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Screening for viral hemorrhagic septicemia virus in lumpfish (*Cyclopterus lumpus*) from Eyjafjörður, Iceland with real-time RT-qPCR

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Yfirlýsingar

Ég lýsi því hér yfir að ég er ein höfundur þessa verkefnis og að það sé afrakstur eigin rannsókna.

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Það staðfestist hér að verkefni þetta fullnægir að mínum dómi kröfum til prófs í námskeiðunum LOK1126 og LOK1226.

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Kristinn P. Magnússon, leiðbeinandi

Útdráttur

Árið 2015 greindist viral hemorrhagic septicemia veiran (VHSV) í fyrsta skipti á Íslandi. Veiran var greind í hrognkelsum (*Cyclopterus lumpus*) úr Breiðafirði, með aðferðum þar sem frumulínur voru notaðar. Tilgangurinn með þessu verkefni var að kanna hvort veiruna væri að finna í hrognkelsum úr Eyjafirði. Til þess var notuð sameindaerfðafræðileg RT-qPCR aðferð sem hefur ekki verið notuð hér á landi í þeim mæli, er æskilegt gæti talist, til slíkrar skimunar.
Tuttugu og þrír fiskar voru veiddir, krufðir og ástandsgreindir. Við krufningu sýndu þrír fiskar svipuð einkenni og VHS veirusýktir fiskar. Eftir skimun með RT-qPCR aðferðinni á RNA úr fiskunum, reyndust allir fiskarnir vera neikvæðir fyrir VHSV.
Í ritgerðinni er rætt mikilvægi þess að skima eftir veirunni í hrognkelsum og öðrum fisktegundum við Ísland í ljósi þess að veiran hefur greinst í nágrannalöndum þar sem hún hefur breiðst hratt út síðustu ár, sér í lagi í fiskeldum þar sem hún hefur valdið miklu tjóni. Í dag eru gerðar tilraunir með að nota hrognkelsi við að hreinsa lýs af eldisfiski og þykir sú aðferð umhverfisvæn og árangursrík. Hins vegar gæti stafað ógn af þeirri aðferð þar sem að VHSV gæti mögulega borist inn í fiskeldin þegar notaðar eru aðrar fisktegundir til hreinsunar á þennan máta. Real-time RT-qPCR aðferðin er því kjörin aðferð til þess að skima eftir veirunni hér á landi sem og annars staðar.

Lykilorð: Viral hemorrhagic septicemia virus, real-time RT-qPCR, hrognkelsi (*Cyclopterus lumpus*), fiskisjúkdómar, aflúsun

Þakkarorð

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Takk öll sömul!

Akureyri, 7. Apríl, 2016
Birta Líf Fjölnisdóttir

Abstract

Late last year, in 2015, the viral hemorrhagic septicemia virus (VHSV) was detected for the first time in Iceland whereas the virus was discovered in lumpfish (*Cyclopterus lumpus*) in Breiðafjörður, Western Iceland, with cell culture methods.

The purpose of this study was to find out if the VHS virus was present in lumpfish collected in Eyjafjörður, Northern Iceland. The screening was carried out with real-time RT-qPCR, a molecular genetic method, which has not ben used frequently in Iceland for that matter. Twenty-three specimens of lumpfish were collected in nets, dissected and analyzed, of which, three showed similar symptoms as found in VHSV infected fish. The results from RT-qPCR screening proved all specimens to be negative for VHSV.

In this thesis, the importance of screening for VHSV is discussed due to the fact that it is present in neighbouring countries and has been spreading out fast, particularly within aquaculture where it is responsible for serious commercial losses. Currently, delousing experiments within fish farming, which include the use of lumpfish as cleaner fish, has gained a lot of interest, and has shown great potential since this method is considered to be environmentally friendly and effective. However, with this method the risk of transmitting VHSV could increase and therefore be a big threat to the aquaculture industry. Real-time RT-qPCR method is considered efficient and accurate to screen for VHSV in Iceland, as well as other countries.

Keywords: Viral hemorrhagic septicemia virus, real-time RT-qPCR, lumpfish (*Cyclopterus lumpus*), fish diseases, delousing

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Abbreviations

OIE – World organization for animal health

DNA – Deoxyribonucleic acid

RNA – Ribonucleic acid

rRNA – Ribosomal RNA

cDNA – Complementary DNA

ssDNA – Single stranded DNA

dsDNA – Double stranded DNA

PCR – Polymerase chain reaction

RT-qPCR – Reverse transcription quantitative polymerase chain reaction

VHS – Viral hemorrhagic septicemia

VHSV – Viral hemorrhagic septicemia virus

EEU – Eurasian economic union

ESA – European free trade association (EFTA) surveillance authority

TBE buffer – Tris/Borate/EDTA buffer

EDTA – Ethylenediaminetetraacetic acid

FRET – Fluorescence resonance energy transfer

GOIs – Genes of interest

C_T – Cycle threshold

ELISA – Enzyme-linked immunosorbent assay

DEPC – Diethylpyrocarbonate

DSMO – Dymethyl sulfoxide

1. Introduction

In Iceland, fisheries have been one of the largest industries for decades, and with fish as the main export product, covering approximately 40% of exports in 2014, there seems to be no retreat in that business whatsoever (Hagstofa Íslands, n.d.)

Subsequently, with increased demand and globalization, the Icelandic aquaculture industry has grown significantly, whereas employment within fish farming has increased for about 20% since 2014 (Rúnarsson, CEO of the National federation of Icelandic aquaculture, e-mail communications, 22.March 2016).

However, in aquaculture, as well as other industries, there can be problems affecting the production where the most common problem within fish farming is the salmon louse (*Lepeophtheirus salmonis*). However, there is another problem that has been causing serious disease outbreaks all over the world for the last decades, the viral hemorrhagic septicemia virus. Together these problems in their different ways, hold responsibility for serious commercial losses within the industry all over the world each year (Hamre et al., 2013; Anna A. Schönherz, Lorenzen, Guldbrandtsen, Buitenhuis, & Einer-Jensen, 2016)

Screening for VHSV is obligatory since it is a reportable pathogen according to the world organization for animal health (OIE), and it has been done every year in Iceland since 1985. The screening has been performed at Keldur, research center for the Icelandic food and veterinary authority whereas it has mainly been carried out with two types of cell culture methods (Árni Kristmundsson, head of fish diseases at Keldur research center, e-mail communications, 12.january, 2016).

However, the virus had never been detected until October last year where it was found in wild lumpfish that was caught in Breiðafjörður, Western Iceland. This caused a bit of a turmoil in the management of Icelandic fisheries industry, since this indicated the loss of an EEU/ESA certification as well as

the loss of an aquaculture nations award that stated that Iceland was free of reportable pathogens (Matvælastofnun Íslands, 2016a, 2016b).

The fact that VHS has now been detected in lumpfish in Iceland could be a major problem and could mark the beginning of a synergy between biological control of salmon louse and the implantation of serious infections in fish farming. This synergy could occur because recent methods used to eliminate the salmon louse in aquaculture trays have consisted of the use of other fish species, namely, lumpfish as "cleaner fish", which could therefore increase the risk of VHS in aquaculture due to the fact that the virus can transmit rather easily. Besides that, this could also indicate that VHSV is spreading further around the world, which can be a huge threat to the aquaculture industry as well as the commercial fishing industry in Iceland (Imsland et al., 2014; Matvælastofnun Íslands, 2016b).

1.1 Salmon louse (*Lepeophtheirus salmonis*)

The salmon louse, an ectoparasitic copepod of the family Caligidae, is one of the major problems within fish farming in Northern Europe whereas the first outbreak was observed at salmon farms in Norway in 1960s (Pike & Wadsworth, 1999). However, it is also responsible for serious commercial and economical losses all over the world, each year (Roth, Richards, & Sommerville, 1993; Skern-Mauritzen, Torrissen, & Glover, 2014).

Although the louse is extremely ubiquitous, their growth rate, fertility and evolution rely heavily on environmental factors such as temperature, host distribution and adequacy as well as the competition between and within species. For the salmon louse the temperature seems to be the most important factor where they appear to thrive better at colder environments while their generation time can decrease significantly at warmer areas (Costello, 2006).

Many researchers have described the developmental cycle of *L. salmonis* as five different phases with either ten or eight different stages depending on the author, when in fact the number of stages has been controversial since the ecdysis steps can be hard to separate. However, the five phases are called nauplius, copepodid (infectious stage), chalimus, preadult and adult stage, where the mature adult females produce eggs for life after the final molt of ecdysis. This constant production makes elimination of the louse

extremely hard since they can produce up to 11 pairs of egg strings during their life span (Eichner et al., 2008; Hamre et al., 2013; Heuch, Nordhagen, & Schram, 2000; Kabata, 1972).

As reviewed by K. Whelan, the louse attaches to a host fish, namely salmon and fresh sea trout, and scrape their skin, mucus and other tissue resulting in problems such as tissue necrosis, epithelium loss, bleeding and other skin related manifestations. This can lead to an increase of the risk of bacterial infections as well as the loss of innate immunity protections. Another consequence is that the host can lose appetite, resulting in reduced growth and physical functions. However, injuries caused by the salmon louse is usually not fatal for the host unless it reaches a level that the host can not fix the damage by itself with their natural defenses (Pike & Wadsworth, 1999; Whelan, 2010)

1.1.1 Biological control of the Salmon louse

Multiple methods, such as chemotherapeutic control with various chemicals such as hydrogen peroxide and organophosphates, have been used in aquaculture to get rid of the salmon louse as well as in-feed treatments and synthetic pyrethroids (Imsland et al., 2015; Roth et al., 1993).

However, most of these methods have not been thoroughly successful, which can be due to the fact that the lice is becoming resistant to some of them. Because of that, a recent biological control method has gained a lot of interest, which is sustainable and effective and therefore interesting for the sake of public acceptance. This new method indicates the use of "cleaner fish", where other fish species, such as the Ballan wrasse (*Labrus bergylta*) and lumpfish (*Cyclopterus lumpus*) are put into the aquaculture trays where they feed on the lice and therefore serve as biological cleaners. However, the wrasse is more sensitive to temperature since it thrives better at higher temperatures (>8°C), while the lumpfish can resist colder environments and thus the lumpfish is more feasible for biological delousing control in aquacultures located at the northern hemisphere (Imsland et al., 2014).

As described by Imsland et al., the use of lumpfish as biological control can decrease the lice infestations significantly with a mature female delousing efficiency over 90%, and adult male delousing over 50% after merely three

months. However, the size of the lice seems to matter whereas it looks like it is harder for the lumpfish to feed on smaller louses (Imsland et al., 2014).

1.2 Viral hemorrhagic septicemia virus

Viral hemorrhagic septicemia (VHS) is a serious disease, with mortality rate of up to 90%, caused by the viral hemorrhagic septicemia virus (VHSV), a reportable fish pathogen with four different genotypes. It was first identified in Danish rainbow trout around 1950 in Egtved, Denmark. Since then it has been detected in over 80 freshwater and marine fish species in Europe, North America and Northeast Asia, indicating that the virus is capable of adapting to new host species and environments (Ito, Kurita, Mori, & Olesen, 2016; Jensen, 1965; Olesen, N.J. & Skall, 2015).

Wilhelm Schäperclaus first described VHS in 1954, where he noted that the disease had the same symptoms as he had detected in trout at a fish farm in Germany, several years before. Schäperclaus examined many rainbow trout which all seemed to have the same or similar symptoms, that were, in most cases, protruded eyes, fluid in stomach cavity, excessive fat around intestines, dark spots on the pancreas as well as that the rear part of the kidneys were swollen (Jensen, 1965; Schäperclaus, 1954). However, after multiple experiments the symptoms have been described as clinical and/or behavioral where the clinical symptoms can be inactivity, brisk mortality, hemorrhages and other visual manifestations while the behavioral symptoms can be weird swimming, such as spiraling, as well as that the fish will not try to escape when put in net as usual (Olesen, N.J. & Skall, 2015).

Transmission of the virus can occur in various ways and seems to affect fish at all ages, but the most known transmission pathway is by urine or sexual fluids, such as eggs, whereas it seems to be a great possibility of infection between wild fish and farmed fish (Garver et al., 2013; McAllister, n.d.). However, it has been examined that the virus can be transmitted quite easily by applications such as muscle and peritoneum injection, feeding, coupling as well as brushing it on gills (Castric & Kinkelin, 1980; McAllister, n.d.; A. A. Schönherz et al., 2012).

Therefore, there seems to be an urgent need for VHSV prevention or control methods, since the virus has great potential of being one of the major problems within the aquaculture industry because of its adaption potential and fast spreading. Because of that, it is essential to rapidly identify the virus with efficient and accurate methods.

1.2.1 VHSV control and prevention

Multiple methods have been tried to control or prevent VHS within fish farming, whereas just few of them turned out to be successful. Those methods consist mainly of aquaculture fallowing, reinfection or destruction of fish farms and therefore not considered beneficial for the industry (Olesen & Korsholm, 1997; Olesen, 1998).

Other methods have been tried such as vaccination, immunostimulation, resistance breeding, restocking with resistant species and disinfection of eggs and larvae. However, none of these methods have been reported efficient enough in the direct control or prevention of VHS except disinfection of eggs and larvae, which is very expensive. Although, resistance breeding and vaccination show a great potential but need further experiments (Giuseppe Bovo, Barry Hill, Asbjørn Husby, Tore Håstein, Christian Michel, Niels J. Olesen, 2005; Lorenzen & LaPatra, 2005; Olesen, N.J. & Skall, 2015; Peddie, McLauchlan, Ellis, & Secombes, 2003).

1.2.2 Detecting VHSV

Since VHSV is a reportable fish pathogen that is spreading fast around the world, it is essential to be able to detect it rapidly and accurately as fast as possible. Researchers have mainly used direct methods to detect and identify the virus, such as microscopic histological examination, agent isolation and identification with cell cultures or artificial media. Other applications such as antibody-based antigen methods such as enzyme-linked immunosorbent assay (ELISA) and fluorescent tests, as well as serological tests have also been used to detect VHSV (Olesen, N.J. & Skall, 2015).

However, to detect VHSV, molecular techniques such as RT-PCR and real-time RT-PCR have gained a lot of interest over the last years because of its accuracy in detecting all viral strains of VHSV, as well as it is more time

efficient than the conventional methods. With these molecular methods there is no need for cell cultures, and the results are available in real time with high accuracy. Therefore these methods are extremely convenient since the virus is reportable and it can spread fast, and thus, cause a serious loss for those who it may concern (Garver et al., 2011; Jonstrup, Kahns, Skall, Boutrup, & Olesen, 2013; Snow et al., 2004).

1.3 Real-time RT-PCR method

Over the last decades molecular experiments have been used extensively where methods such as the polymerase chain reaction (PCR), and real-time polymerase chain reaction (real-time PCR) have been one of the leading experimental applications used (Klug, Cummings, Spencer, & Palladino, 2010).

Kary Mullis was the first to describe the PCR in 1986, whereas it marked a major breakthrough in molecular studies all over the world. With this method researchers were able to investigate genetic material in a brand new way that required just small amounts of DNA. They were now able to clone without the use of host cells, determine mutations and genetic defects, identify viruses and bacterial species and they were also able to fix and amplify damaged DNA fractions by using the correct primers (Klug et al., 2010).

Polymerase chain reaction has been divided into three steps:

- Denaturation: Double DNA strands are denatured into two single strands. This is done by increasing the temperature to 92-95°C for about 1 minute.
- Hybridization/Annealing: Temperature is decreased slowly to 45-65°C so primers can bind to the single stranded DNA. Keep in mind at this stage the length of the primers, base composition and the primer/single strand match when choosing the temperature.
- Extension: Temperature is increased to 65-75°C, DNA polymerase starts making new DNA strands by adding bases at the end of the primers in 5' – 3' direction. Keep in mind here which polymerase is used, since not all proteins can handle the temperature changes like in this reaction. In most cases *Taq polymerase* is used, a polymerase derived from the bacteria *Thermus aquaticus*.

In each cycle of the reaction, which takes about 2-5 minutes, DNA strands are doubled so after less than 3 hours the yields are over million-fold. For this reaction, the DNA sequence needs to be rather short and it has to be known so it is possible to design suitable primers (Klug et al., 2010).

Another method that was derived from PCR is the real-time RT-PCR (real-time reverse transcription polymerase chain reaction). This method was also a breakthrough since it made it possible to watch the polymerase chain reaction in real time, as well as it was also possible to quantify the products with specific methods such as absolute and relative quantification where specific chemistries are applied. Real-time RT-PCR is highly sensitive but extremely flexible with multiple different aspects (Bustin, 2000; Fleige & Pfaffl, 2006). An important step before carrying out the reaction is to have high quality, non-degraded RNA from tissue or cells. Quality, inhibitory components in sample and storage condition may also have impact on the results (Bustin, 2000).

The next step includes reverse transcription (figure 1), where *reverse transcriptase* polymerase is used to transform RNA into cDNA. There are three different ways to generate cDNA, with specific primers, random hexamers or oligo-dT primers whereas it is extremely important at this point to maintain good practice skills so the cDNA reflects the RNA as it should (Bustin, 2000)

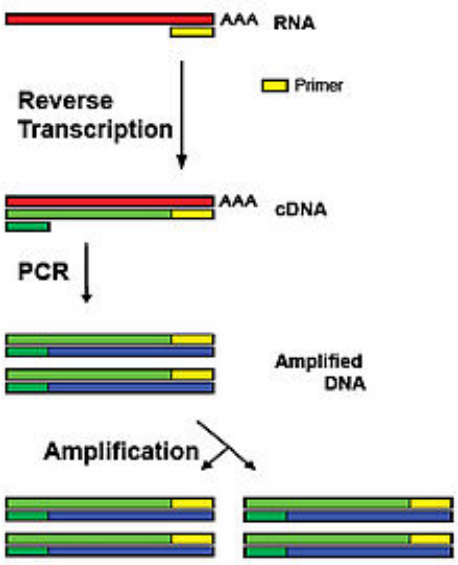

Figure 1. Reverse transcription (Wikimedia Commons, 2016)

After cDNA synthesis, the real-time qPCR can be carried out with the preferred fluorescent chemistry application, where fluorescent dyes are used. The dyes bind specifically to double stranded DNA (dsDNA), not single stranded DNA (ssDNA), resulting in fluorescent signals that specific instruments detect from the surface of each sample tube. The light that reflects from the surface indicates the quantity of the PCR amplified product after each cycle. It also indicates an accurate number of molecules per sample since the fluorescent signal increases at the same time as the molecules. The collected results are imported to appropriate software at real time so the results can be read at the same time that they occur. (Bustin, 2000).

Real-time qPCR has four phases, which have been describes as linear-ground phase, exponential phase, linear phase and plateau/end state (figure 2):

- Linear-ground phase: At this phase the reaction is at a starting point and fluorescent signal is still below the background fluorescent level. This phase is usually 10-15 cycles.
- Exponential phase: At this point the fluorescent signals reach a threshold, which is much higher than the first linear-ground phase. When the threshold has been reached the current cycle is marked as the cycle threshold (C_T), that is, how many cycles have occurred when threshold is reached. In the exponential phase the product doubles in each round if the conditions are ideal. This reaction phase is extremely accurate and specific.
- Linear phase: At the linear phase the reaction slows down since substrates are being used up and the products stop being amplified. This phase is usually 10-15 cycles.
- Plateau state/End state: Amplification has stopped and fluorescent measurements are not significant. If the reaction is kept on going the products start to break down resulting in a plateau state of the curve (Bustin, 2000; Glick, Pasternak, & Patten, 2009; Wong & Medrano, 2005)

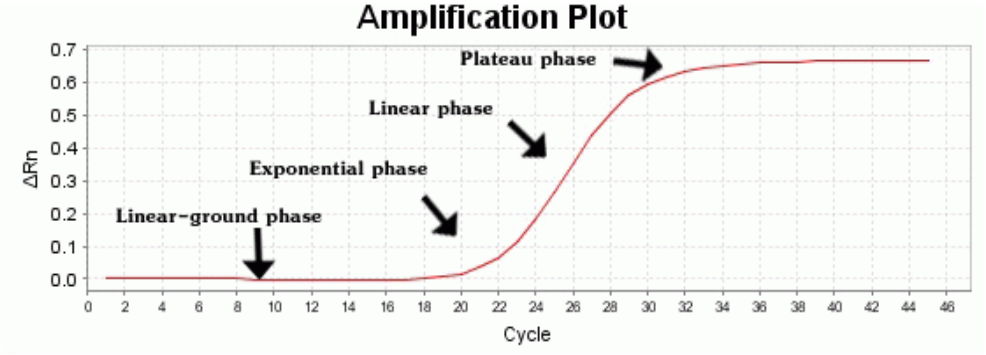

Figure 2. Reaction phases of real-time qPCR (Source Molecular, n.d.)

However it is also an option to perform the reaction at the same time as the reverse transcription in a single tube, and then it is referred to as one-step reaction, while the traditional way is a two-step procedure where cDNA is synthesized separately prior to the real-time reaction (Bustin, 2000)

1.3.1 Real-time RT-PCR detection chemistries

Four known fluorescent chemistries are used for the real-time RT-PCR with different approaches (figure 3), where the main difference is the binding specificity of the probes (Mackay, Arden, & Nitsche, 2002).

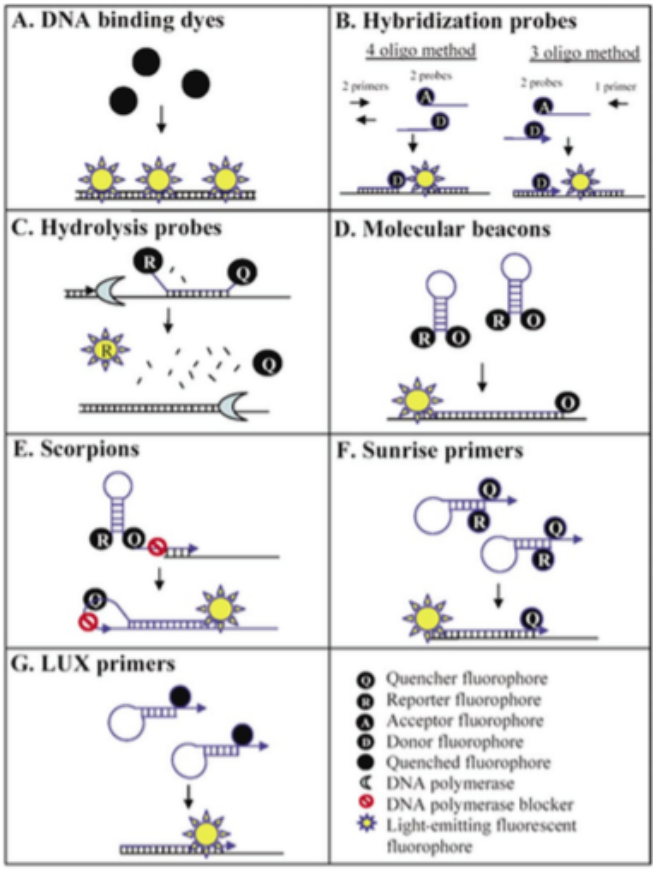

Figure 3. Detection chemistries of real-time PCR (Wong & Medrano, 2005)

At first it is worth to mention the use of unspecific binding dyes, such as SYBR® green I and ethidium bromide, where the dyes bind non-specifically to dsDNA, resulting in the emission of fluorescent signal regardless of what sequence is being amplified. When using binding dyes it is essential to perform a melt curve analysis to determine the primer efficiency (Wong & Medrano, 2005).

The other three methods consist of fluorescence resonance energy transfer (FRET), where hybridization, hydrolysis and hairpin probes are used for the specific binding of fluorescent probes to the correct, distinct dsDNA fragments. The hybridization probe chemistry includes the binding of labeled primers and probes at each end of the strand so the background fluorophore on the dsDNA excites the donor fluorophore when they come across each other, resulting in a fluorescent signal of the amplicons. Hydrolysis probe chemistry on the other hand, namely TaqMan® chemistry, includes the use of probes consisting of a 25-30 nucleotide sequence, that is labeled at the 5' end with a reporter fluorochrome and a quencher at the 3' end, which is degraded by DNA polymerase when annealed to the specific sequence, resulting in high emission of fluorescent signal. The third FRET chemistry consist of hairpin probes such as molecular beacons and scorpion primers as well as Sunrise™ and LUX™ primers as reviewed by Wong and Medrano (Schaad & Frederick, 2002; Wong & Medrano, 2005).

1.3.2 Quantification methods

Real-time PCR can be used in three ways, that is, quantitatively (quantitative real-time PCR) which is frequently used in gene expression studies, semi-quantitatively where certain amount of DNA (above/below) is observed (semi quantitative real-time PCR) or qualitatively (qualitative real-time PCR) where quality is determined as well as it is used to observe which genes are present or not (Glick et al., 2009)

Two known methods are used for quantification determination, absolute quantification and relative quantification where it is often a question which method is convenient whereas the type of the observed sample is what eventually matters. With absolute quantification a standard curve is used to calculate the total copy number of the PCR amplicons, which is good for

absolute results, such as viral screenings. This is done by plotting the logarithm of dilution series of known samples, which indicate an initial quantity, versus the cycle threshold. With this plot it is possible to generate a derivate from the linear regression equation and therefore quantify the copy number for each screened sample. However it is important to run standards for each run to keep the data reliable and in contrast, although that may often not be the case where many scientists use the same standard for multiple experiments which can affect their accuracy (Bio-Rad Laboratories, 2006; Boulter et al., 2016; Ferre, 1992)

With relative quantification on the other hand, one or more householding or reference genes are used as calibrators or controls, whereas it is essential that they have a gene expression on similar or same levels as the genes of interest (GOIs). This method is convenient when the aim is to explore physiological changes such as gene expression and thus quantify it with specific mathematical applications. With relative quantification the aim is to get a PCR efficiency at about 1 where the efficiency, C_T and sample difference are the key parameters when exploring gene expression of a GOI that is normalized to a reference gene (Boulter et al., 2016; Pfaffl, Horgan, & Dempfle, 2002)

1.4 Purpose

The aim of this study was to screen for viral hemorrhagic septicemia virus in lumpfish from selected areas in Eyjafjörður, Northern Iceland. For that purpose, real-time RT-qPCR VHSV specific detection method was used since current methods used for VHSV detection in Iceland is mainly cell culture based methods and enzyme-linked immunosorbent assay (ELISA).

The following research questions were proposed:

- Is VHSV present in Eyjafjörður. Iceland?
- How efficient and accurate is real-time RT-qPCR to detect all genotypes of VHSV?
- Are current methods used in Iceland to detect VHSV outdated?

2. Materials and Methods

2.1 Sampling

Lumpfish were collected in nets at two different locations (66° 12.52”N, 018° 39.3“W and 66° 24.37”N, 018° 24.37”W) by fishermen in Eyjafjörður, Northern Iceland between 18-25. February, 2016. The fish was kept on ice before it was dissected. To prevent RNA degradation, spleen and kidney samples were collected in eppendorf tubes from each fish and either put straight into -20°C freezer, or freezed in liquid nitrogen and stored on dry ice until it was stored in -20°C freezer.

2.2 RNA extraction

Diethylpyrocarbonate (DEPC) treated water was prepared for RNA extraction by adding 0.1mL of DEPC in 100mL MilliQ water (0,1% v/v) and mixed vigorously, then it was incubated at 37°C for 12 hours, and at last, autoclaved for 1 hour before it was used.

After sample collection, total RNA was extracted with TRizol reagent (ThermoFisher, #15596026) according to manufacturer’s protocol with the following modifications; 4μL of RNA extraction control from VHSV kit (Techne, #TKIT12087M) was added to the extraction buffer before homogenizing the samples to determine if the extraction was successful since the VHSV kit included separate primers and probes to detect the exogenous RNA. RNA integrity was also observed by gel electrophoresis on 1.2% agarose gel. This was done by dissolving 1.2g of agarose in 100mL TBE buffer (Tris/Borate/EDTA buffer) in a microwave oven. 2μL of SYBR® safe, 10.000X DSMO concentrate (Thermo Fisher, #S33102), was added into 75mL of the 1.2% heated liquid gel and poured in a tray until it was solid.

Meanwhile, the samples were prepped by adding 2μL of RNA sample with 8μL of DEPC treated water and 2μL of 6X concentrate gel loading solution type I (New England Biolabs, #B7021S) in a 0.2mL MicroAmp™ reaction tubes (ThermoFisher, #N8011540). The tubes were then incubated in a PCR machine at 65°C for 5min before added into wells. At last, 2μL of 1KB

DNA ladder (New England Biolabs, #N3232L) was put into one well and 10μL of each RNA sample was put in separate wells on the 1.2% agarose gel. The gel was run for 35min at 115V and then analyzed under an UV light with Syngene InGenius LHR gel documentation system (Synoptics, Ltd., Cambridge, England). Pictures were taken with GeneSnap software for further examination.

2.3 Real-time RT-qPCR

One-step quantitative real-time RT-PCR was performed with StepOne™ real-time PCR system (Applied Biosystems, Foster City, USA) in a 0.1mL MicroAmp fast optical 48-well reaction plate (ThermoFisher, #4375816). Viral hemorrhagic septicemia virus kit was used according to manufacturer's protocol (Techne, #TKIT12087M) with TaqMan® chemistry. To determine the absolute quantification a standard curve was included and performed according to manufacturer's protocol. Endogenous and exogenous controls were also included and run at the same time (see appendix I for plate setup).

2.4 Data analysis

RNA integrity was determined by examining the 1.2% agarose gel runs, as well as observation of amplification curves from the exogenous control included in the VHSV kit (Techne, #TKIT12087M). Real-time RT-qPCR Data was collected and analyzed with StepOne™ real-time PCR system (Applied Biosystems, Foster City, USA), where a standard curve method was used to determine the absolute quantity of VHSV and if it was present or absent in the collected samples.

3. Results

3.1 RNA integrity and quality

Figure 4 indicates all of the extracted RNA samples where each band indicates total RNA of that concentration. A 1KB DNA ladder was used for the analysis where the significant top bands at approximately 1.0KB should be 28S rRNA while the significant bands at about 0.5kb should be the 18S rRNA, the third significant band below that should indicate 5S rRNA.

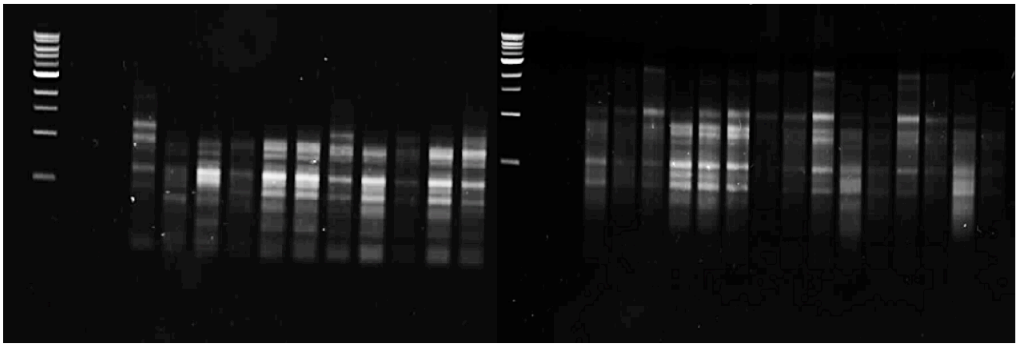

Figure 4. Merged image of RNA samples on 1.2% agarose gels taken with GeneSnap software

Most of the samples did not degrade below 5S rRNA and therefore considered to give reliable results when carried out in a real-time RT-PCR reaction.
Amplification plots from the RNA extraction control from the VHSV kit was not considered reliable to detect the quality of the extraction due to a mistake when mixing the materials provided by manufacturer. Therefore the results obtained from the StepOne™ system were omitted from this study.

3.2 Absolute quantification results

All of the 23 samples were run and analyzed with StepOne™ real-time PCR software where all of the absolute negative samples were omitted to make the figures clearer for analysis. Each sample was run in a single tube, and since none of them turned out to be positive it was decided to make that count as definite results without repetition.

Like the standard curves on figure 5 indicate, all of the samples turned out to be negative, that is, there was no significant amplification. The one point (green) within the standards on both of the curves is the positive control.

On the right standard curve some of the sample seem to reach the threshold after more than 35 cycles, which indicates late amplification, and thus not considered significant results.

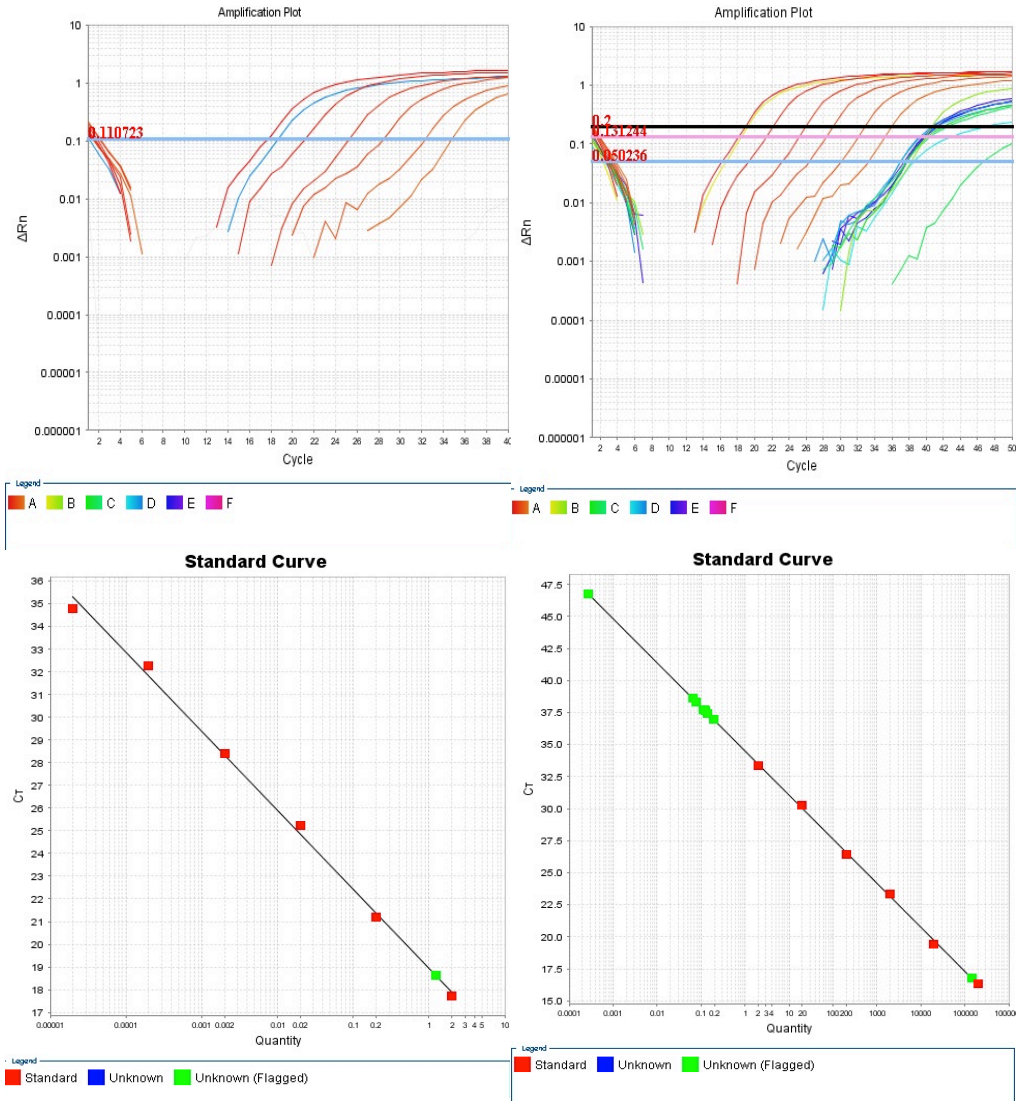

Figure 5. Amplification plots and standard curves of analyzed samples

3.3 Copy number determination

To determine with a fair certainty that the late amplicons (figure 5, right standard curve) were negative, even though they reached the threshold before the last cycle of the reaction, a copy number quantification was performed by using a derivation of the linear regression equation, y = mx + b. Determination of quantity for these samples was done by plotting the logarithm of the initial copy numbers versus the C_T values so:

$$C_T = m(\log \text{quantity}) + b$$

And thus, the quantity of the unknown samples could be calculated with the following equation:

$$\text{Quantity} = 10\left(\frac{C_T - b}{m}\right)$$

The quantity could then be calculated based on the given slope, C_T and y-intercept values, as shown in table 1, that was obtained from the StepOne™ software.

Table 1. Slope, y-intercept and C_T values with R^2 obtained from StepOne™ software for each standard curve

	A	B
Slope	-3.477	-3.443
Y-intercept	36.344	34.502
C_T	18.62	16.76
R^2	0.997	0.999

When copy number had been calculated the samples were compared with the positive controls by a column graph (figure 6).

As figure 6 indicates, none of the samples had a significant number of copies per μL (<0.5) if compared with the positive controls and therefore not considered to have the viral hemorrhagic septicemia virus.

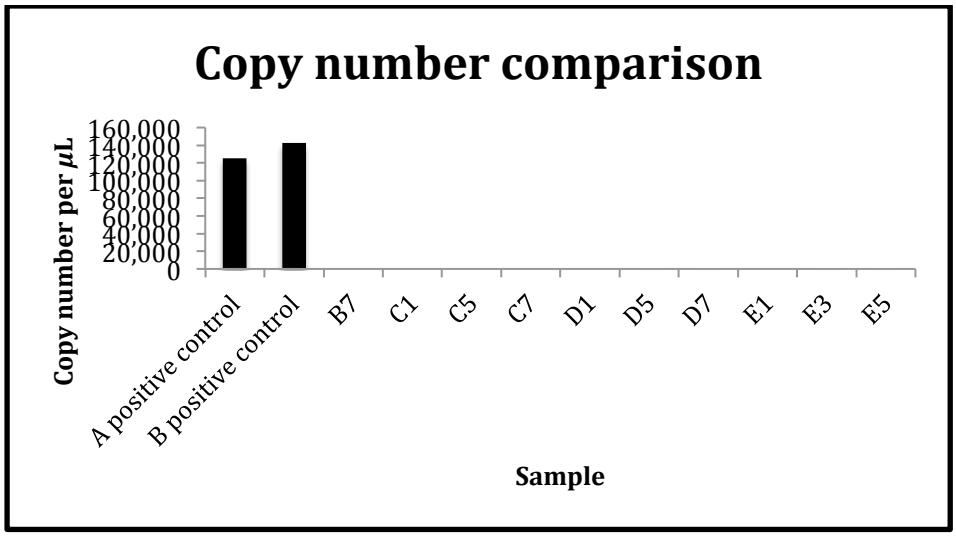

Figure 6. Comparison of copy numbers between positive control and screened samples

3.4 Endogenous control results

Endogenous control reaction was run in a separate tube, at the same time as the VHSV reaction, to determine if the extraction of valid biological template was successful whereas an endogenous specific primer and probe mix was included in the VHSV kit to detect an endogenous gene. All of the samples had significant amplification curves for that matter except five samples, which were omitted from the amplification plot in figure 7.

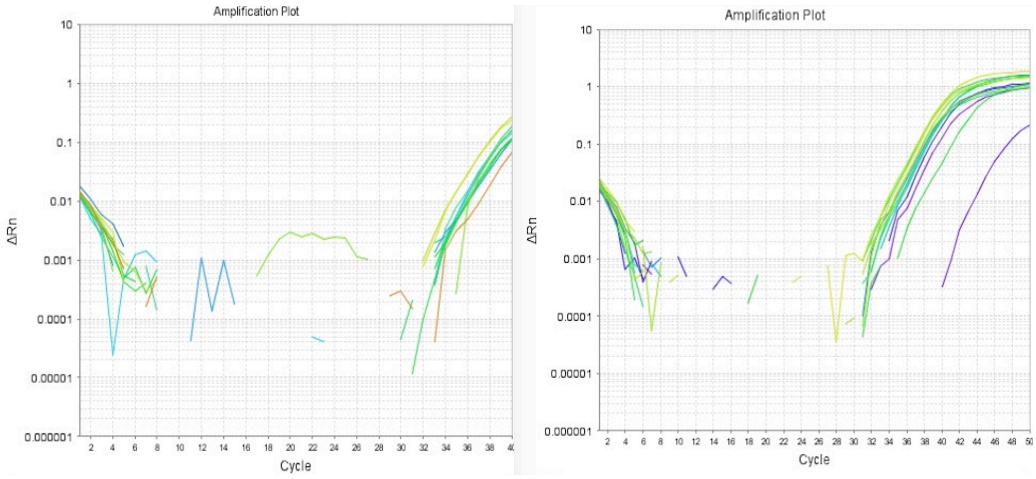

Figure 7. Amplification plots for endogenous control. Left: run A, Right: run B

4. Discussions

With this study, 23 kidneys in total, from 23 separate lumpfishes, were screened for VHSV with real-time RT-qPCR method. This was done to prove, with a sensitive and an accurate method, that the detection of VHSV late last year in Breiðafjörður is not an unique incident, but to prove that the virus has become prevalent in Iceland, and therefore a significant threat to the Icelandic aquaculture- and commercial fishing industry (Matvælastofnun Íslands, 2016a).

At first, RNA integrity and quality was determined with gel electrophoresis to ensure it was possible to use it for further steps. Gel images taken with GeneSnap software indicated that the RNA was not degraded and therefore usable for next steps. However, it would have been preferred to have more significant bands on the gels with better contrast on 28S, 16S and 5S. It would also have been preferred to compare the agarose gels with the RNA exogenous control, which failed, for accurate final conclusion.

All of the screened samples were analyzed with StepOne™ software with a standard curve method, where the copy number was calculated for the absolute quantification of all genotypes of VHSV. Results obtained from these analyses indicated that all of the specimens turned out to be negative, that is, none of the samples were amplified when carried out with real-time PCR.
However, some of the amplicons seemed to have reached late cycle threshold and hence the copy number was calculated for those samples. Copy number calculations however, showed no significant results where all of the amplicons had copy number per μL below 0.5 and therefore not considered to be positive due to the fact that the positive controls had copy numbers above 120.000 per μL.

However, after consideration it was estimated that these results do not indicate in a significant matter that the virus is really not prevalent in Iceland. This is due to the fact that the sample size for this study was extremely small, as well as the fact that the samples were just collected at two different times in Eyjafjörður alone. To get reliable results it would be necessary to collect samples of a greater size at multiple various places, not to mention, at those

areas that the virus has already been detected. Thus, it is considered essential to precisely screen for VHSV all around Iceland in a much greater manner that it has already been done over the last decades.

This study however, turned out to be in contrast with other studies and showed that the real-time RT-qPCR method is extremely good to screen for the virus because of its sensitivity and rapidity (Jonstrup et al., 2013). For a study like this it is good to be able to detect the absolute quantity whereas the standard curve method is also very "user friendly" and easy to apply on data such as obtained for virus studies like this one. The detection kit that was purchased from Techne for this study, turned out to be extremely specific, supposedly able to detect all genotypes of VHSV, which is a great advantage for virus screening where the virus has multiple strains. Current methods used to detect the virus in Iceland consist mainly of cell culture based methods as well as ELISA, based on this study as well as other studies it is recommended to update the detection methods since these methods are more time consuming and less accurate (Árni Kristmundsson, head of fish diseases at Keldur research center, e-mail communications, 12.january; 2016; Jonstrup et al., 2013)

For this study, TaqMan® chemistry was applied because of its specificity, however it would have been nice to apply other real-time RT-PCR chemistries such as SYBR® green chemistry to compare the specificity of the primers and the amplification obtained from both chemistries.

If the virus however, would turn out to be prevalent in Iceland, namely in lumpfish, it could have serious consequences like previously mentioned since recent experiments in aquacultures have focused on using lumpfish as biological control for the salmon louse, a serious occurring fish farming problem all over the world. Since the virus seems to be spreading fast, as OIE report indicates, the use of different fish species as a biological control for the salmon louse could cause the transmission of VHSV into aquaculture trays not to mention since the transmission seems to occur via multiple pathways. This is something that has to be taken into consideration when applying "cleaner fish" to the trays where it is essential to screen for the virus prior to the insertion of them to prevent a synergy between the biological control and virus

transmission, especially in colder environments since the virus optimal temperature is 2-20°C and the salmon louse thrives better in colder environments as previously mentioned (Costello, 2006; Olesen, N.J. & Skall, 2015).

Prevalence of VHSV in Iceland could also be a major threat to the Icelandic aquaculture- and commercial fishing industry since it had never been a problem in Iceland before it was detected in Octobers last year, as reported by Matvælastofnun Íslands (Matvælastofnun Íslands, 2016a). With that said, there is no reason to believe that the virus is not capable of adapting to Icelandic environments and it is quite easy to estimate that it already has or at least is close to it because of its rapid spreading and serious outbreaks all over the world (Olesen, N.J. & Skall, 2015).

5. Conclusions

Twenty-three samples from lumpfish in Eyjafjörður were screened for VHSV, where all specimens turned out to be negative for VHSV. However, it is essential to have samples that reflect the reality for a study like this, that is, a greater sample size collected at multiple different areas around Iceland. There is no reason to believe that the virus is not present in Iceland since it has been detected all over the world whereas it seems to adapt to various environments as reported by OIE (Costello, 2006; Olesen, N.J. & Skall, 2015)

It is also essential to screen for VHSV before applying other fish species into aquaculture trays for biological control since that could increase the risk of VHSV within fish farming, making a major problem a worse. Therefore, the detection and data analysis of the virus should be rather easy but at the same time, sensitive, accurate and rapid, which real-time RT-qPCR is and thus it is a feasible option as VHSV detection method.

The use of other fish species as biological control, in fish farming, is extremely smart, and not to mention supporting the increase in environmental awareness with a lot of interest in "white" biotechnology. With that said it would be seriously harming if this application marked the beginning of a synergy between biological control and transmitting viral diseases.

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7. Appendix I

For both plates, wells labeled with odd numbers had VHSV primer/probes, while wells labeled with even numbers included endogenous primer/probes.

Table 2. Plate setup for run A. S: Standards, NTC: Negative control, + control: positive control

	1	2	3	4	5	6	7	8
A	S1	S2	S3	S4	S5	S6	NTC	NTC
B	2K1	2K1	2K1-1	2K1-1	2K2	2K2	2K3	2K3
C	2K4	2K4	2K5	2K5	2K6	2K6	2K7	2K7
D	2K8	2K8	2K9	2K9	2-Test	2-Test	+ Control	+ Control
E								
F								

Table 3. Table setup for run B. S: Standards, NTC: Negative control, + control: positive control

	1	2	3	4	5	6	7	8
A	S1	S2	S3	S4	S5	S6	NTC	NTC
B	+ Control	+ Control	1K1	1K1	1K2	1K2	1K3	1K3
C	1K4	1K4	1K5	1K5	1K6	1K6	1K7	1K7
D	1K8	1K8	1K9	1K9	1K10	1K10	1K11	1K11
E	1K12	1K12	1K13	1K13	1K14	1K14	1-Test	1-Test
F