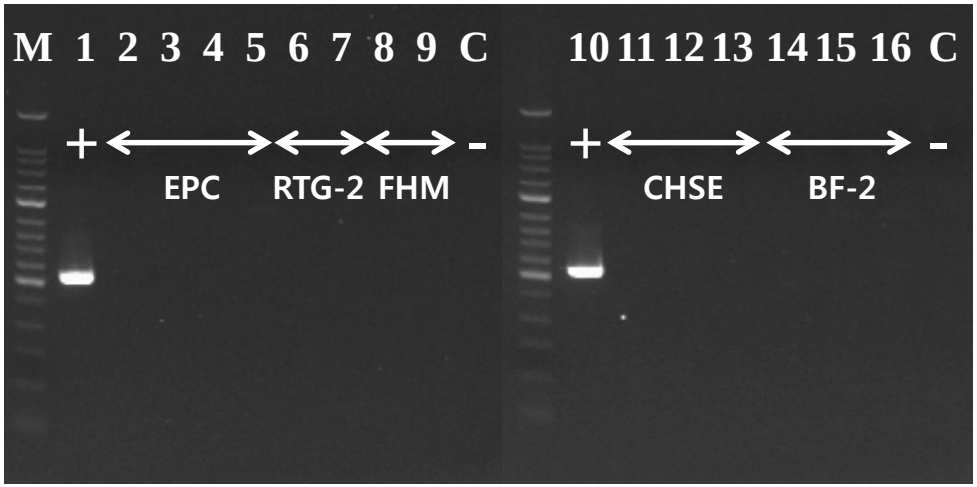
Lane 1 : Positive control, Lane 2 : IHNV F-32/87
Lane 3 : IHNV I-4008, Lane 4 : IHNV DW, Lane 5 : IHNV BC
Lane 6 : Birnavirus II, Lane 7 : LGV, Lane 8 : PFR
Lane 9 : SVC 56/70 Fijan, Lane 10 : KHV H361

Background-2

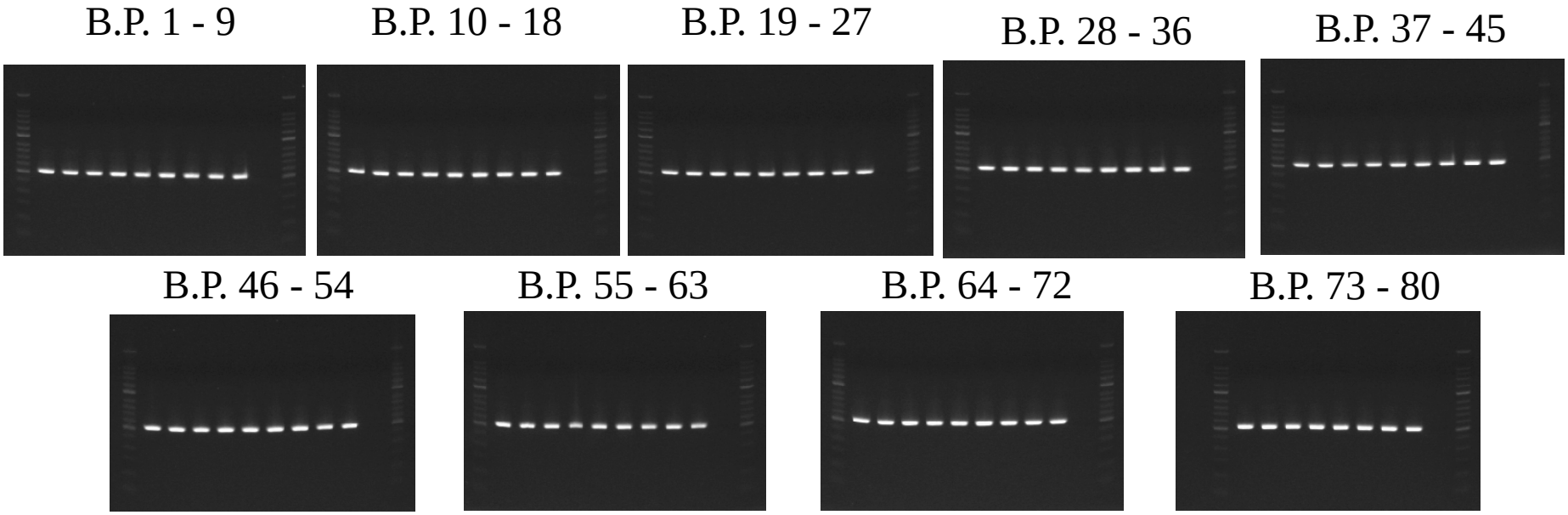
- The novel 3F2R method showed the **same sensitivity and specificity** as **cell culture and real-time RT-PCR**.

- **No specific responses** were observed in **heterologous viruses, tissue of several fish species** (rainbow trout, atlantic salmon, olive flounder) and **normal fish cell lines using 3F2R method**.

Cell lines



Background-2



- The novel RT-PCR was following tested on **80 VHSV isolates** representing a worldwide collection of all known genotype and subtypes, where **it produced clear and unique amplicons for all 80 isolates.**

AIM

- To assess why a low sensitivity is observed when detecting VHSV genotype IVa by the current RT-PCR given in the OIE manual.
- To confirm the specificity of the novel 3F2R method on organ materials from VHS infected rainbow trout and Atlantic salmon
- To assess the reproducibility and robustness of the 3F2R conventional RT-PCR by an inter-laboratory proficiency test among 9 selected laboratories.

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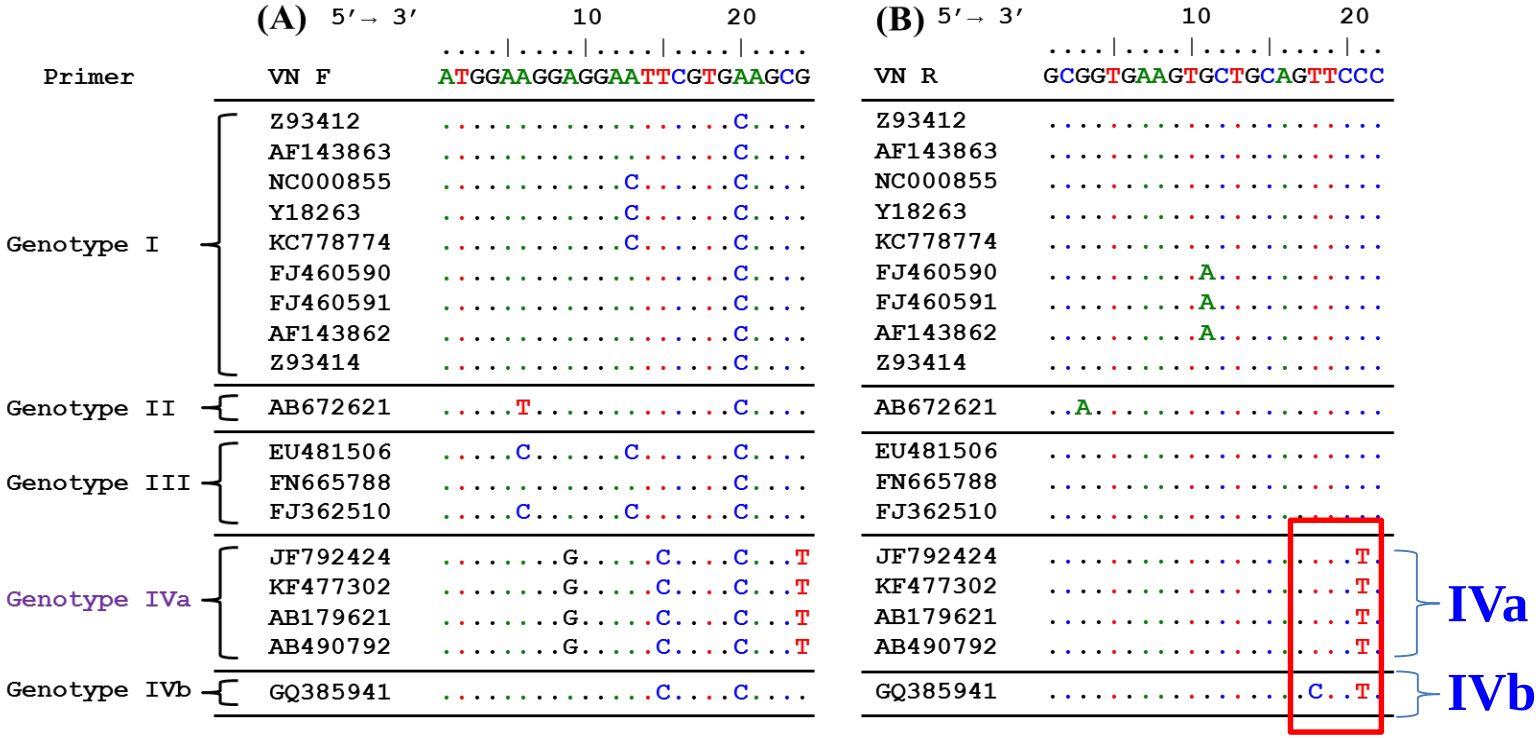
The reason for the low sensitivity of VHSV IVa using the OIE primer set



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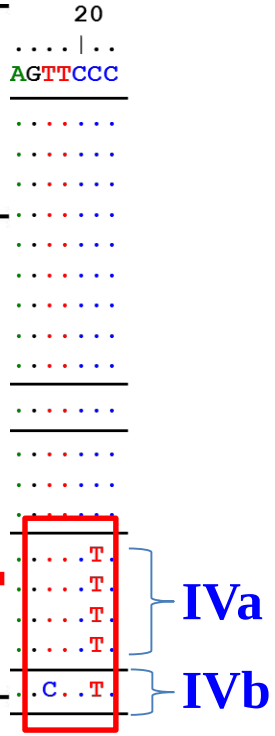

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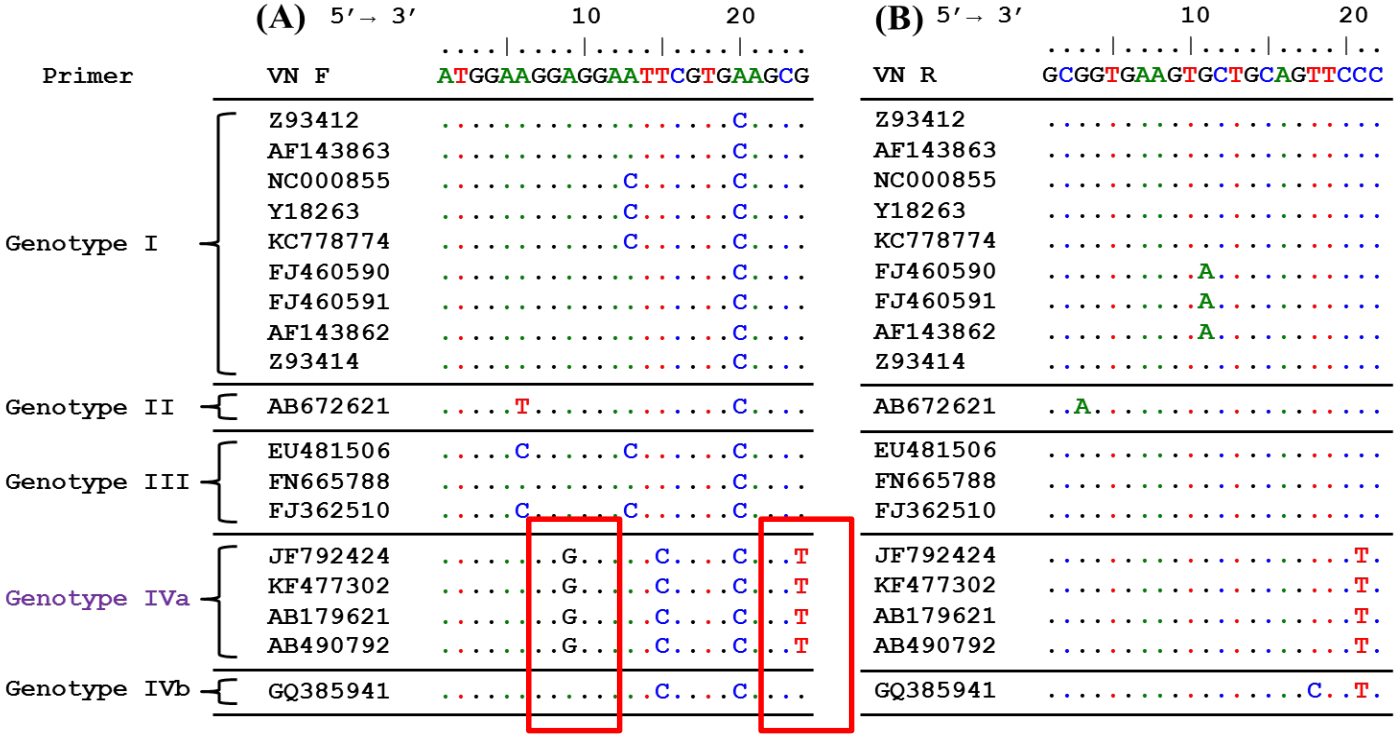
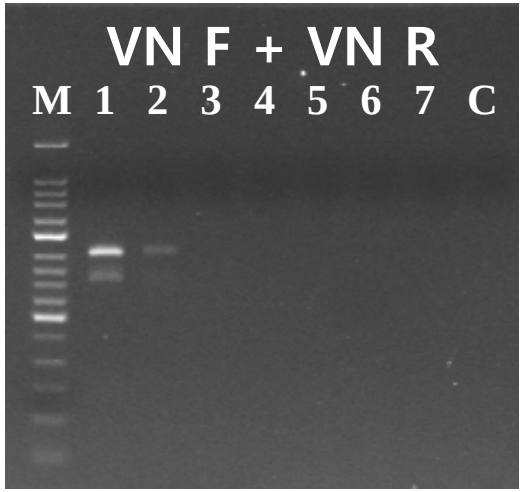
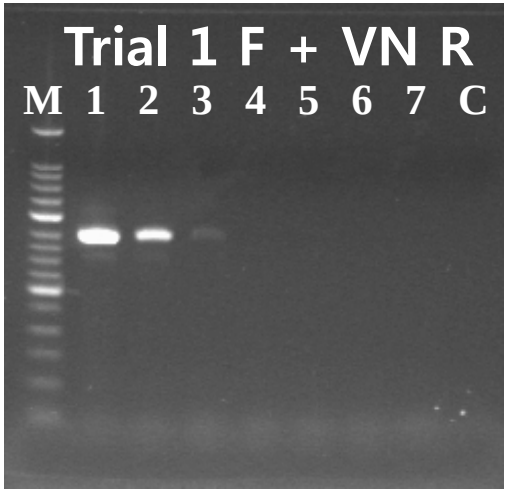
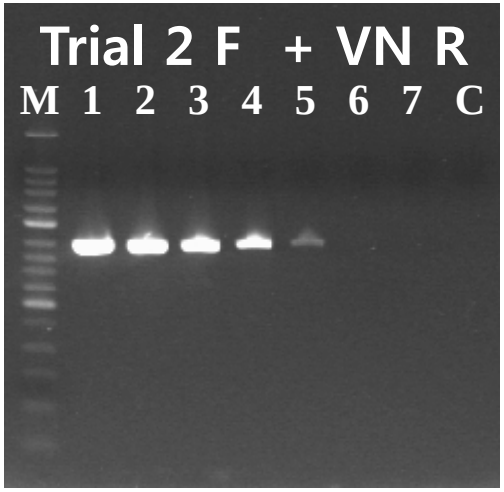


Primer Name	Primer Sequence (5′→3′)
VHSV Trial 1 F	ATGGAAGGGGGAATTCGTGAAGCG
VHSV Trial 2 F	ATGGAAGGAGGAATTCGTGAAGCT

Results using template as VHSV IVa type







M : marker, Lane 1 : 10^{-2} dilution of RNA, Lane 2 : 10^{-3} dilution,
Lane 3 : 10^{-4} dilution, Lane 4 : 10^{-5} dilution, Lane 5 : 10^{-6} dilution
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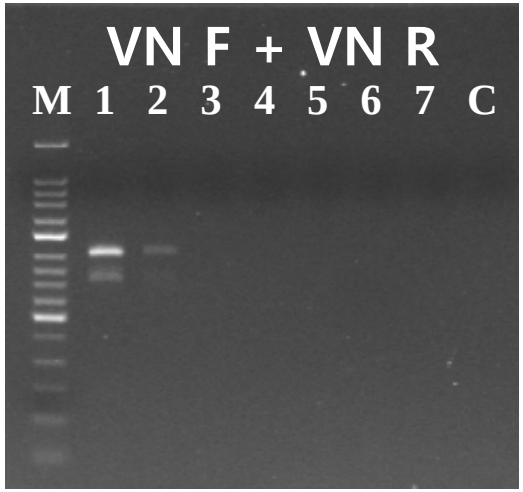

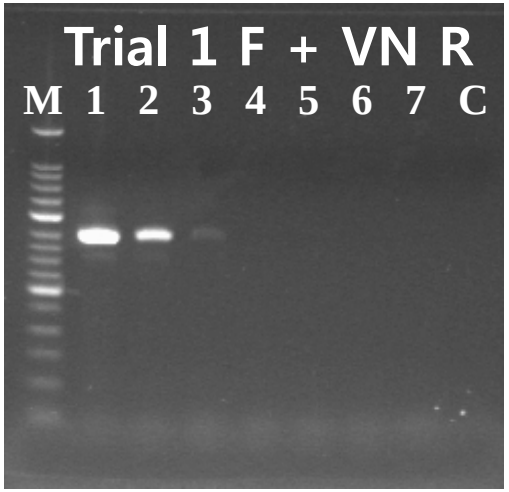

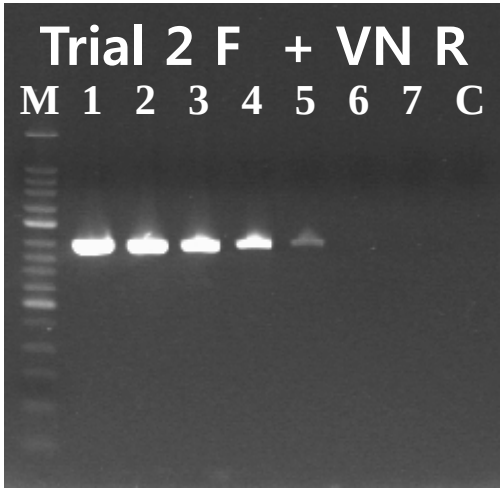

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Caused low sensitivity on IVa type

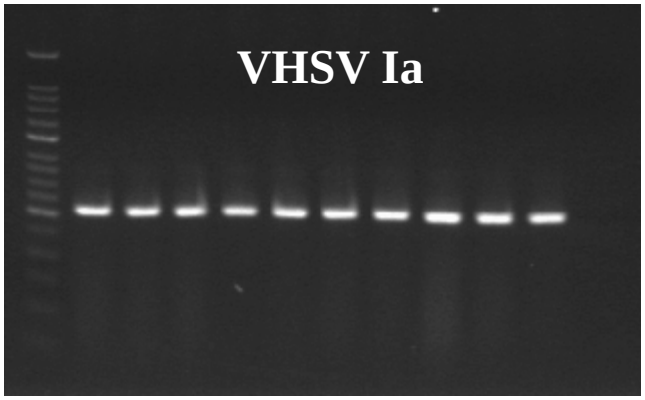
AIM

- To assess why a low sensitivity is observed when detecting VHSV genotype IVa by the current RT-PCR given in the OIE manual.
- **To confirm the specificity of the novel 3F2R method on organ materials from VHS infected rainbow trout and Atlantic salmon**
- To assess the reproducibility and robustness of the 3F2R conventional RT-PCR by an inter-laboratory proficiency test among 9 selected laboratories.

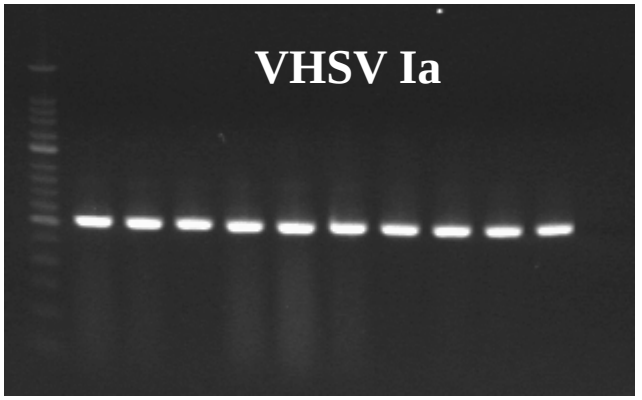
RT-PCR using 3F2R primer on samples fromVHSV infected fish

VHSV Ia challenged fish

Atlantic salmon IP infection

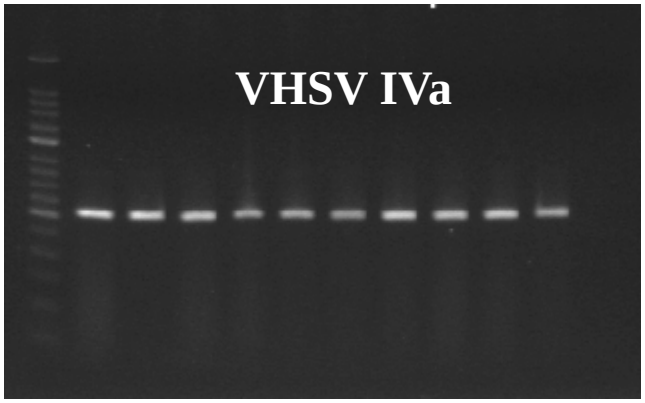


Rainbow trout bath infection

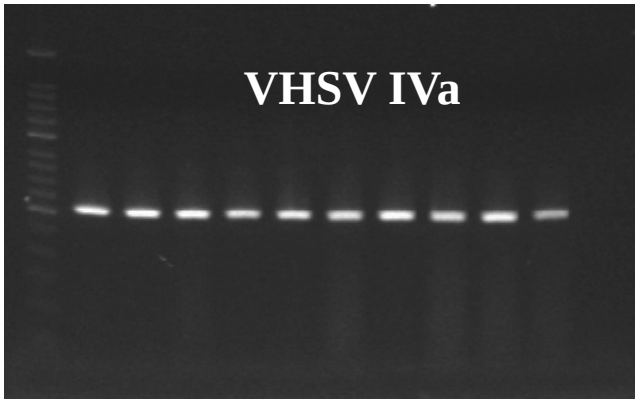


VHSV IVa challenged fish

Atlantic salmon IP infection

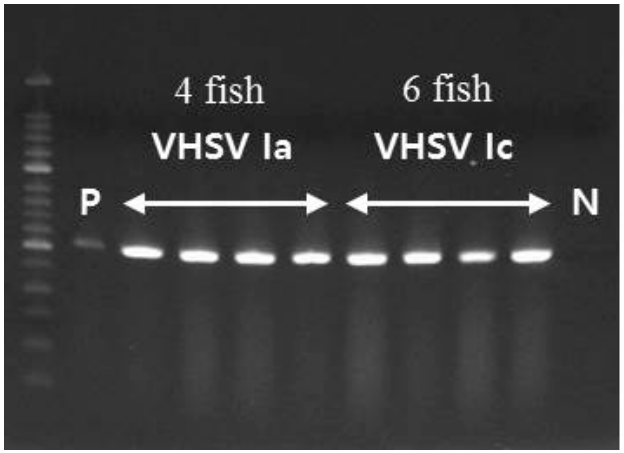


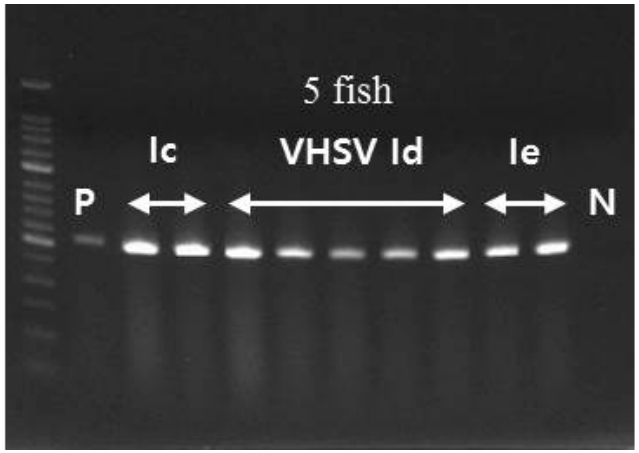
Rainbow trout IP infection

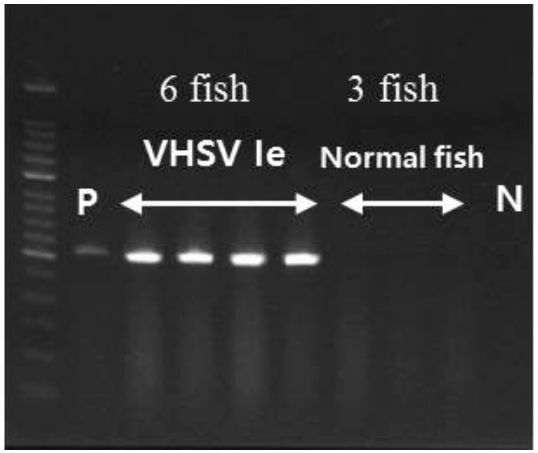


→**It was confirmed that only specific bands were observed using the 3F2R primer set on VHSV fish infected samples.**

RT-PCR using 3F2R primer on samples fromVHSV I subtypes infected rainbow trout







→It was confirmed that only specific bands were observed using the 3F2R primer set on samples from rainbow trout infected with VHSV sub-type Ia, Ic, Id and Ie.

AIM

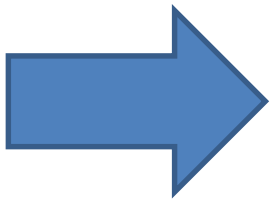
- To assess why a low sensitivity is observed when detecting VHSV genotype IVa by the current RT-PCR given in the OIE manual.
- To confirm the specificity of the novel 3F2R method on organ materials from VHS infected rainbow trout and Atlantic salmon

- **To assess the reproducibility and robustness of the 3F2R conventional RT-PCR by an inter-laboratory proficiency test among 9 selected laboratories.**

[France(ANSES), UK(CEFAS), Denmark(DTU), Germany(FLI, two laboratory), Italy(IZSVe), Korea(NFQS), Japan(Two OIE ref. lab., KHV, RSIVD)]

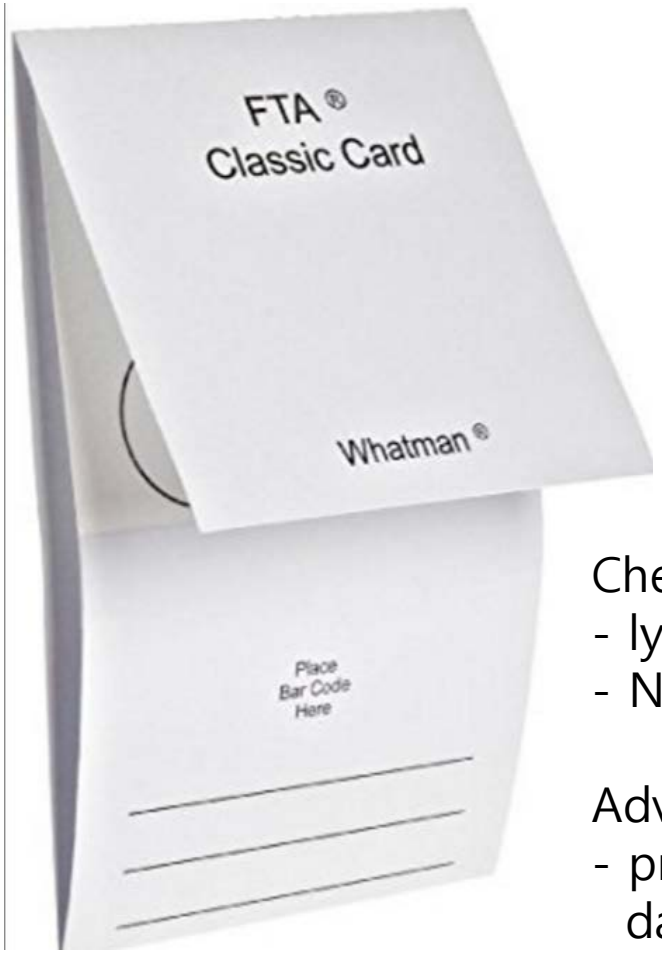
Sample preparation (10) on FTA cards

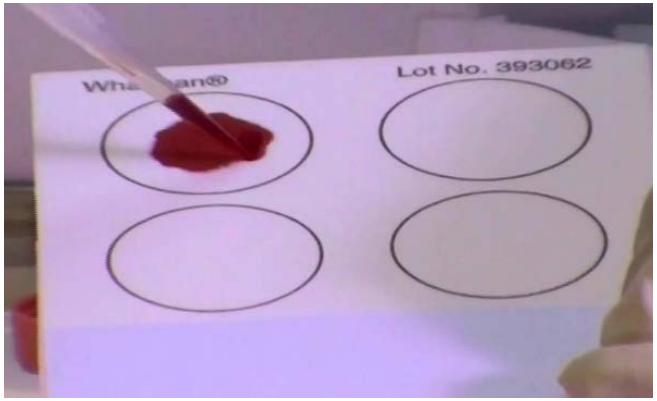
- 6 VHSV samples : VHSV I, Ib, II, III, IVa, IVb
- 3 heterologous virus : IPNV, HRV, IHNV
- 1 control : only normal cell culture medium



The viral supernatants were dropped on FTA cards (Whatmann Company).

What is FTA cards ?





Chemical formula on the cards
- lysis cell membrane and denature protein on contact
- Nucleic acids : entrapped, immobilised and stabilised

Advantage of FTA cards
- protect nucleic acids from nucleases, oxidation, UV damage and microbial and fungal attack
- inactivation: infectious pathogens
- stable for storage at room temperature

SOP for 3F2R

SOP for detection of VHSV by the "3F2R" conventional RT-PCR

AIM

To assess the reproducibility of a novel conventional RT-PCR for detection of viral hemorrhagic septicemia virus (VHSV) by an inter-laboratory proficiency test among 6 selected laboratories using the Kim3F2R primer set.

BACKGROUND

Conventional RT-PCR is typically used for detecting VHSV and for genotyping the virus. However, using the primers and procedures given in the VHSV chapter of the OIE Aquatic Manual we found a low sensitivity for detection of VHSV IVa isolates. In addition, non-specific reaction with fish cell lines was often observed when using the OIE RT-PCR. Thus, there was a need for improvement of the VHSV conventional RT-PCR given in the OIE Diagnostic Manual with regard to specificity and sensitivity in order to detect all VHSV genotypes and to remove the non-specific reactions due to fish cell lines.

Candidate primers from 5 regions of the VHSV nucleoprotein (N) gene were tested, and a highly sensitive primer set (Kim3F2R) was selected among these. The reaction conditions of the selected primer set were established and no non-specific reactions in fish, fish cell lines or with heterologous viruses were observed. The sensitivity of new RT-PCR was tested in parallel with cell cultivation, the "Jonstrup et al." RT-qPCR, and the conventional OIE VN RT-PCR. It was concluded that the sensitivity for all VHSV genotypes wasere at the same level when using cell culture, qPCR, and the new conventional RT-PCR except for conventional OIE VN RT-PCR. The novel RT-PCR was following tested on 80 VHSV isolates representing a worldwide collection of all known genotype and subtypes, where it produced clear and unique amplicons for all 80 isolates.

REAGENTS

1) Isolation of RNA

   Qiagen RNeasy minikit from Qiagen, 70% ethanol, 2-mercaptoethanol

2) New conventional RT-PCR

   Qiagen Onestep RT-PCR kit, Forward primer (VHSV 3F), Reverse primer (VHSV 2R), Takara 50bp marker, loading dye, agarose gel

   ☞ Primer sequence : VHSV 3F 5' - GGG-ACA-GGA-ATG-ACC-ATG-AT - 3',

   VHSV 2R 5'- TCT-GTC-ACC-TTG-ATC-CCC-TCC-AG - 3'

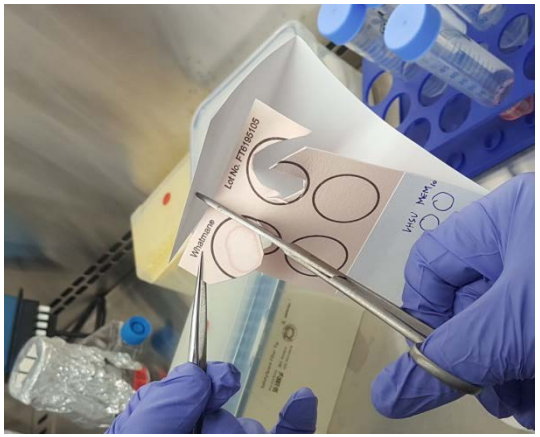
METHODS

All procedures should be carried out on ice or in a cooler in a laminar airflow cabinet.

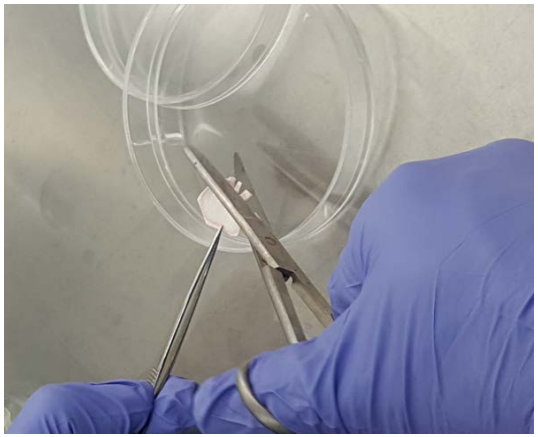
RNA EXTRACTION from FTA cards

1. For the RNA extraction, all work should be performed on ice, using gloves.
2. With help of scalpel blade or scissors cut out a small piece (approximately 0.5 cm in diameter) from the area where the sample has been adsorbed (within the large circle drawn on the card) and place it in a 1.5 mL tube
3. Add 500 µl RLT buffer (lysis buffer) and 5 µl of 2-mercaptoethanol in the tube* and mix thoroughly by pipetting up and down at least 5 times. Hereafter place the tube on a tilt table for one hour at room temperature.

Analysis methods



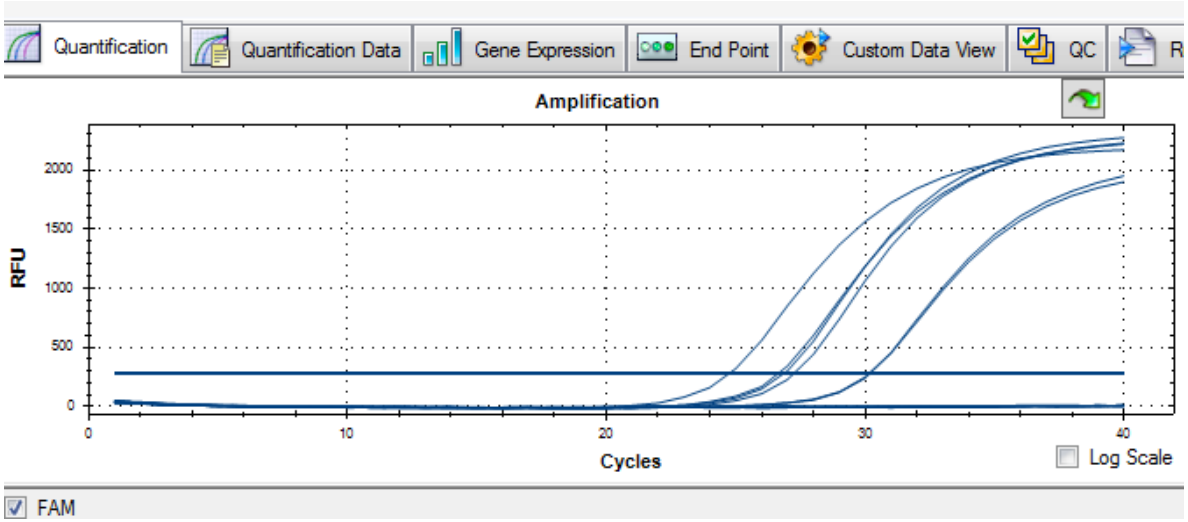
Cutting of FTA cards



Cut into small pieces
and mixed lysis buffer

- RNA extraction on FTA cards
- Real-time RT-PCR for VHSV detection
- RT-PCR using VN (OIE) primer set for VHSV detection
- RT-PCR using 3F2R primer set for VHSV detection

qRT-PCR results using Jonstrup et al. method

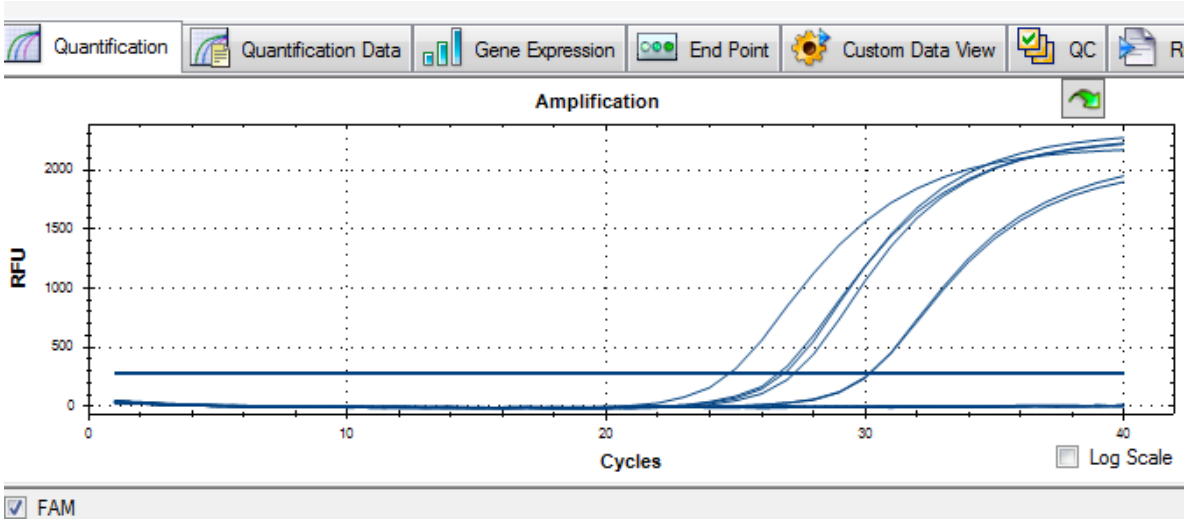


Sample	Cq
FTA 1	24.72
FTA 2	N/A
FTA 3	30.17
FTA 4	N/A
FTA 5	26.82
FTA 6	27.22
FTA 7	N/A
FTA 8	26.61
FTA 9	N/A
FTA 10	30.13

CT value	Sample	Isolate	VHSV genotypes
	S 1	DK-F1	**Genotype I**
	S 2	IPN SP	
	S 3	DK-1p52	**Genotype II**
	S 4	HRV8401	
	S 5	Goby 1-5	**Genotype IVb**
	S 6	JF-JF00Ehi	**Genotype IVa**
	S 7	IHN 32/87	
	S 8	DK-4p168	**Genotype III**
	S 9	Medium (cell control BF-2)	
	S10	DK-1p8	**Genotype Ib**

Positive results : Sample 1, 3, 5, 6, 8, 10

qRT-PCR results using Jonstrup et al. method



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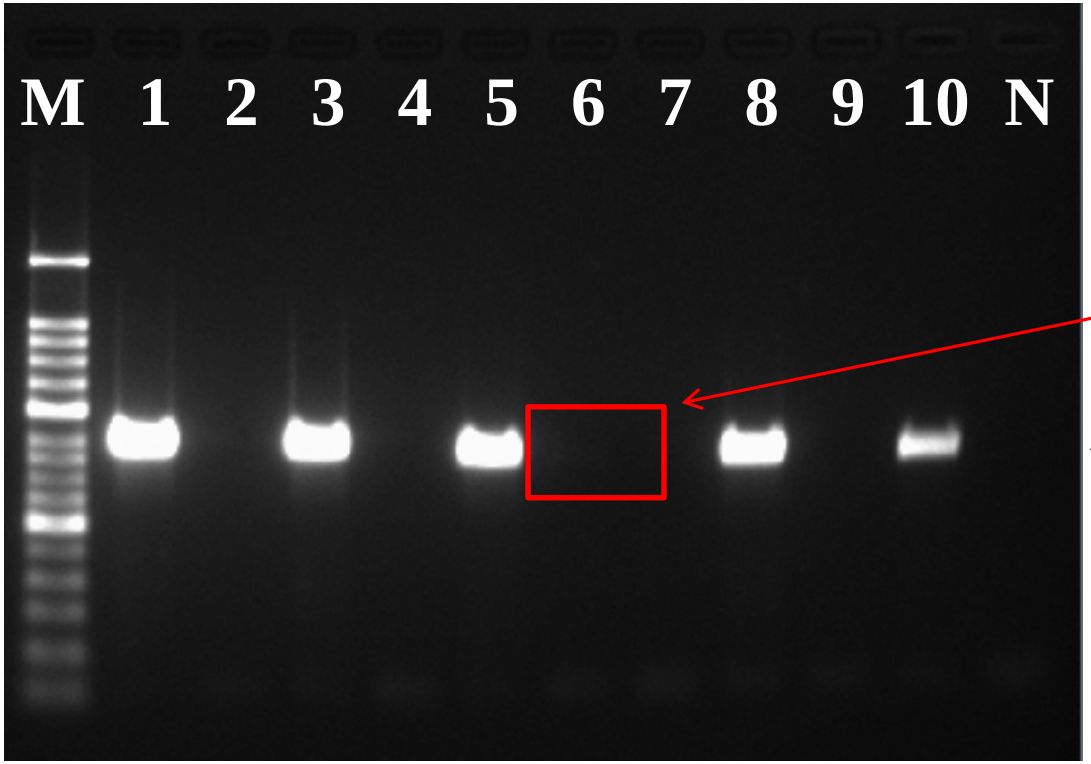
Almost same level of viral RNA : 5, 6, 8

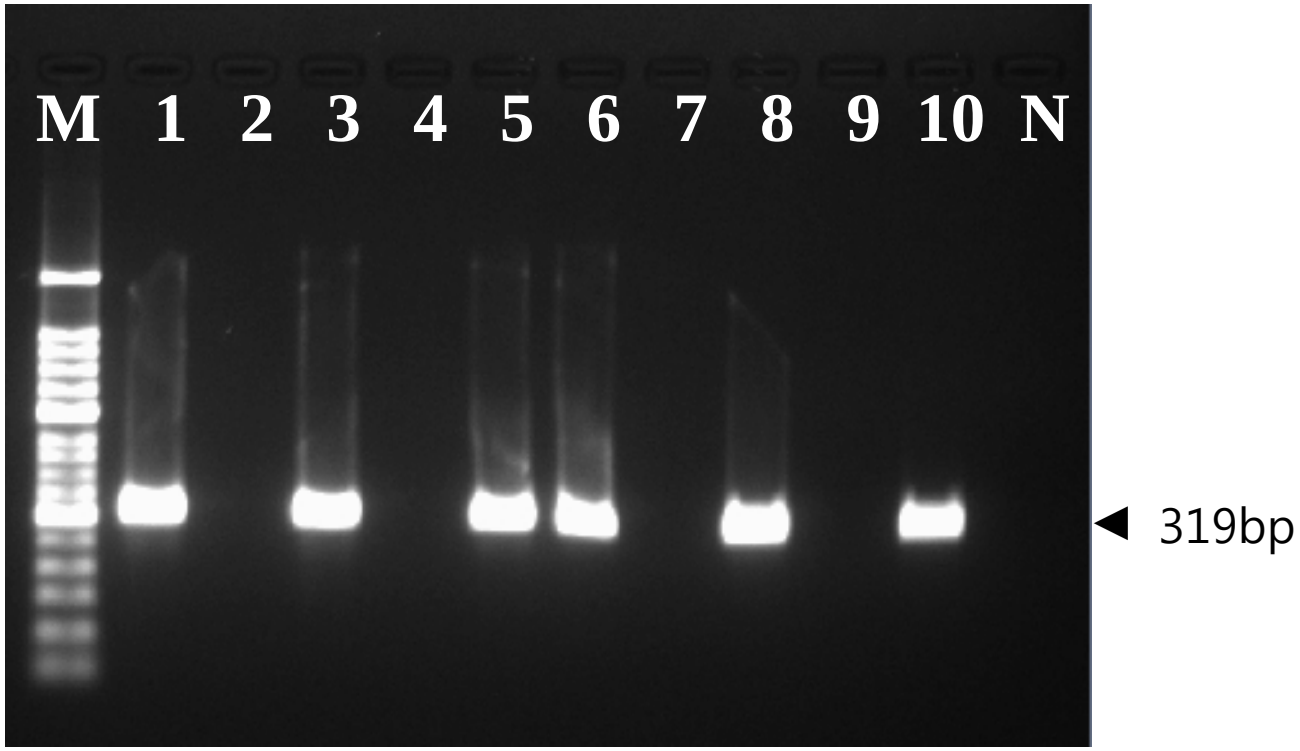
Conventional RT-PCR results using OIE VN primer



M : 50 bp DNA marker
1. DK-F1(**Genotype I**) 2. IPN SP 3. DK-1p52 (**Genotype II**) 4. HRV8401
5. Goby 1-5(**Genotype IVb**) 6. JF-JF00Ehi(**Genotype IVa**) 7. IHN 32/87
8. DK-4p168(**Genotype III**) 9. Medium (cell control BF-2)
10. DK-1p8(**Genotype Ib**)

Positive results : Sample 1, 3, 5, ~~6~~, 8, 10

Conventional RT-PCR results using 3F2R primer



M : 50 bp DNA marker
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5. Goby 1-5(**Genotype IVb**) 6. JF-JF00Ehi(**Genotype IVa**) 7. IHN 32/87
8. DK-4p168(**Genotype III**) 9. Medium (cell control BF-2)
10. DK-1p8(**Genotype Ib**)

Positive results : Sample 1, 3, 5, 6, 8, 10

Summary-1 of the PCR results from 9 institutes

Institute Number	3F2R Conventional PCR	qPCR or Sequencing (option)
1	**Success** (Macherey Nagel Nucleospin Virus & Invitrogen superscript III one-step RT-PCR)	Success (qPCR, Jonstrup et al method) (Invitrogen superscript III one-step qRT-PCR)
2	**Success** (Qiagen Rneasy Mini Kit & Qiagen Onestep RT-PCR Kit)	Success (qPCR, Jonstrup et al method) (Qiagen QuantiTect RT Kit)
3	**Success** (QIAamp Viral RNA mini kit & Qiagen Onestep RT-PCR Kit)	Success (Sequencing and genotyping)
4	**Fail** (EZ-1 RNA tissue mini kit & EZ-1 BioRobot & Two step RT-PCR using MMLV and Go-Taq)	ND

Summary-2 of the PCR results from 9 institutes

Institute Number	3F2R Conventional PCR	qPCR or Sequencing (option)
5	**Success** (QIAamp Viral RNA mini kit & Qiagen Onestep RT-PCR Kit)	ND
6	**Success** (Macherey Nagel Nucleospin Virus & Qiagen Onestep RT-PCR Kit)	Success (qPCR, Jonstrup et al method) (Qiagen QuantiTect RT Kit)
7	**Success** (Qiagen Rneasy Mini Kit & Qiagen Onestep RT-PCR Kit)	Success (qPCR, Jonstrup et al method) (Qiagen QuantiTect RT Kit)
8	**Success** (Qiagen Rneasy Mini Kit & Invitrogen superscript III one-step RT-PCR)	ND
9	**Success** (Qiagen Rneasy Mini Kit & Invitrogen superscript III one-step RT-PCR)	ND

Thus, the **reproducibility of 3F2R** was confirmed by several institutes and kits.

Conclusions

- We found **the cause of low sensitivity on VHSV IVa** type by OIE manual.
- **Specificity of the novel 3F2R method** was confirmed on organ materials from fish samples.
- The **reproducibility of 3F2R method** was confirmed by several institutes.
- Finally, **we suggest that the 3F2R primer set shall replace the current primer set recommended in the OIE manual for detection of VHSV**.

Acknowledgements

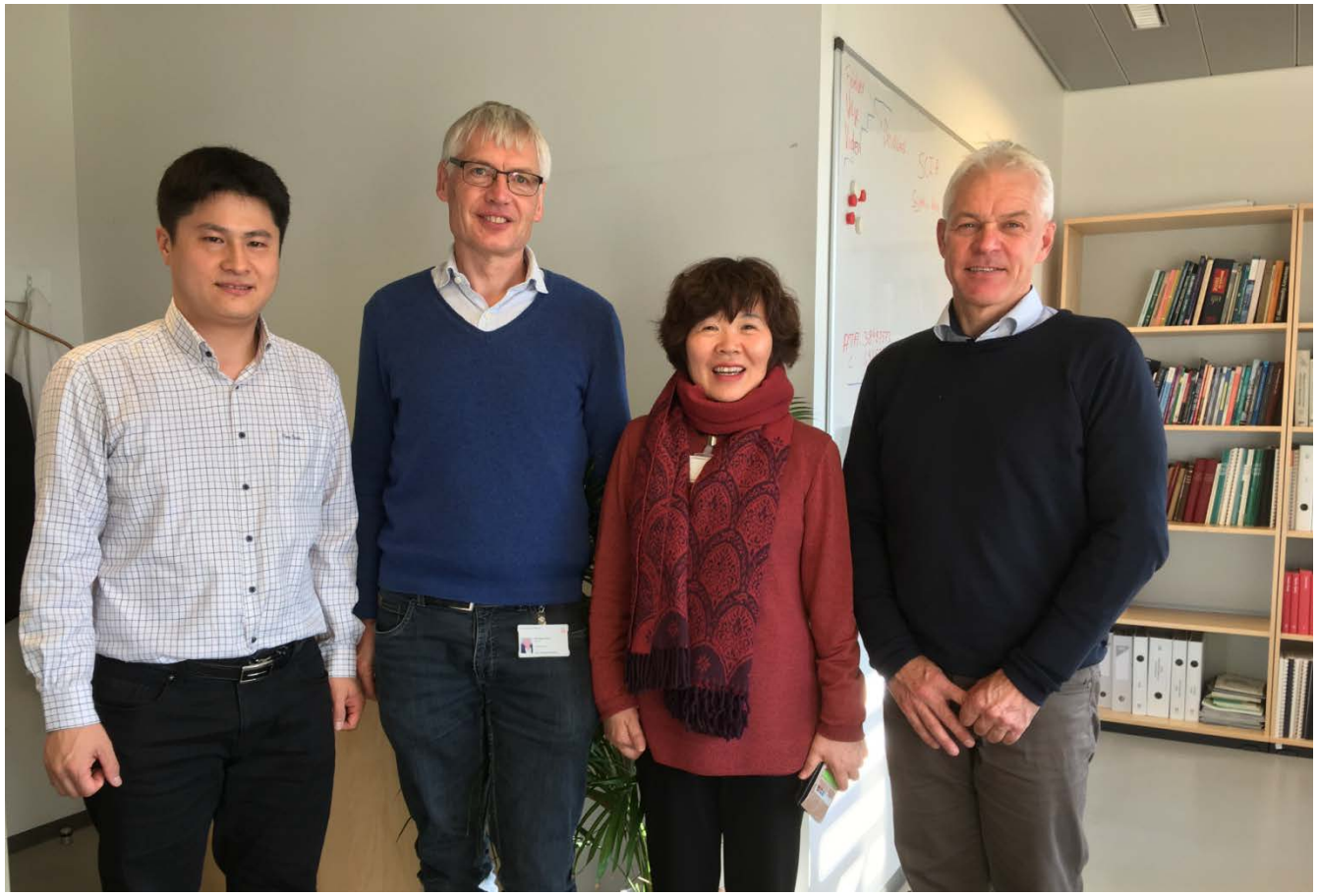
- **Denmark (DTU)**

  : Troels, Niccolo, Teena, Tine Moesgaard, Argelia, Betina, Christina, Didde, Alencar, Susie

- **Korea (NFQS)**

  : Kwon, Lee, Oh

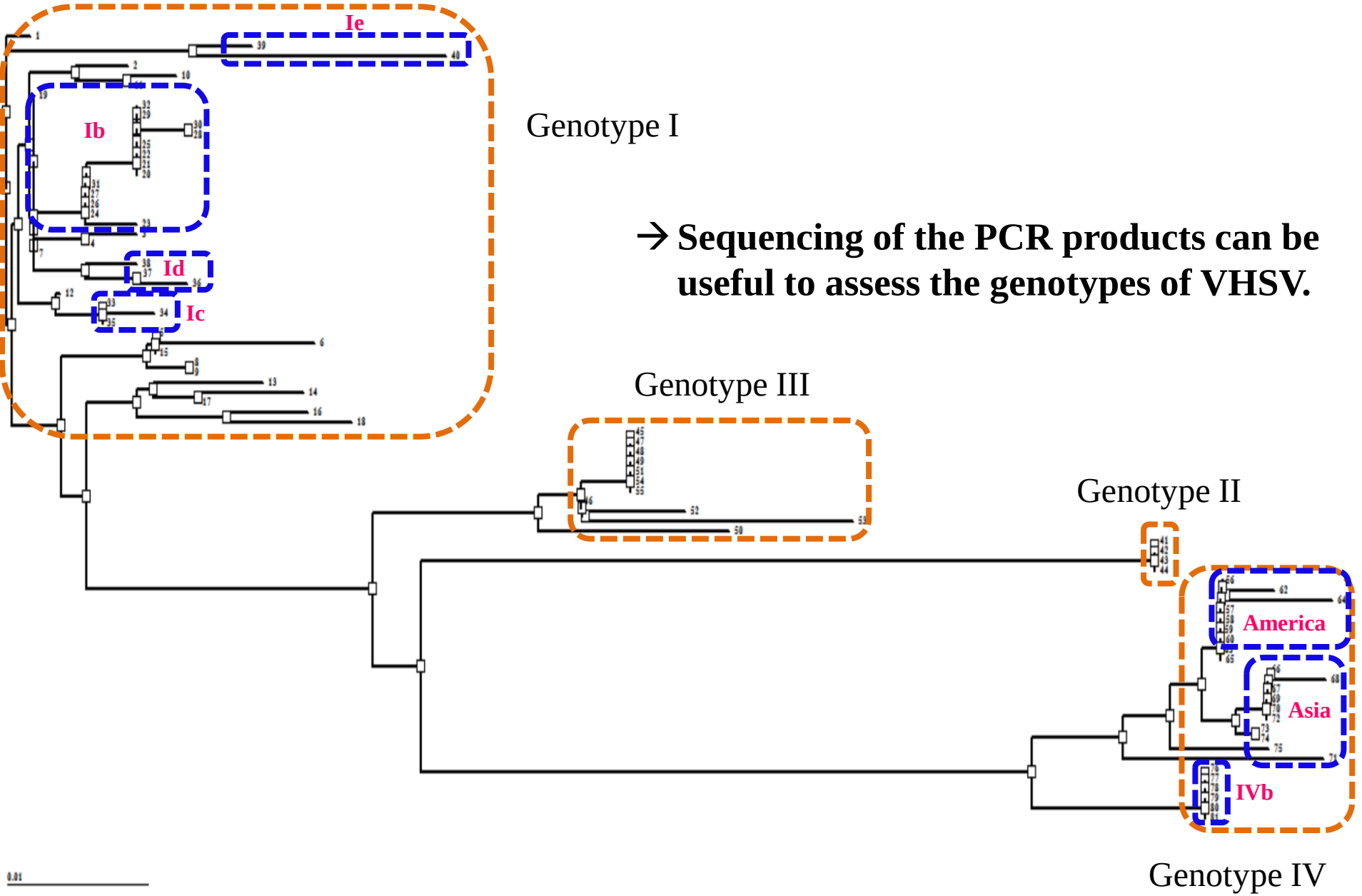
- **Experts from 9 laboratories** for proficiency test



Thank You !

Team of OIE Twinning project on VHS

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



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