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Protocols

Development of an *in-situ* hybridization assay using riboprobes for detection of viral haemorrhagic septicemia virus (VHSV) mRNAs in a cell culture model

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ABSTRACT

An *in situ* hybridization (RNA-ISH) assay has been developed and optimized to detect viral haemorrhagic septicemia virus (VHSV), an OIE listed piscine rhabdovirus, in infected fish cells using fathead minnow (FHM) as a model cell line. Two antisense riboprobes (RNA probes) targeting viral transcripts from a fragment of nucleoprotein (N) and glycoprotein (G) genes were generated by reverse transcription polymerase chain reaction (RT-PCR) using VHSV specific primers followed by a transcription reaction in the presence of digoxigenin dUTP. The synthesized RNA probes were able to detect viral mRNAs in formalin fixed VHSV infected FHM cells at different time points post inoculation (pi). To correlate the signal intensity, a time dependent quantitation of the viral mRNA transcript and infectivity titer was done by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) and 50% tissue culture infectivity dose ($TCID_{50}$), respectively, from the infected cells and culture supernatants. Further, we compared the diagnostic sensitivity of ISH assay with immunocytochemistry (ICC). Both the riboprobes used in the ISH assay detected VHSV as early as 6 hpi in the FHM cells inoculated with a multiplicity of infection (moi) of 2. Also, the signal detection in ISH was at an early stage in comparison to ICC, wherein, signal was first detected at 12 hpi. Our results clearly highlight that current ISH assay can be of value as a diagnostic tool to localize and detect VHSV in conjunction with conventional virus isolation in cell culture.

1. Introduction

Viral diagnostics has made significant advances over the years and today we have a repertoire of techniques used for the laboratory diagnosis of viral infections which includes detection of viral antigens, nucleic acids, and antibodies (Peaper and Landry, 2014). However, viral isolation by cell culture is always carried out in virological laboratories to ascertain the presence of viral pathogen (Gregory, 2000). Even with advent of molecular techniques like RT-PCR and qRT-PCR, virus isolation using cell culture is still the gold standard and method of choice for diagnosis of viral infections (Hematian et al., 2016). The technique relies on visualization of cytopathic effect (CPE) caused by the viral damage to cells. The degree of visible damage to cells depends upon viral strain, type of host cells, multiplicity of infection (MOI), and other factors. However, virus isolation by cell culture has a major drawback as the appearance of CPE usually takes weeks and interpretation of results requires a professional experienced eye. In case of delayed or non appearance of CPE in cell culture, viral visualization is done by hemadsorption or *in situ* by viral antigen or nucleic acid detection (Suchman and Blair, 2007). Such an approach is important in *in vitro* viral diagnosis particularly at early stages when no visible CPE is evident and when there is paucity of time to identify the viral pathogen. Visualization of viral particles *in situ* coupled with its isolation and interpretation of results by CPE can lead to a robust initial diagnosis and substantiate the preliminary confirmation about the viral aetiological agent.

Viral haemorrhagic septicemia (VHS) is a serious fish disease which infects more than 80 fish species world-wide (Sandlund et al., 2014). The aetological agent of the disease is a negative-sense single stranded RNA virus belonging to the family *rhabdoviridae* and genus *novirhabdvirus.* In natural or experimental infections, the disease is manifested as chronic, subacute or acute. Accordingly, clinical pathology of VHS also varies with the chronic form generally characterized by nervous manifestations along with low level mortality while as the acute disease being the most rapid resulting in greater mortality. Chronically infected fish often acts as virus carrier which shed VHSV virus particles into the surrounding environment. The nervous form is often associated with erratic swimming behavior characterized as spiraling and/or

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flashing. Clinical signs, although non-specific generally appear as dermal petechial hemorrhages, hemorrhages at the base of fins, pale gills, periocular hemorrhage, lethargy, and abnormal to erratic swimming (Kim and Faisal, 2011). Target organs are kidney, heart and spleen while as in chronic stages virus titre can become high in the brain. Phylogenetic analysis deciphers VHSV into four genotypes, which appear to be distributed geographically, rather than by host or year of isolation. Genotypes I, II, and III are found mainly in Europe and Japan, while isolates of genotype IV have been found only in North America, Japan, and Korea (Lvov et al., 2015).

Currently, the detection of VHSV relies mainly on molecular methods like nucleic acid detection by RT-PCR and qRT-PCR, protein based techniques such as enzyme linked immunosorbent assay (ELISA), immunoflorscent antibody test (IFAT) and immunohistochemistry (IHC), and by cell culture method ($TCID_{50}$) (OIE, 2018). Whereas, all these techniques can detect VHSV and particularly the molecular techniques like qRT-PCR are sensitive enough to detect even small copies of the virus. However, none of these diagnostic methods can detect the virus *in-situ* in infected cells which limits their use in studies involving viral localization, visualization and qualitative description.

in situ hybridization (ISH) has a role to play in diagnostics as it offers great scope to localize the viral infection, identify sites of gene expression and analyze mRNA transcription and distribution (Nouri-Aria, 2008). ISH assay is based on hybridization of labeled probes complementary to specific viral target sequences in fixed cells. Cell culture models have long been used as a platform in studies involving viral infections given their versatility and flexibility to study and optimize the assay interactions in a controlled environment (Hudu et al., 2016). Also, development and validation of diagnostic method in *in vitro* cell culture model is cost effective when compared with *in vivo* models, where, economical and ethical constraints are to be taken into consideration (Duell et al., 2011). In case of fish viruses, only one *in vitro* study so far by Alonso et al. (2004) has focused on development of an ISH assay in a cell culture system to detect sole aquabirnavirus virus in infected cells using DNA probe.

In this line, the study here describes an ISH assay using digoxigenin (DIG)-labeled riboprobes for the detection and localization of VHSV in infected fish cell line. In addition, ISH and immunocytochemistry (ICC) assays were compared for their relative sensitivity in detecting VHSV mRNAs and antigens, respectively, in a cell culture platform. Further, the signal detection in both the assays (ISH and ICC) correlated with viral mRNA transcript and infectivity titer in a time dependent manner by quantitative (q)RT-PCR and $TCID_{-50}$, respectively, in the infected cells and culture supernatants. Fathead minnow (FHM) infected cells were used as a cell model for further work involving fixation, probe hybridization and signal detection. The present study for the first time reports the development and validation of RNA-ISH assay to detect VHSV in FHM cells and also this is the first study to localize VHSV genotype IVa using RNA-ISH.

2. Materials and methods

2.1. Virus and cell culture

An isolate of VHSV, FYeosu05 (genotype IVa), isolated from a VHSV infected olive flounder farm at Yeosu in 2005 (Kim et al., 2009) was propagated in FHM cell line in 75 cm^2 tissue culture flasks at 15 ± 0.5 °C. Culture was maintained with Dulbecco's minimum essential medium (DMEM, Gibco, USA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco, USA), 150 IU/mL penicillin G, and 100 µg/mL streptomycin (Gibco, USA). After the development of complete CPE, cell culture supernatant was centrifuged (4000 rpm, 15 min, 4 °C) and viral culture fluid was subdivided into small aliquots and stored at −80 °C until use.

2.2. Experimental layout and sampling

FHM cells were seeded on four well cell chamber slide (SPL life sciences, Korea) and incubated at 15 °C until confluency. Afterwards, VHSV was inoculated at a multiplicity of infection (moi) of 2, after which cells and culture supernatants were sampled at 3, 6, 9, 12, 24, 48, and 72 hpi. Infected cells were gently scraped with a cell scraper and pipetted out along within culture supernatant in a 1.5 ml micro centrifuge tube. Collected samples were centrifuged (4000 rpm, 30 min, 4 °C) and supernatant was transferred to another micro centrifuge tube following which cell pellet was washed twice with phosphate buffered saline (PBS) and re-suspended in DMEM. The samples were stored at −80 °C and later used for quantification analysis of viral genome (N and G gene segments) by qRT-PCR and titration in FHM cells.

For ISH and ICC assays, a parallel sampling was done at similar time points as described above. After removing supernatant, cell fixation in case of ISH was carried out with 4% formalin solution (Junsei, Japan) for 15 min, whereas for ICC, cells were fixed in absolute methanol for 10 min. Slides were then rinsed three times with diethyl pyrocarbonate (DEPC) treated water. Afterwards, ISH slides were maintained in absolute methanol at –20 °C while as ICC slides were maintained in PBS containing 0.02% (w/v) sodium azide at 4 °C, until use. All samples were statistically analyzed in triplicates.

2.3. Probe preparation and labeling

DIG-labeled antisense RNA probes complementary to positive-stranded viral mRNAs were generated from PCR products targeting the nucleocapsid (N) and glycoprotein (G) genes of VHSV. Viral RNA was extracted using RNeasy Mini Kit (Qiagen, Germany) following the manufacturer's instructions. cDNA synthesis was carried out by incubating total RNA at 37 °C for 15 min in a 10 µL reaction mixture containing 2 µL of 5 × RT buffer, 0.5 µL of Enzyme Mix, and 0.5 pmol/µL of open reading frame (ORF) primer set. Synthesized cDNAs were stored at – 20 °C until use. For N gene probe preparation, already available primer set for RT-PCR (Snow et al., 2004) was used to generate riboprobe, while as for G gene, a new primer set was designed for probe synthesis using ncbi primerblast online tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) using the genome sequence from GenBank with accession number KF477302.1 (Kim et al., 2013). The details for the primer sequences and product size are mentioned in Table 1.

RT-PCR was performed in a *MyGenie*™ 96/384 thermocycler (Bioneer, Korea) using gene specific primers with T7 promoter sequences. A 20ul reaction mixture was used to amplify the cDNA containing 2 µL of 10 × Ex Taq buffer, 2 µL of 2.5 mM dNTP mixture, 0.5 µL of Ex Taq (5 U/µL), and 20 pmol of open reading frame (ORF) primer set. RT-PCR for N-gene segment was performed using the following amplification protocol: pre-denaturation at 94 °C for 5 min, 36 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 68 °C for 1 min, followed by a final extension at 68 °C for 7 min, whereas, for G-gene segment following amplification protocol was used: pre-denaturation at 95 °C for 5 min, 30 cycles of denaturation at 95 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min, followed by a final extension at 72 °C for 5 min. Amplified product for each gene was purified using QIAquick PCR Extraction Kit (Qiagen, GmbH-Germany) according to the manufacturer's protocol. The concentration of purified template was determined using a NanoDrop 1000 spectrophotometer (Thermo scientific, USA). Purified sample was stored at –20 °C until use. The labeled riboprobes were generated by in vitro transcription of the PCR product (1 µg of purified DNA) using the DIG RNA labeling kit (Roche, Germany) following the manufacturer's instructions. A small volume of the *in vitro* transcribed riboprobe was run on 0.5% agarose gel electrophoresis to confirm the presence of RNA after DNase treatment (Fig. 1). The synthesized riboprobes were stored at −20 °C while protecting from light.

Table 1
Oligonucleotide primers used in the present study.

Primer	Sequence (5′–3′)	Gene target	Purpose	Product
VHSV-N-T7-F	ATGGAAGGAGGAATTCGTGAAGCG	Nucleoprotein (N)	ISH	512
VHSV-N-T7-R	TAATACGACTCACTATAGGG GCGGTGAAGTGCTGCAGTTCCC			
VHSV-G-T7-F	AGAACTGAGTGCCTGGATGC	Glycoprotein (G)	ISH	514
VHSV-G-T7-R	TAATACGACTCACTATAGGG AGCAGAGCACGAGCAGAAG			
VHSV-N-F	GAATCCGTGCAGCTTTTTCAGG	Nucleoprotein (N)	qRT-PCR	< 200
VHSV-N-R	CAAGTGCATCCACGATCACCTTC			
VHSV-G-F	AGAAACGCTTAAGGGGCATCTG	Glycoprotein (G)	qRT-PCR	< 200
VHSV-G-R	ACCTTGCATGCCATTGTGAGC			

Underlined sequences are T7 promoter sequences.

2.4. In situ hybridization (ISH)

All the reagents and buffers used for ISH assay including graded methanol and PBS-T were prepared with RNase-free diethyl pyrocarbonate (DEPC) treated water. The step wise detailed procedure for the present RNA-ISH assay is described here under.

2.4.1. Post-fixation and probe hybridization

FHM cells were rehydrated in graded methanol and treated with proteinase K (10 µg/mL in DEPC treated H_2O) at room temperature for 10 min. Afterwards, they were post-fixed in 4% formaldehyde for 10 min at room temperature and rinsed three times with PBS-T (phosphate buffered saline with 0.1% Tween-20). Pre-hybridization was performed at 65 °C for 1 h with hybridization buffer [50% deionized formamide, 5 × SSC (Saline sodium citrate buffer- Welgene, Korea), 0.1% Tween-20 (Sigma, USA), 50 µg/mL heparin sodium salt (Sigma, USA), 500 µg/mL transfer RNA (Sigma, USA), and 0.1% 1 M critic acid]. Labeled RNA probe was diluted (1:1000) in hybridization buffer and added on each slide followed by incubation at 65 °C overnight in a humid chamber.

2.4.2. Post hybridization washes

After hybridization, slides were washed twice (10 min each) in pre-warmed buffer (at 65 °C) containing 70% hybridization mixture (without heparin, transfer RNA and RNA probe) and 30% 2 × SSC at 65 °C. This was followed by washing twice in 0.2 × SSC (10 min each) at 37 °C, and once with MAB (maleic acid buffer) with 0.1% tween-20 (1 M maleic acid, 0.15 M NaCl and 0.1% tween-20, pH 7.5) for 10 min at room temperature. The cells were then blocked with blocking solution containing 10% blocking stock solution (Roche, Germany), 10% normal goat serum (Cell signaling, USA), and 1 × MABT at 37 °C for 1–2 h.

2.4.3. Detection and visualization

Detection was carried out by incubating the slides with anti-DIG IgG Fab fragments conjugated with alkaline phosphatase (AP) (Roche, Germany) in MABT at dilution of 1:5000 at 37 °C for 1–2 h. Unbound Fab fragments were removed by rinsing with MABT 3 times (10 min each) after which samples were incubated with coloration solution (0.1 M NaCl, 0.1 M Tris-HCl, pH 9.5, 50 mM $MgCl_2$ and 0.1% Tween-20) at room temperature for 15 min.

Staining was performed with NBT/BCIP (Roche, Germany) following the manufacturer's instructions. Once desired color development was observed the reaction was stopped with stop solution (10 mM Tris-HCl, pH 9.5, 1 mM EDTA, pH 8.0) two times (10 min each) and afterwards rinsed twice with PBS (10 min each). The slides were then counterstained with bismarck brown (Sigma Aldrich, USA) or fast green (Sigma Aldrich, USA) and mounted with HistoChoice mounting media (Amresco, USA). Stained VHSV inoculated FHM cells were then examined and photographed using a light microscope BX53 (Olympus, Japan).

2.4.4. ISH assay optimization

As ISH technique requires stringency at various steps, therefore, the development of RNA-ISH assay in a cell culture model requires optimization as maintenance and adherence of cell monolayer is difficult when compared to a tissue section. As probe hybridization and washing are critical steps in an ISH assay, therefore, we optimized these two parameters in our RNA-ISH assay protocol. Also, we tested two different temperatures for probe hybridization viz., 37 °C and 65 °C. The number of stringency washes and different wash buffer concentration combinations were also tested to better optimize the ISH assay. For detection

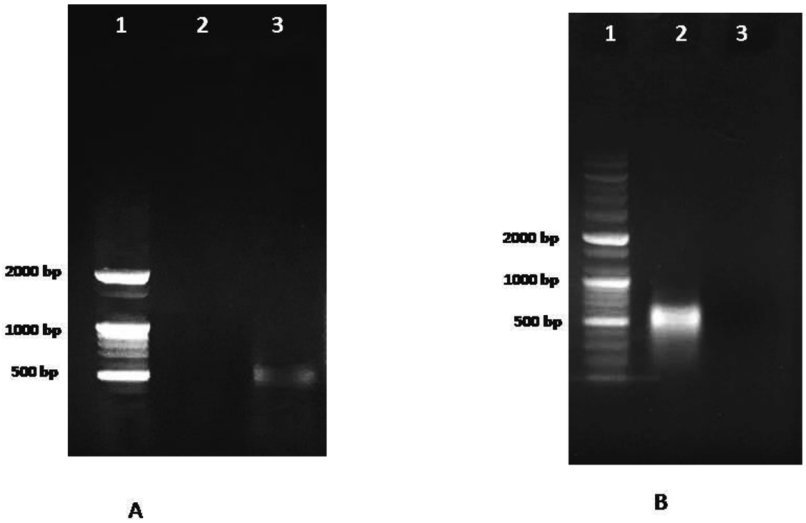

Fig. 1. Agarose gel electrophoresis (0.5%) showing *in-vitro* transcribed VHSV N and G gene riboprobes. (A) *In-vitro* transcribed and DIG labelled N gene riboprobe (Lane 3); Lane 1 = 100 bp ladder; Lane 2 = Negative control (B) *In-vitro* transcribed and DIG labelled G gene riboprobe (Lane 2); Lane 1 = 100 bp ladder; Lane 3 = Negative control.

Table 2
RNA-ISH assay optimization parameters.

Parameter	Signal intensity		Background
Hyb. temp.(°C)	N riboprobe	G riboprobe	
37	+ +/+ + +	+ +	n.a
65	+ + +	+ + +	n.a
Ab incb. time (h)			
1-2	+ +/+ + +	+ +/+ + +	–
overnight (> 12)	+ +/+ + +	+ +/+ + +	–
Wash buffer			
comb (a)	+ +/+ + +	+ +/+ + +	–
comb (b)	+ +/+ + +	+ +/+ + +	–
Color dev.			
15 min.	+/+ +	+	–
30 min.	+ +/+ + +	+ +	–
1 h	+ + +	+ + +	-/+
> 1 h	+ + +	+ + +	+/+ +

Abbreviations: Hyb.temp.: hybridization temperature, Ab incb. time: Anti-DIG antibody incubation time, Color dev.: color development, n.a: not applicable, comb: combination, Signal intensity: weak(+), moderate(+ +), high(+ + +); Background: nil (-), negligible (+), moderate (+ +); Wash buffer: comb(a): with t-rna and heparin, com(b): without t-rna and heparin (For demarcation of signal intensity check supplementary file).

step, different time intervals were tested for color development to test "signal to noise" development. The details for the same are presented in Table 2 and score demarcation is attached in Supplementary file 1.

2.5. Immunocytochemistry (ICC)

Methanol fixed FHM cells were incubated in 3% H_2O_2 (in PBS) at room temperature for 10 min to block the endogenous peroxidase activity. Afterwards, slides were washed three times in PBS (10 min each). Non-specific protein binding sites were blocked by incubating cells in 10% normal goat serum (Cell signaling, USA) for 1 h. Cells were then incubated overnight with primary antibody (at a dilution of 1:1000) raised in mice against glycoprotein (G) of VHSV as described by Jeong et al. (2017). On the second day, slides were washed three times (10 min each) in PBS (pH 7.4). For detection step, slides were incubated with 1:500 dilution of secondary antibody viz., goat anti-mouse IgG horseradish peroxidase (HRP) conjugated (AB frontier, Korea) in PBS at room temperature for 1 h and then rinsed with PBS (pH 7.4) three times (10 min each). After washing, colorimetric detection was performed using 3.3`–diaminobenzidine (DAB) substrate Kit (Abcam, UK). After color development, the reaction was stopped by adding PBS and finally cells were counterstained with Mayer's hematoxylin (Sigma-Aldrich, USA), mounted with HistoChoice mounting media (Amresco, USA) and photographed using a light microscope BX53 (Olympus, Japan).

2.6. Quantitative analysis

2.6.1. Quantitative RT-PCR (qRT-PCR)

The qRT-PCR to quantify viral mRNA transcript from infected cells and culture supernatants was carried out in an Exicycler 96 Real-Time Quantitative Thermal Block (Bioneer, Korea) following the already published protocol of Kim et al. (2014) using SYBR green mixture, AccuPower Greenstar qPCR Premix (Bioneer, Korea). Two primer sets were used for N and G gene segments and the details for the same are mentioned in Table 1. The plasmid DNA for standard curve was constructed by separately cloning the VHSV ORF of each gene (N and G) into pCR2.1-Topo vector (Invitrogen). The serial 10-fold dilutions of the cloned plasmid were amplified in duplicate by qRT-PCR to determine the sensitivity of the qRT-PCR assay. The slope and R^2 values of the primer set for N gene was −0.2928 (96% efficiency) and 0.9979, respectively, whereas, slope and R^2 values of the primer set for G gene was −0.2894 (95% efficiency) and 0.9995, respectively. Briefly, the reaction conditions were set as follows: a 10 min pre-denaturation cycle at 95 °C, 40 cycles of 20 s denaturation at 95 °C, and a 40 s extension at 58 °C. The specification of the qRT-PCR reaction was analyzed through melting curve analysis and the baseline was determined automatically by the Exicycler Analysis Software (Bioneer, Korea). All samples were scaled up to give a count of copies per mL.

2.6.2. VHSV infectivity titer

VHSV titration was performed in 96-well cell culture plates with confluent FHM cells. Sampled VHSV infected FHM cells and supernatants were serially diluted 10-fold (10^{-1} to 10^{-8}). A 50 µL of each sample was inoculated into each well and incubated at 15 ± 0.5 °C for 10 days. After 10 days post inoculation, 50% tissue culture infection dose ($TCID_{50}$) was determined using the method of Reed and Muench, (1938) based on the number of wells displaying viral cytopathic effect (CPE). All samples were scaled up to give a count of $TCID_{50}$/ mL.

2.7. Statistical analysis

Statistical analysis for the quantitative data was performed using one-way ANOVA (analysis of variance) to determine the significant differences between the means. The level of significance was determined at $P < 0.05$ and Post hoc analysis was followed by Duncan's multiple range test. Data are presented as mean ± SEM.

Whereas, for qualitative data, Cohen's Kappa was used to compare virus detection between techniques (ISH and ICC). A test sample was considered positive by a technique if at least two replicates at one time point presented a color precipitate (positive signal). Similar approach has been used earlier by Arzul et al., (2002) and later followed by Martenot et al. (2016). The interpretation of Kappa value is based on the numeric scale proposed by Landis and Koch, (1977): < 0, poor agreement; 0 to 0.20, slight agreement; 0.21 to 0.40, fair agreement; 0.41 to 0.60, moderate agreement; 0.61 to 0.80 substantial agreement, and 0.81 to 1: almost perfect agreement). All the statistical analysis was carried out using SPSS v.16.0 (IBM Corp., USA).

3. Results

3.1. Probe synthesis and in vitro transcription

Agarose gel electrophoresis results for RNA probes are shown in Fig. 1. The size of the *in vitro* transcribed RNA probes correspond to 500 base pair ladder which is near the expected size of nucleotides for both N and G gene segments. After *in vitro* transcription, remaining DNA template was efficiently digested using DNase solution (recombinant grade) which is evident in the gel picture (Fig. 1A and B) as no traces of DNA template can be found other than a single band of RNA probe.

3.2. RNA in situ hybridization (RNA-ISH)

To detect viral mRNAs in infected FHM cells, formalin fixed slides were subjected to RNA-ISH and the results for the same are presented in Figs. 2 and 3. Signal was at first detected as early as 6 hpi using both the digoxigenin labeled RNA probes (N and G). Although, mRNA transcripts were weakly stained at earlier time points but with time the signal intensity got increased and an intense signal was observed at 24 hpi followed by 48 hpi and 72 hpi using both the riboprobes. Also, we observed that increasing the coloration time from 30 min to 1 h in the final detection step, the signal was more prominent in case of weakly stained slides viz., 6 hpi and 9 hpi, without any visible background. Even though, no cytopathic effect was evident in infected cells until 48 hpi but signal detection at 6 hpi using RNA-ISH, which then increased gradually, underlines the sensitivity of the assay.

For the ISH assay optimization, different parameters were optimized as listed in Table 2. Probe hybridization at 37 °C gave moderate signal in case of G gene riboprobe, and moderate to high signal using N gene riboprobe after overnight incubation but occasionally, the results were

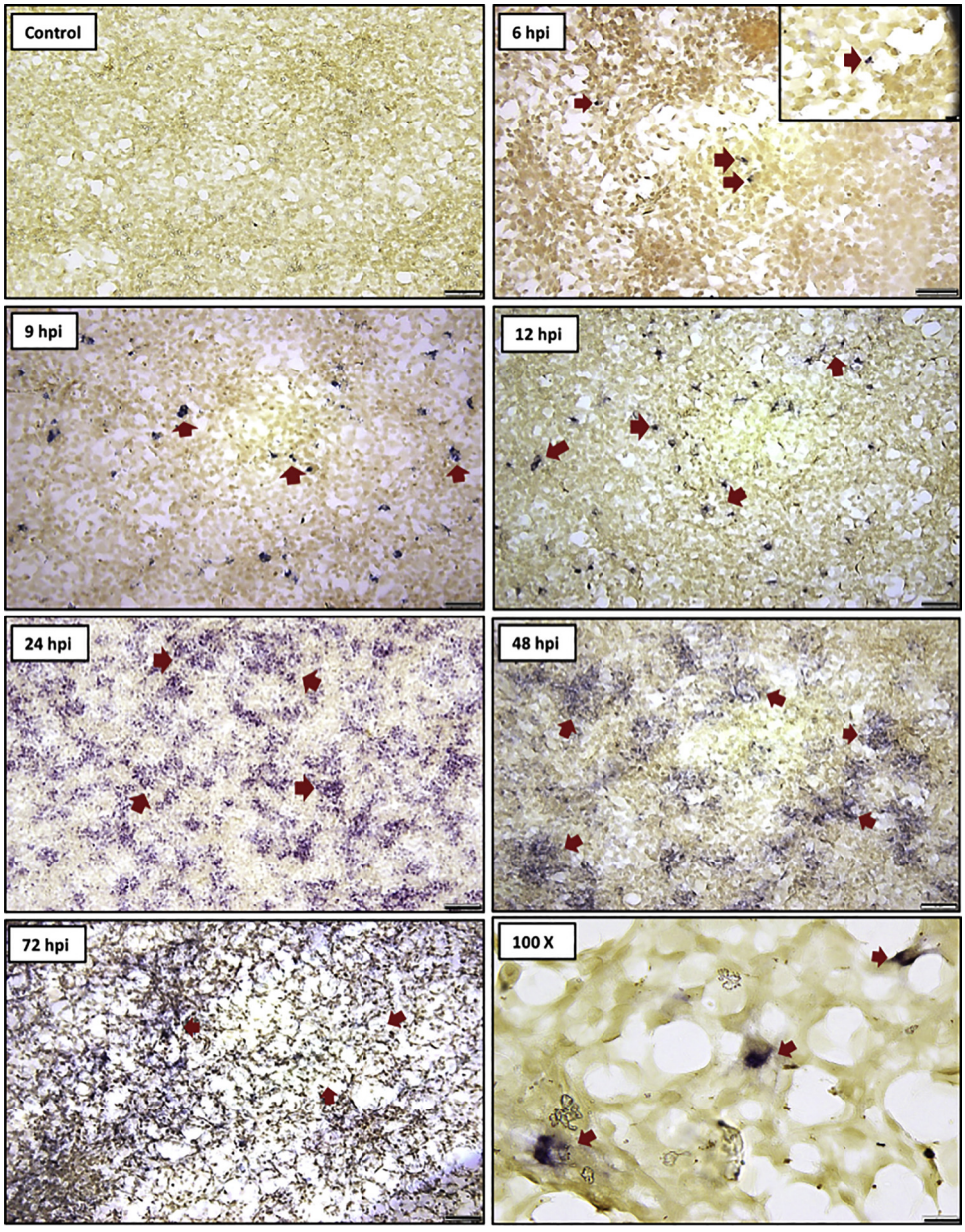

Fig. 2. *In situ* hybridization of FHM cells post VHSV infection at different time points using nucleoprotein (N) gene riboprobe. Cells were counterstained with bismarck brown and dark blue color (pointed by red arrows) represents hybridization signal (Bar represents scale = 50 μm ; 100 X = Representative image at 100X magnification showing cytoplasmic staining) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

inconsistent at 37 °C. However, high signal intensity was observed at all time points using both the probes after overnight hybridization at 65 °C. So, for the present ISH assay probe hybridization was carried out at 65 °C. Care should be taken to maintain a humidified environment, particularly at 65 °C, to prevent slide drying which is critical as it often leads to high background in detection step. Also, in comparison to overnight incubation at 37 °C, some cells can loose adherence at 65 °C and wash out if not handled carefully during washing steps. Also, primary antibody (anti DIG) incubation time was optimized at two different time intervals viz., 2 h and overnight (> 12 h) and in both the cases similar results were obtained. As such, 2 h incubation was followed as it reduced the overall assay gap by one day. We also tested two combinations of wash buffers, one containing the same hybridization mixture (comb. a) used for probe hybridization and the other with similar components (comb. b) except for t-RNA and heparin. Interestingly, no difference was observed using both the combinations and in our case we stuck to comb (b) for the current RNA-ISH. Color development was also tested at different time intervals for both the probes in the final detection step. This is important as any prior detection or delay can give varying results or color background. Although, difficult to optimize but in our hands color development from 45 min to 1 h yielded good results without any or negligible (if any) background. Further, we found that probe denaturation prior to hybridization as an optional step, as it did not make much of a difference at the end of the assay.

Based on our in vitro RNA-ISH assay, VHSV detection was possible using both the digoxigenin labeled riboprobes. Although, VHSV mRNAs were detected using both the probes, however, signal intensity was slightly more pronounced using N gene riboprobe but nevertheless, the sensitivity of both riboprobes was similar as viral mRNAs were detected at similar time points.

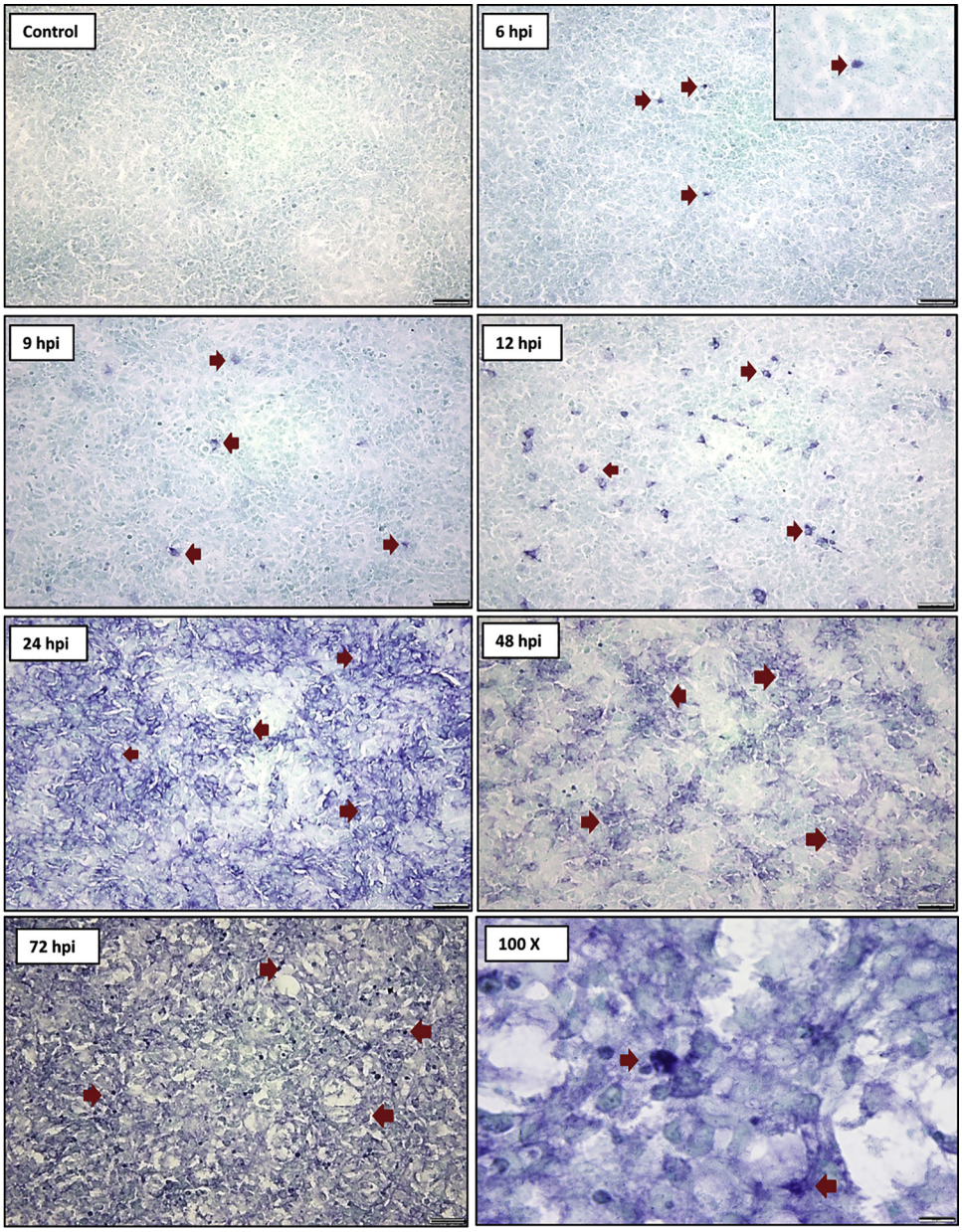

Fig. 3. *In situ* hybridization of FHM cells post VHSV infection at different time points using glycoprotein (G) gene riboprobe. Cells were counterstained with fast green and dark blue color (pointed by red arrows) represents hybridization signal (Bar represents scale = 50 µm ; 100 X = Representative image at 100X magnification showing cytoplasmic staining) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3.3. Immunocytochemistry (ICC)

Methanol fixed cells were subjected to ICC using monoclonal antibodies directed against viral glycoprotein to detect VHSV. For the primary antibody, out of three dilutions tested (1:100, 1:200 and 1:1000) from the stock, the dilution at 1:1000 was used for further assay as no visible background was observed at this dilution in contrast to other dilutions. As for the signal detection, the viral antigens were first detected first at 12 hpi. This was followed by a gradual increase in signal intensity for the corresponding time points and signal intensity peaked at 24 hpi followed by 48 hpi and 72 hpi. The results for the ICC assay are presented in Fig. 4.

A comparison was made between ISH and IHC (Table 3) to measure the degree of agreement between the two techniques using Cohen's kappa to take into account any chance agreement. In total 21 test samples (3 replicates per time point) were tested for each technique. A test sample was considered positive if at least two out of three replicates showed positive signal (color precipitate) with respect to control. The Kappa value (k) was 0.364 and corresponded to a fair agreement between the two techniques. Also, a system to score levels of ISH staining in cells was established: (−) no signal, (+) low signal, (++) moderate signal, and (+++) high signal. This approach was used earlier by Monaghan et al., (2015) and Martenot et al., (2016) to rate the signal intensity in case of ISH and IHC assay, respectively.

3.4. Viral mRNA transcript and infectivity titer

The mRNA transcript for N and G gene segments of VHSV along with the infectivity titer from cells and culture supernatants are presented in Fig. 5. The mRNA transcript for both the genes (Fig. 5A,B) were statistically similar until 12 hpi in cells as well as culture supernatants. Afterwards, the viral mRNA transcript for N gene (Fig. 5A) started to show an increasing trend and peaked at 24 hpi and 48hpi. Whereas, for G gene segment (Fig. 5B) a similar trend was observed,

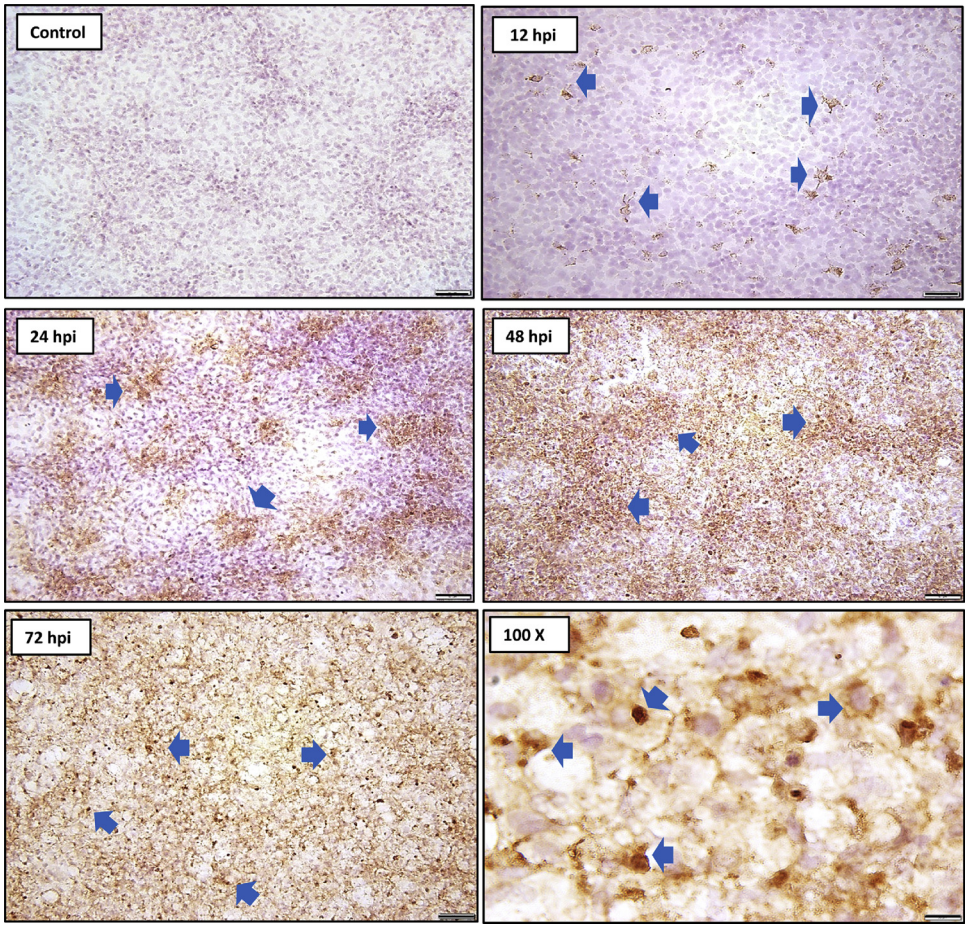

Fig. 4. Immunocytochemical (ICC) staining of FHM cells at different time points post VHSV infection. Cells were counterstained with hematoxylin and dark brown color (pointed by blue arrows) represents positive signal (Bar represents scale = 50 µm ; 100 X = Representative image at 100X magnification showing cytoplasmic staining) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Table 3
Comparison of sensitivity between ISH and ICC.

Technique	Time point (h)						
	3	6	9	12	24	48	72
ISH							
N-riboprobe	–	+	+ +	+ +	+ + +	+ + +	+ + +
G-riboprobe	–	+	+/+ +	+ +	+ + +	+ + +	+ + +
ICC	–	–	–	+/+ +	+ + +	+ + +	+ + +

Signal intensity: nil (-), weak (+), moderate (+ +), high (+ + +) [For demarcation of signal intensity check supplementary file].

wherein, the mRNA transcript started to increase at 24 hpi and peaked at 48 hpi and 72 hpi.

On the other hand, viral infectivity titer (Fig. 5C) in infected cells and culture supernatant also showed an increasing trend over time. Shortly, after virus inoculation the titer started to increase gradually in cells with time and highest peaks were observed at 24 hpi, 48 hpi and 72 hpi. Also, viral titer in the culture supernatants was statistically similar from 6 hpi to 12 hpi and, afterwards titer peaked at 24 hpi, 48 hpi and 72 hpi with highest peak observed at 48 hpi. It can be observed from the results that viral copy number and infectivity titer followed a similar trend until 12 h and after wards both the viral copy number as well as infectivity titer increased sharply at and after 24 h in both the cases.

4. Discussion

The present work reports the development and optimization of an *in situ* hybridization assay to detect VHSV in a cell culture platform using two antisense riboprobes complementary to viral mRNAs. Our aim was to exploit the potential of RNA-ISH in detection and visualization of viral particles *in situ* in infected fish cells to complement the routine viral detection in cell culture based isolation. *in situ* detection of viral particles by hybridization of complementary probes offers applications in clinical research and diagnostic pathology (Franco Mesa, 2013). Detection of viral particles by ISH does not demand strict professional expertise as the virus is visualized directly in the cells. On the other hand, indirect diagnosis by studying cellular morphological changes due to CPE requires an experienced eye as the spectrum of degenerative change is broad which hinders the precise interpretation about the viral presence (Leland and Ginocchio, 2007). Therefore, physical positioning of viral particles along with observation and interpretation of CPE can together augment *in vitro* viral detection. Also, colorimetric *in-situ* hybridization (CISH) has many advantages over fluorescent in situ hybridization (FISH) as signals do not fade over time, can be stained permanently, is less expensive and widely used by pathologists because visualization can be done using a simple light microscope (Jensen, 2014). Nucleic acid probes for ISH can be made of either DNA or RNA molecules, however, RNA probes have many advantages over DNA probes as cRNA-mRNA hybrids are more stable than cDNA-mRNA complexes and also excess probe can be easily digested via RNase, thereby lowering non-specific signal (Carter et al., 2010; Kumar, 2010).

In the recent past, most studies have focused either on development or comparison of quantitative detection of VHSV by qRT-PCR (Hope et al., 2010; Garver et al., 2011; Phelps et al., 2012; Jonstrup et al., 2013; Kim et al., 2014). Whereas, improvements over the conventional cell culture based diagnostics has received less attention despite the fact that virus isolation using cell culture has long been the mainstay in clinical virology even with technological advancements over time (Hodinka et al., 2013). The use of ISH technique to study viral

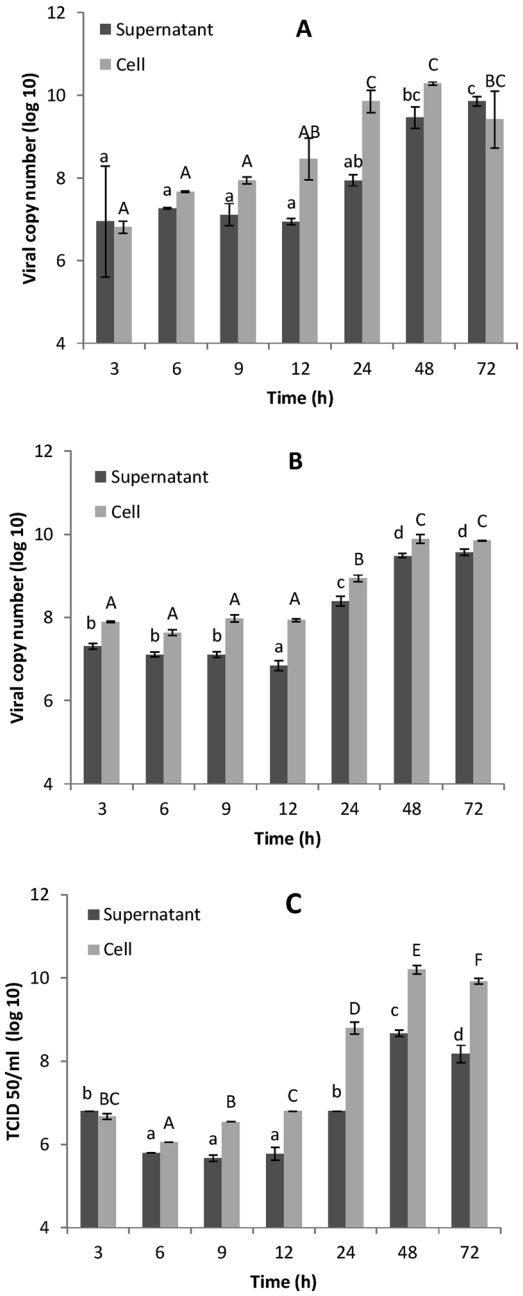

Fig. 5. Quantification of viral mRNA transcript of nucleocapsid gene (A) and glycoprotein gene (B) from the infected cells and culture supernatants at different time points post infection (pi) using qRT-PCR (C) VHSV infectivity titer (log-10 $TCID_{50}$/ml) from infected cells and culture supernatants at different time points post infection (pi). Mean with different superscript letter per factor indicates significant difference. ANOVA was followed by Duncan's multiple range test ($P < 0.05$) and values are presented as mean ± SE.

infections *in vitro* has been frequently employed in human clinical research (Vyboh et al., 2012; Zhang et al., 2017) but such an approach is scarce in case of fish viruses. Although, previous studies by Al-Hussinee and Lumsden, (2011) and Do et al. (2013) have used ISH technique to study *in vivo* VHSV infection but using DNA probes and with different aim to study viral tissue tropism. Till date, no study so far has focused on the use of RNA-ISH in clinical aspect to augment the detection of VHSV in cell culture based routine diagnostics. Our present study for the first time established the use of RNA-ISH assay for VHSV detection in a cell culture model using fat head minnow (FHM), an OIE approved (OIE, 2018) fish cell line. This is also for the first time that use of RNA probes for *in vitro* detection of fish viruses has been carried out and correlated with other available diagnostic methods. When combined with conventional virus isolation and characterization by cell culture, the combo can together increase the overall diagnostic sensitivity of *in vitro* detection which has been very often criticized (Jonstrup et al., 2013; Pierce et al., 2013).

Based on our RNA-ISH assay results, the viral mRNAs were detected early at 6 hpi using both the riboprobes, which was prior to appearance of CPE, thereby, highlighting the potential and sensitivity of RNA-ISH. Previous studies (Bodewes et al., 2014; Pfankuche et al., 2016, 2018) have also underlined the significance of ISH assay as an important diagnostic tool and a first line of confirmation for detection of viral pathogens. Also, the modern era of molecular biology has witnessed exhaustive studies on the use of real-time PCR as a powerful diagnostic tool and the technique has been frequently used in aquaculture for detection of fish pathogens. In relation to VHSV, the application of qRT-PCR was first reported by Chico et al., (2006) for detection of VHSV in experimentally challenged rainbow trout and this was followed by a subsequent study by Liu et al., (2008) to determine the potential of qRT-PCR for simultaneous detection of three piscine rhabdoviruses. The technique was reported to be sensitive in detecting known genotypes of VHSV (Cutrín et al., 2009) coupled with its application in estimation of viral load from VHSV infected tissues (Oidtman et al., 2011). The present results of RNA-ISH assay were consistent with viral quantification (N and G gene) analysis by qRT-PCR in terms of signal intensity. Although, the detection by the later was more sensitive which is expected as sensitivity of qRT-PCR cannot be challenged when compared to cell culture based diagnosis (Hope et al., 2010; Ammayappan et al., 2011; Madani et al., 2014). Even though the difference in copy number was not significant until 12 hpi but interestingly RNA-ISH could localize individual mRNAs in cells at earlier time points following which the signal intensified with corresponding time points. Also, viral infectivity titer increased with time, although, the difference was significant at each time point but titer showed a sharp increase at 24hpi which correlated with qRT-PCR results and RNA-ISH signal intensity. Both the riboprobes were sensitive in early detection of viral particles but N gene riboprobe was slightly more pronounced in terms of signal strength. The reason for the same could be the positioning of N gene on the transcriptional frame as it is located ahead of G gene and as such is transcribed first. The justification is supported by a recent report by Dietzgen et al. (2017), wherein, the authors reported that during the rhabdoviral assembly, transcription of the negative-stranded genome is facilitated by a transcriptase complex and occurs progressively on a decreasing molar gradient based on gene distance from the genomic 3′ end (N→P→M→G→L).

Also, we compared the diagnostic sensitivity of RNA-ISH with ICC. Although, the two techniques are based on different principles viz., nucleic acid detection (ISH) and protein detection (ICC) but nevertheless they have been used together to complement each other in localization studies (Xiu et al., 2014; Yue et al., 2014; Wu et al., 2018; Cooper et al., 2018). However, our comparison here was to check the diagnostic sensitivity of both the techniques in earlier detection of VHSV. It is clear from our results that ISH was able to detect VHSV earlier (at 6 hpi) when compared to ICC, wherein, detection by the later was first at 12 hpi. The delay in signal detection using ICC can be attributed to assembly of individual viral particles to form complete virions and translation of mRNAs into proteins. Therefore, ISH has an advantage over ICC in early detection of viral particles as viral nucleic acids can be directly localized. Previous studies (Sur et al., 2003; Furtado et al., 2015) also reported greater sensitivity of ISH compared to IHC. Also, there is minor structural alteration during fixation in ISH (Alonso et al., 2004) while as there can be cross linking of the proteins due under or over fixation in ICC, which can result in minimum access to detect the viral antigen (protein) of interest. Further, the inter-rater agreement using Cohen's kappa gave a fair agreement ($k = 0.364$) between the two techniques viz., ISH and ICC, which was over and above

any chance agreement. Fair implies reasonable differences between the two techniques which can be justified as the detection by ISH was earlier than ICC at least by three time points. However, the disparity can also be due to effect of various treatments on the quality of the cells like cross linking of proteins due to formalin treatment and nucleic acid degradation which can have an effect on the end result (Bancroft and Gamble, 2008).

As regards the specificity of our RNA-ISH assay, the N riboprobe can localize known genotypes of VHSV as the probe was designed based on OIE approved primers specific to all known VHSV genotypes. However, the N riboprobe may not be specific to VHSV genotype IV b as it is mentioned in the OIE manual that genotype IVb isolates may not be recognized using the OIE approved primers. Whereas, for G riboprobe synthesis, a new primer set was designed for the current study based on the glycoprotein gene sequence of VHSV genotype IVa (FYeosu05). Also, G riboprobe apart from localizing VHSV genogroup IVa, can in addition localize other VHSV genotypes as probe designing was focused on conserved region of G gene among VHSV isolates. As such, both the synthesized riboprobes have broader application in localization of VHSV.

In conclusion, our study for the first time developed and optimized RNA-ISH assay to detect VHSV in a cell culture model. Two synthesized riboprobes (N and G) efficiently localized viral mRNAs in infected FHM cells prior to appearance of cytopathic effect. The signal intensity at different time points post inoculation correlated with viral quantification assays (qRT-PCR and TCID-50). Also, our RNA-ISH assay was more sensitive when compared to ICC in detecting VHSV. Overall, our results indicate that RNA-ISH assay in conjunction with VHSV isolation in cell culture can increase the sensitivity of *in-vitro* viral diagnosis and the duo can be employed as a two-step initial VHSV confirmation procedure. In addition to *in vitro* viral diagnostics, the synthesized riboprobes also offer applications to study tissue tropism of VHSV, however, its optimization and sensitivity in such kind of *in vivo* studies needs to be further validated as host dynamics *per se* can be variable.

Declaration of interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jviromet.2018.11.003.

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