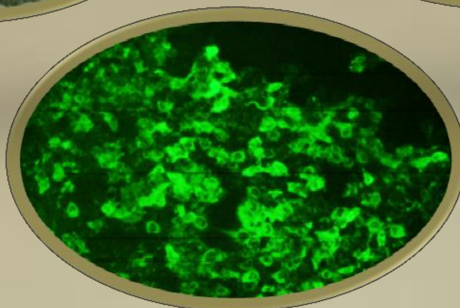
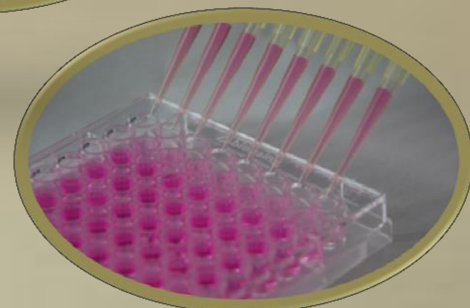
Aquabirnavirus

European Eel and Rainbow trout





CENTRAAL VETERINAIR INSTITUUT

WAGENINGEN UR

Aquabirnavirus

European Eel and Rainbow trout

T.H.P.M. Janssen

3259226

Research project Central Veterinary Institute, WUR

Veterinary Medicine, University Utrecht

14-06-2013

Project Tutors:

CVI of Wageningen UR	Dr. Ir. Marc Engelsma
University of Utrecht	Dr. J.E. Rofina

Table of contents

Introduction 1

Aquabirnaviruses of fish 1

European eel and rainbow trout 1

The clinical- and pathological signs of aquabirnavirus 2

The diagnostic possibilities of aquabirnaviruses 2

Aim of this study 3

Materials and Methods 4

Cell culture 4

Viruses 4

Buffers and cocktails used for the antibody tests 4

Indirect immunoperoxidase monolayer assay and indirect immunofluorescence test 5

Polymerase chain reaction (PCR) 7

IPNV and EVE purification with TRIzol 9

IPNV and EVE purification with sucrose 11

Results 12

The comparison of iIPMA and the iIFT 12

RNA purification 15

Discussion 17

The comparison of IIPMA and the IFT 17

Polymerase chain reaction (PCR) 18

RNA purification 18

Conclusions 20

References 20

Introduction

In general, viruses are a threat for fish culture and may cause a decline in fish stocks. Some of these viruses may be responsible for the decreasing production in the fish farming industry. This report focuses on *aquabirnavirus* occurring in the European eel (*Anguilla anguilla*), the rainbow trout (*Oncorhynchus mykiss*) and the brook trout (*Salvelinus fontinalis*).

<u>Aquabirnaviruses of fish</u>

Viruses of the genus *aquabirnavirus* belong to the family of the *Birnaviridae.* These viruses are non-enveloped viruses and the genome of the *aquabirnavirus* contains bi-segmented, double standed RNA [1-3]. Segment A is the largest segment (3097 base pairs) and encodes for polyprotein VP2-NS-VP3 which is expressed as three separated proteins namely VP2, NS and VP3 [4]. The smaller segment B (2784 base pairs) encodes for the protein VP1, the RNA-polymerase [5]. The protein VP2 is high variable and encodes for the capsid protein. Because of the high variable of the genome from VP2 there has been a lot of research to the genome of the VP2 protein [6]. Infectious pancreatic necrosis virus (IPNV) was the first *aquabirnavirus* isolated in cell culture from fish. In 1940 the disease was observed in cultivated trout but was not yet described as IPNV. It was only in 1950 that the disease was diagnosed as IPNV based on histopathological findings. From that time onwards several IPNV strains and closely related viruses have been found by salmonids with and without clinical signs and by other fish species all over the world [5]. The first a*quabirnavirus* isolated from marine fish is yellowtail ascites virus (YAV) which is an illustration of a pathogenic *marine aquabirnavirus* (MABV) [5]. In 1969 an a*quabirnavirus* was diagnosed for the first time in Japanese eel whereby the mortality increased up to 50%. The pathogen that caused these clinical conditions was serological connected with the IPNV type Ab. The *aquabirnavirus* that was found in eel was named eel virus European (EVE) [6]. IPNV and EVE are both strains of the same *aquabirnavirus* but they occur in different hosts [7]. The *aquabirnavirus* strains can be divided in several genogroups using a phylogenetic tree as shown in figure 1. The phylogenetic tree is based on the protein VP2 and consists of seven genogroups whereby the *marine aquabirnaviruses* are clustered as a separate genogroup [2].

Figure 1: Phylogenetic tree of aquabirnaviruses based on the VP3 protein from aquabirnavirus [2].

<u>European eel and rainbow trout</u>

The European eel has a complex life cycle and is a catadromous species [3]. Since 1970 the population of the wild eel decreased based on scientific evidence and data from the commercial fishery [8-10]. Possible causes that declare the decline are environment pollution, overfishing, ocean changes, diseases and barriers that disturb migration [8, 9]. The three most common pathogenic eel

viruses are the eel virus European (EVE), the eel virus European X (EVEX) and the anguillid herpes virus 1 (AngHV-1) [3]. Prevention of diseases is hard because commercial eel vaccines aren't available and it is difficult to treat a stock of diseased eel [3, 11]. For the commercial production of eel it is still necessary to capture wild glass eels. Grown up eel doesn't spawn well in captivity so it is difficult to breed eel. These wild glass eels may potentially carry pathogens that cause disease during farming. The worldwide production of European eel is estimated to be more than 10.500 tonnes. Most of the European eel is produced in the Netherlands, Italy and Denmark [12]. The trout is just like other salmonids a freshwater fish [4]. The population of salmonids has decreased over the last century which has resulted in the emergence of hatcheries and fish farms to supplement wild populations and to meet the global food stock [4]. IPNV is highly contagious and IPNV was initially reported in fry but later on the disease also appeared in older trout in hatcheries and fish farms [1].

The clinical- and pathological signs of aquabirnavirus

In sensitive eel from older age *aquabirnavirus* may cause mortality up to 100%, especially when these fish are exposed to a stress factor. Glass eel is less sensitive and the mortality may increase to 5% - 7%. The clinical signs are laziness, swimming at the water surface, abnormal shape of the trunk, anaemia, redness of the skin and oedema of the head and operculum as shown in figure 2 [3]. The pathological signs of infected eel are ascites with haemorrhagic transudate, pale and swollen abdominal organs and bleedings in the intestinal wall and abdominal wall. When *aquabirnavirus* was diagnosed for the first time the affected eel had nephritis and inflamed gills. These pathological signs where described as brachio-nephritis [7]. In fry of rainbow- and brook trout *aquabirnavirus* causes clinical signs. The clinical signs are darker color, swimming at the surface, distinctive shimmering movements, exophthalmos and hyperventilation. The mortality depends of the stock intensity and may increase up to 90%. The mortality in fingerlings is less high but can increase to 70%, normally the mortality is 10% - 20%. The pathological signs of infected trout are swollen abdomen, pale liver and petechiën of the viscera as shown in figure 3 and 4 [13].

Figure 2: the European eel which is affected with EVE. CVI©

The diagnostic possibilities of aquabirnaviruses

The diagnostic tests that are used to diagnose *aquabirnavirus* in fish are the real time polymerase chain reaction (PCR), the indirect immunofluorescence test (iIFT) and the indirect immunoperoxidase monolayer assay (iIPMA). The iIFT and iIPMA are both antibody tests, only the way of detection is different. The iIPMA uses a immunological staining whereby antibodies are labelled through a peroxidase-catalysed reaction. The iIFT uses antibodies which are labelled with fluorescent markers. A specific antibody that is directed against the virus binds on the epitope of the virus. A second fluorescent labelled antibody that is directed to the first antibody binds whereby the fluorescent label is attached to the first antibody. Besides the fact that the tests differ in method they differ also in the ultimate intended use. The CVI uses the iIPM as a standard diagnostic test which is labour intensive. The advantage of antibody tests is that the intact virus is detected. A positive test is

indicative for the presence of active virus. In most cases antibody tests have a low sensitivity in comparison with other diagnostic test such as a PCR. The PCR is another diagnostic test to detect *aquabirnavirus* from fish. Currently there is a real time PCR available to detect EVE in eel and IPNV in trout. These real time PCRs are based on targeting the polymerase gene of EVE and IPNV. The real-time PCR is not yet validated and the sensitivity and specificity of the test is not yet known. The currently used real time PCR is based on the SYBR® Green method. SYBR® Green is a dye which binds to double stranded DNA. During the PCR the target sequence is amplified by DNA polymerase. Every cycle more DNA is produced en more SYBR® Green is bound which has an effect on the intensity of the staining. The PCR device measures the intensity of the staining and converts it in a diagram. Several aspects are not clear about the sensitivity and specificity of this real-time PCR. SYBR® Green is a good PCR method to diagnose EVE but is less specific than the Taqman PCR. The Taqman PCR uses a probe which makes the change of not targeted double stranded cDNA less possible.

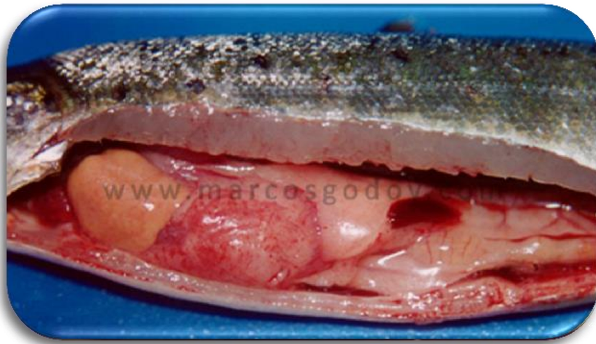


Figure 3: pathological signs of an infected salmon with IPNV. The liver is pale and several petechiën are present in the viscera (14).

The European eel virus is a RNA virus and therefore, it mutates rapidly. From IPNV of salmonid it is known that several mutations from specific proteins influence the virulence of IPNV. By sequencing several EVE virus strains the presence of these mutations of the specific proteins in is investigated. Subsequently through sequencing of several EVE virus strains, it can be investigated whether the viruses are detected by the PCR [14].

Figure 4: pathological signs of an infected salmon with IPNV. Several petechiën are present in the viscera and the liver is pale and haemorrhagic (14).

Aim of this study

The aim of this study is to improve the diagnostic possibilities of the detection of *aquabirnaviruses* from fish. The partial aims of this study are:

- Converting the standard diagnostic test of the CVI from an iIPMA to an iIFT.
- Improving of a real-time Taqman PCR for the detection of EVE and IPNV.
- High throughput sequencing of EVE strains native from outbreaks in the Netherlands since 1990.
    - Identification of the genogroups
    - Checking nucleotide sequences at the primers and probe site from the real-time Taqman PCR.
    - Investigate the sequence of the *aquabirnavirus* from eel whether there are mutations present which are related to the virulence of the virus.

Materials and Methods

Cell culture

Eel Kidney-1 (EK-1) cell lines originate from kidney cells of the eel and are used to culture eel viruses. Rainbow Trout Gonad-2 (RTG-2) cell lines originate from gonad cells of the rainbow trout and are one of the cell lines to culture trout viruses [15]. These cell lines are cultivated (Figure 5) and passaged in plates and flasks with sterile cell growth medium. During passage, the cells are being trypsinized, split, and resuspended to inoculate new flasks and plates, in new medium. To store the cell lines, they are kept in liquid nitrogen where they can be kept for a long time, and reused. The used cell lines are regularly checked for growth habits and the absence of infection with Mycoplasmas. The cell grow medium was freshly prepared for each passage, in an flow cabinet. The medium for EK-1 prepared for an F75 flask (25 ml) consists of 25 ml Leibovitz's L-15 medium (L-15) with 5 % (v/v) fetal bovine serum, 1% (v/v) glutamine, 1 % (v/v) sodium carbonate, 0,1% (v/v) gentamycin and 0,08% (v/v) beta mercapto-ethanol. The medium for IPNV prepared for an F75 flask (25 ml) consists of 25 ml Eagle's Minimal Essential Media (EMEM) with 10 % (v/v) fetal bovine serum, 1% (v/v) antibiotic cocktail 1, 1% (v/v) glutamine and 1 % (v/v) sodium carbonate[15].

Figure 5: An incubator for cell culture and virus culture. CVI©

Viruses

To perform the antibody tests, PCR and RNA purification several *aquabirnavirus strains* were used as shown in table 1. The reference strains originated from various institutes such as the American Type Culture Collection (ATCC), the Technical University of Denmark (DTU) or the National Reference Laboratories (NRL) for fish diseases. The field strains were samples selected from the virus collection of the CVI which consists of samples from outbreaks of *aquabirnaviruses* in fish in the Netherlands since 1989. The ring test samples were created by European Union Reference Laboratory (EURL) for fish Diseases from Denmark and distributed among the different laboratories. The negative control consisted of virus growth medium without virus which was applied to the cell. The virus grow medium was freshly prepared for each passage in an flow cabinet. The medium for EK-1 cells prepared for 96 well plates consisted of Leibovitz's L-15 (L-15) medium with 2 % (v/v) fetal bovine serum, 1% (v/v) glutamine, 3,5 % (v/v) sodium carbonat and 1% (v/v) antibiotic cocktail 2. The virus grow medium for RTG-2 cells was the same only Eagle's Minimal Essential Media (EMEM) was used instead of L-15 medium [15].

Buffers and cocktails used for the antibody tests

The buffers and cocktails for the antibody tests were made in advance and were used to perform all tests that are included in this research. The antibiotic cocktail 1 consisted of 205.5 ml sterile Milli Q with $2.25*10^6$ IE Penicillin and 1000 mg Kanamycin. The antibiotic cocktail 2 consisted of 116.5 ml sterile Milli Q with $6.75*10^6$ IE Penicillin and 3000 mg Kanamycin. Both antibiotic cocktails were mixed at 20 degrees Celsius for 15 minutes and stored at -20°C . The Hyper Immuun Serum (HIS) buffer consisted of 200 ml super Q with 7.3 g NaCl, 0.25g NaN_3 and 25 ml 10% Tween 80. The pH was adjusted to pH 7.6 and the buffer was supplemented up to 250 ml. The conjugate buffer consisted of

225 ml Super Q with 7.3 g NaCl and 25 ml 10% Tween 80. The pH was adjusted to pH 7.6 with 1 M NaOH and the buffer was supplemented up to 250 ml. The substrate buffer consisted of 250 ml Super Q with 1.025 g sodium acetate. The pH was adjusted to pH 5.0 with 50 mM acetic acid. The 3-amino-9-ethylcarbazale (AEC) stock solution consisted of 20 ml dimethylsulfoxide (DMSO) with 80 mg AEC. Wash buffer A consisted of 500 ml Super Q with 4.4g NaCl which was mixed properly. Wash buffer B consisted of 475 ml super Q with 4.4 g NaCl and 25 ml 10 % Tween 80. The iIFT uses PBS as a buffer which is commercial available and doesn't have to be made in advance.

The viruses which are used in the research

Virus	no.	Strain	I/F	PCR	TRI	Virus	no.	Strain	TRI	S
IPNV Ab (reference strain)	V1	IPNV Ab	X	x		DSU 130899	V 13	EVE	x	
IPNV Sp (reference strain)	V 2	IPNV Sp	X	x		DSU 154354	V 14	EVE	x	
IPNV VR299 (reference strain)	V 3	IPNV Vr299	X	x		DSU 228309	V 15	EVE	x	
DSU 1200734	V 4	EVE	X		x	DSU 347025	V 16	EVE	x	
DSU 11010989	V 5	EVE	X		x	DSU 421218	V 17	EVE	x	
DSU 1104270	V 6	EVE	X			DSU 480386	V 18	EVE	x	
DSU 11006739	V 7	IPNV	X			DSU 498487	V 19	EVE	x	
DSU 11006033	V 8	IPNV	X			DSU 520533	V 20	EVE	x	x
DSU 11006951	V 9	IPNV	X			DSU 569599	V 21	EVE	x	
Ring test 2012 amp.1	V 10	IPNV	X			DSU 617670	V 22	EVE	x	
Ring test 2011 amp.5	V 11	IPNV	X			DSU 4019560	V 23	EVE, HVA	x	
DSU 115742	V 12	EVE			x	DSU 5011486	V 24	EVE	x	x

Table 1: The virus strains which are used to perform the iIPMA and iIFT test (I/F), the PCR test (PCR), the RNA purification with TRIzol (TRI) and the RNA purification with sucrose (S). EVE strains are *aquabirnaviruses* from the eel shown in green and IPNV are *aquabirnaviruses* from the trout shown in blue. All virus strains are numbered to simplified the discussion about these viruses.

Indirect immunoperoxidase monolayer assay (iIPMA) and indirect immunofluorescence test (iIFT)

The protocol for performing the iIFT and the iIPMA takes four days. The protocol for the first three days is almost equal for both assays, only the cell line and the cell growth medium and virus growth medium were different. At day one the confluence of the cell lines were checked under the microscope and the required media were placed at 26°C. The cell lines were passaged 1:2 and the sterile cell growth medium was prepared as described above[15]. The cell growth medium of the old F75 flask was discharged with a sterile vacuum device. Add 7 ml PBS to the old flask, rinse the monolayer and discharge the PBS from the monolayer. Add 7 ml Trypsine Versene (pH 7.2, 20°C) to the old flask and spread it over the monolayer by putting the flask in an angle. Discharge the Trypsine Versene from the monolayer and leave 1 ml behind. Incubate the monolayer at room temperature and beat the flask carefully to loosen the cells. Resuspend the cells in 7 ml PBS by aspirating them into a pipette a few times up and down and add the cell suspension in 50 ml cell growth medium. Pipette 100 µl cell suspension per well in the 96 well plates and incubate at 15°C for 24 hours as shown in figure 6. At day 2 the newly created monolayer was viewed under the microscope and the virus growth medium was prepared as described above. From the reference strains and the field strains 50 µl was added to a ~ 80% confluent EK-1 or RTG-2 monolayer [15]. The plate was incubated at 15°C for 1 hour and after incubation 100 µl of fresh viral grow medium was added to each well [15]. The well was incubated at 15°C for 48 hours and from this point on the antibody testing protocols were different.

The indirect immunofluorescence test (iIFT)

After incubation the medium was decanted and the 96 well plate was rinsed with 100 µl PBS. The plates are placed at 20°C for 2 hours and then frozen for 1 hour -20°C. Subsequently 100 µl 10% formalin in PBS (pH7.2) was added to each well and incubated at 20°C for 10 minutes. The formalin was decanted from the plates and the plates were rinsed once with PBS. Fifty microliters of rabbit-anti-IPNV antiserum in a dilution range of 1:100 in PBS was added to each well and incubated at 37°C for 1 hour. Next the plates were rinsed 3 times with 100 µl PBS. Fifty microliters of fluorescein-isothiocyanate (FITC) conjugated swine-anti-rabbit polyclonal antibody diluted 1:100 was added to each well and were incubated at 37°C for 30 minutes. Eventually the plates were rinsed 3 times with 100 µl PBS and are examined under the 495 nm fluorescence microscope. Cells positive for *aquabirnavirus* are identified by the cytoplasm which shows a bright green granular fluorescence surrounding a black nucleus. An approved positive test shows a decreasing number of fluorescent cells with a decreasing anti-serum dilution. When no green fluorescent cells were observed the results were diagnosed negative. Because the results of the iIFT was sometimes difficult to interpretate, the tests were read by two persons [15].

The indirect immunoperoxidase monolayer assay (iIPMA)

After incubation with virus, the medium was decanted and the 96 well was rinsed with 100 µl wash buffer A. The plates were placed at 20°C for 2 hours and then frozen for 1 hour at -20°C. Subsequently 100 µl 10% formalin in PBS (pH7.2) was added to each well and incubated at 20°C for 10 minutes. The formalin was decanted from the plates and the plates were rinsed once with 100 µl wash buffer A. Fifty microliters of a dilution range 1:100 of rabbit-anti-IPNV antiserum was added to each well and incubated at 37 °C for 1 hour. Next the plates were rinsed three times with 100 µl wash buffer B. Fifty microliters of Horse Radisch Peroxidase (HRPO) conjugated swine-anti-rabbit polyclonal antibody diluted 1:100 was added to each well and was incubated at 37°C for 30 minutes. Eventually the plates were rinsed three times with 100 µl wash buffer B. Fifty microliters of substrate was added, consisting of 1 ml 3-amino-9-ethylcarbazole (AEC), 20 ml substrate buffer and 100µl 3%H_2O. Incubate the plates 45 minutes at 20°C. The results were examined under the microscope. When the conjugate was bound to the cells it will interact with H_2O_2 which will convert colourless chromogenic into red precipitate. Cells which were infected with IPNV had red coloured cytoplasm and colourless nucleus. Cells which were not infected with IPNV had both an uncoloured nucleus and uncoloured cytoplasm. Because the results of the iIPMA is sometimes difficult to interpretate, the tests were read by two persons [15].

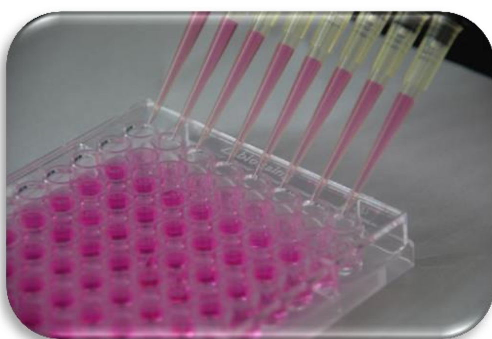


Figure 6: 96 well plate, adding VGM. CVI©

Polymerase chain reaction (PCR)

RNA extraction

RNA extraction was performed with the kit and the protocol from Qiagen. The RNA was extracted from 10% organ suspensions or from cultivated *aquabirnavirus* which was stored at -80 degrees. Fifty microliters of sample was added to 350 µl RLT buffer which contains 10 µl β-Mercaptoethanol (β-ME) per millilitre RLT buffer which was mixed properly. With every four samples a negative control sample was included. Four hundred microliter 70% ethanol was added, the solution was homogenized by pipetting and was poured into a Rneasy column. The Rneasy column was centrifuged for 15 seconds at 14.000 rpm and the flow-through was discarded. Add 700 µl RW 1 buffer to the Rneasy column, centrifuge for 15 seconds at 14.000 rpm and use a new collection tube. Repeat this step with 500 µl RW 1 buffer and discard the flow-through. Five hundred microliter RPE buffer was added to the sample and centrifuged for 2 minutes at 14.000 rpm. The washed and extracted RNA was eluted in 50 µl RNase/DNase free water with 1 µl Ribonuclease inhibitor. The RNA was stored at -20°C [15, 16].

Primer and probe design

The target sequence for the SYBR® Green real-time PCR was based on VP 1 from the IPNV genome. Based on this sequence the forward primer EVE.VP1.F06 (5'–CAAGCTTAAGGAAACGGTCAATG–3') and the reverse primer EVE.VP1.R06 (5'–GGTCGGGTTTGTGGAATGG–3') were designed (unpublished data CVI®). The primers are obtained from Eurogentec. The primers for the Taqman real-time PCR were based on the VP3 region of the polyprotein gene. Based on this sequence the forward primer VP3F: (5'–CGACCGACATGAACAAAATCA–3'), reverse primer 1 VP3R1: (5'–TGT GCGAATACAGCTGCAACT–3') and the VP3 probe: (6-FAM-5'TCTAGCCAACAGTGTGTACGGCCTCCC-3'-TAMRA) were used from a publication [1]. The reverse primer VP3R2: (5'–TCTGCGAACACCTCGACGACT–3') was designed. During the performance of the Taqman real-time PCR three mixes with a different reverse primer were used. Reverse primer 1 (VP3R1), reverse primer 2 (VP3R2) and a mix of reverse primer 1 and 2 (VP3RM) were used. The primers were obtained from Eurogentec and generate an amplicon of 109-base pair (bp) fragment [1, 17]. The forward primer, reverse primer 1 and the probe where designed to detect IPNV strain Sp which is the most common strain in Norway [1]. When the sequences from the specific probe and primers where blasted in GenBank there was a high specificity for IPNV strain Sp. Reverse primer 1 which is described in the article from Orpetveit et al. was slightly changed to detect more genotypes. IPNV Sp, IPNV Ab and IPNV VR 299 are strains which represents different strains of the most important genogroups of *aquabirnavirus* (Table 1). When the primers and probes were balsted in GenBank with these three strains the specificity for the other *aquabirnaviruses* can be increased [1].

Reverse transcription

The Taqman Reverse Transcriptase Reagent kit (Applied Biosystems) was used to carry out the reverse transcription (RT). Fifty microliters RT-mix was used which contains 5 µl RT-buffer, 11 µl $MgCl_2$ (25mM), 10 µl dNTP's (2.5mM), 2.5 µl Random Hexamers (50 µM), 2 µl RNase Inhibitor (20U/µl), 1.25 µl Multiscribe Reverse Transcriptase (50U/µl), 13.25 µl DNase-RNase free water (Sigma-Aldrich) and 5 µl RNA template. Both the preparation of the RT-mix as the whole procedure was performed on ice. Five microliters RNA was mixed with 45 µl RT-PCR-mix was poured into a 96

well PCR plate. Both the RNA samples and a negative control which consists of RT-mix with DNase-RNase free water was included. The whole procedure was performed on ice. The 96 well plate wass placed in the GeneAmp9700 PCR thermocycler. The thermal procedure of the RT program consisted of 10 minutes incubation at 25 °C, subsequently 30 minutes incubation at 49°C and thereafter 5 minutes incubation at 95°C. Next the plate was cooled down until 4°C. The complementary DNA (cDNA) which was stored at 20°C [15].

SYBR® Green real-time PCR

Fifteen microliters SYBER Green-mix was used which contains 10 µl SYBR® Green Mastermix, 0.8 µl Primer For (10µM), 0.8 µl Primer Rev (10µM), 0.25 µl Uracil DNA Glycosylase, 3.15 µl DNase-RNase free water (Sigma-Aldrich). Five microliters cDNA was mixed with 15 µl SYBER Green-mix was poured into a Fast Optical 96 wells plate. Both the cDNA samples and a negative control which consisted of SYBER® Green-mix with DNase-RNase free water was included. The Fast Optical 96 wells plate was sealed with an optical adhesive film, centrifuged for 1 minute at 1000 rpm and placed in the 7500 Fast Real-Time PCR system (Applied Biosystems). The thermal procedure of the SYBR® Green real-time program consisted of 10 minutes incubation at 37°C and subsequently 10 minutes incubation at 95°C. Then 40 cycles of 15 seconds incubation at 95°C and 1 minute incubation at 60°C were running. Next the melting curve was generated [15, 17]. To determine the specificity of the amplification the melting curve and amplification plot was examined after the run was completed.

Taqman real-time PCR

Fifteen microliters Taqman-mix (Table 2) was used which contains 10 µl TaqmanFast Mastermix, 0.8 µl Primer Forward (10µM), 0.8 µl Primer Reverse (10µM), 1.6 µl Probe (1 µM), 0.25 µl Uracil DNA Glycosylase and 1.55 µl DNase-RNase free water (Sigma-Aldrich). Five microliters cDNA was mixed with 15 µl Taqman-mix was poured into a Fast Optical 96 wells plate (Applied Biosystems). Both the RNA samples and a negative control which consisted of Taqman-mix with DNase-RNase free water was included. The Fast Optical 96 wells plate was sealed with an optical adhesive film, centrifuged for 1 minute at 1000 rpm and placed in the 7500 Fast Real-Time PCR system (Applied Biosystems). The thermal procedure (Table 3) of the Taqman real-time program consisted of 10 minutes incubation at 37°C and subsequently 10 minutes incubation at 95°C. Then 40 cycles of 3 seconds incubation at 95°C and 30 seconds incubation at 60°C are running [15, 17]. To determine the specificity of the amplification the fluorescence plot was examined after the run was completed.

Component	p.r (µl)	Standard concentrations
TaqmanFast Universal PCR mix (2x)	10	
Primer IPNV-VP3-For (10µM)	0.8	***[0.4 µM]***
Primer IPNV-VP3-Rev (10µM)	0.8	***[0.4 µM]***
Probe IPNV-VP3-probe (1µM)	1.6	***[0.08 µM]***
Uracil-DNA Glycolase (UDG 5U/µl) NEB	0.25	***[0.5Units]***
Dnase-Rnase free water (Sigma-Aldrich)	1.55	

Table 2: Optimalisation and performance of the primers and probe. The results of the optimal concentration are shown [1].

Duration	Temperature	Cycli
10 min	37°C	-
10 min	95°C	-
3 sec	95°C	40
30 sec	60°C	
10 min	20°C	-

Table 3: Optimalisation and performance of the primers and probe. The results of the optimal annealing temperature of the IPNV Taqman real-time PCR.

IPNV and EVE purification with TRIzol

Virus Culture

For the virus culture two monolayers of EK-1 cells were cultivated in a sterile F-75 (25ml) plastic culture flask. The confluence of the cell lines were checked under the microscope, the required media were placed at 20°C and the virus growth medium was prepared. Twenty-five millilitres virus growth medium was applied to each EK-1 monolayer together with 1000 µl of virus [15]. One plate was incubated at 15 °C, for 24 hours and one plate for 48 hours. No cytopathic effect (CPE) should be evident when performing the purification. The purification was performed after 24 hours and 48 hours. For the PCR with the samples from the RNA purification with TRIzol AngHV-1, EVEX and host primers are used [3, 15].

Buffers

The buffers used during the RNA purification with TRIzol are the proteinase digestion buffer and the RNA extraction buffer. The proteinase digestion buffer consists of 2000 µl 0.2 M Tris-CL (pH 8.0), 500 µl 25mM EDTA (pH 8.0), 3000 µl 0.3 M NaCl, 2000 µl 2% SDS (sodium dodecyl sulphate) and 2500 µl Distilled Water DNase/RNase Free. The RNA extraction buffer consists of 820 µl Distilled Water DNase/RNase Free, 280 µl 0.14 M NaCl, 200µl 0.0015 M $MgCl_2$, 200 µl 10mM Tris Cl (pH 8.6), 100 µl 0.5% Nonidet P-40 (NP-40), 200 µl 1mM dithiothreitol, 200 µl 20mM vanadyl ribonucleoside complexes.

Purifying RNA

Discharge the virus growth medium and wash each monolayer twice with 7 ml ice-cold phosphate-buffered saline (PBS). Remove the cells from the plate in 7 ml ice-gold PBS, transfer the cells into an 50 ml microfuge tube and centrifuged at 2000 rpm at 4°C for 10 minutes. Discharge the supernatant, resuspend the cell pellet in 1000 µl ice-cold PBS which was transferred in an 1.5 ml microfuge tube and centrifuge at 11.372 rpm for 90 seconds at 4°C in an eppendorf 5417R centrifuge. Discard the supernatant, resuspend the cell pellet in 200 µl RNA extraction buffer, vortex the suspension for 15 seconds and incubate 5 minutes on ice. Centrifuge at 11.372 rpm for 90 seconds at 4°C in an eppendorf 5417R centrifuge and transfer the supernatant which contained the RNA to a fresh microfuge tube. Add 200µl of proteinase digestion buffer, mix by vortexing and add 1 µl proteinase K (final concentration 50µg/ml). Mix the solution well and incubate for 30 minutes at 37°C.

Figure 7: ultracentrifuge CVI©

TRIzol reagent and TRIzol LS reagent

Add 0.75 ml of TRIzol LS reagent per 0.25 ml of supernatant sample (5-10*10^6cells). Lyse the cells by pipetting up and down several times and incubate the homogenized sample for 5 minutes at 20°C. Add 0.2 ml of chloroform per 1 ml TRIzol reagent, shake the tube vigorously by hand for 15 seconds, incubate for 2 minutes at 20°C and centrifuge at 11.372 rpm for 15 minutes at 4°C in an eppendorf

5417R centrifuge. Remove the aqueous phase of the sample by angeling the tube at 45°, place the aqueous phase into a new microfuge tube and proceed to the RNA Isolation Procedure.

RNA Isolation Procedure

The RNA isolation procedure was performed with the Zymo-Spin IIC protocol. Add 1 volume ethanol (95-100%) directly to the aqueous phase and mix by vortexing. Transfer the mixture into a Zymo-spin IIC column in a collection tube and centrifuge at 11.372 rpm for 1 minute. Transfer the column into a new collection tube, add 400µl RNA wash buffer to the ZymoSpin IIC column and centrifuge for 11.372 rpm for 1 minute. Work on ice and prepare the DNase I cocktail which consists of 5µl DNase I, 8µl DNase I Reaction Buffer, 3µl DNase/RNase-Free Water and 64µl RNA Wash Buffer for each sample. Add the DNase I cocktail directly to the matrix of the Zymo-Spin IICColumn and incubate at 37°C for 15 minutes, then centrifuge at 11.372 rpm for 30 seconds. Add 400µl Direct Zol RNA PreWash, centrifuge at 11.372 for 1 minute, discard the flow-through and repeat this step. Add 700 µl RNA Wash Buffer, centrifuge at 11.372 for 1 minute, discard the flow-through and transfer the column into an RNase-free tube. Add 25 µl DNase/RNase-Free Water to the column, centrifuge at 11.372 for 1 minute and store the eluted RNA at -80°C.

E-Gel 2% agarose

Prepare 20 µl sample, add 10 µl eluted RNA to 10 µl DNase/RNase-Free Water and prepare 20 µl 500 base pair DNA ladder through add 3 µl DNA ladder to 17 µl DNase/RNase-Free Water. Attach the power of the E-Gel Initrogen iBase, secure the cassette into the E-Gel iBase and PRE RUN the gel. Take out the comb and load the samples, the DNA ladder and 20 µl DNase/RNase-Free Water in an empty well. Run the gel three times for 15 minutes and read the results between the runs.

<u>IPNV and EVE purification with sucrose</u>

Virus Culture

Virus culture as described under IPNV/EVE purification with TRIzol. However the plates were incubated at 15 °C, for 24 hours to 72 hours. More than 80% cytopathic effect (CPE) should be evident when performing the purification.

Buffers

Several buffers are used when performing the RNA purification with sucrose. The Tris-HCL buffer consists of 30.3 g Tris in 250 ml Super Q, pH 7.5. The EDTA buffer consists of 46.5 g disodium ethylene diaminetetracetic acid with 250 ml Super Q, pH 8.0. NaCl buffer consists of 73.1 g NaCl with 250 ml Super Q. The TNE buffer consists of 50 ml 1M TRIS.HCl, 30 ml 5M NaCl and 2 ml 0.5 M EDTA with Super Q to a final volume of 1000ml, pH 7.5. The sucrose solution was prepared from 10-60%, the sucrose solution of 60% is prepared as follow: six parts of sucrose is mixed with four parts of TNE. The solutions are autoclaved.

Purifying RNA

Collect the culture medium in 50 ml flacon tubes and centrifuge 22.000 rpm for 90 minutes at 10°C. Discard the supernatant, suspend virus pellet in 1 ml TNE buffer and mix by vortexing. Prepare a 60%-10% (v/v) sucrose gradient in TNE buffer in a 10 ml ultracentrifuge tube, using steps of 5% with a blunt 1 ml pipet as shown in figure 8. Incubate for 3 hours at 20°C and layer the virus suspension onto the sucrose gradient. Centrifuge with rotor SW41Ti at 22.000rpm for 18 hours at 10°C. Discard the sucrose solution above the virus band and collect the virus band. Dilute the virus band in sucrose with 1:10 TNE buffer in a new 10 ml ultracentrifuge tube and centrifuge with rotor SW41Ti at 30.000 rpm for 3 hours at 10°C. Discard the supernatant en suspend the virus pellet in 100 µl TNE buffer. Store at -80°C until further use.

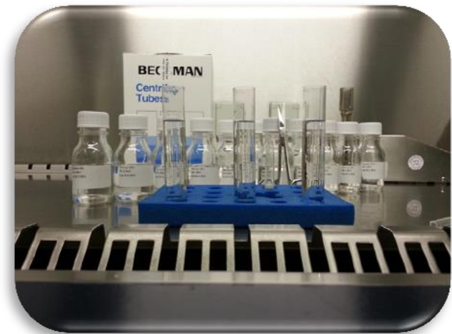


Figure 8: preparation of the RNA purification with sucrose

E-Gel 2% agarose

Is performed as described above.

Results

<u>The comparison of iIPMA and the iIFT</u>

The iIFT and the iIPMA were performed side by side and compared with each other and with the SYBR® Green PCR. The samples from the reference strains (IPNV Ab, IPNV Sp and IPNV VR299) were positive in both assays. The three trout field strains (V7 t/m V9) and the three eel field strains (V4 t/m V6) were positive in the PCR but not all the samples were positive in the antibody assays. Both the iIFT and the iIPMA showed that V6 was positive and that V4, V5, V8 and V9 were negative as showed in figure 9. In the iIPMA V7 was positive but the result of the iIFT was doubtful. The iIFT tested both ring test samples (V10,V11) as positive. When performing the iIPMA the ring test sample 2012, ampul 1 (V10) was positive and the ring test sample 2011, ampul 5 (V11) was negative.

One of the six field strains was positive and four of the six field strains were negative in both tests. One of the two ring test strains was positive in both tests. The results of the iIFT and the iIPMA differed in one field strain sample and one ring test strain as shown in figure 9. The PCR had shown that all the samples where positive while the antibody assays tested 5 out of 11 samples positive.

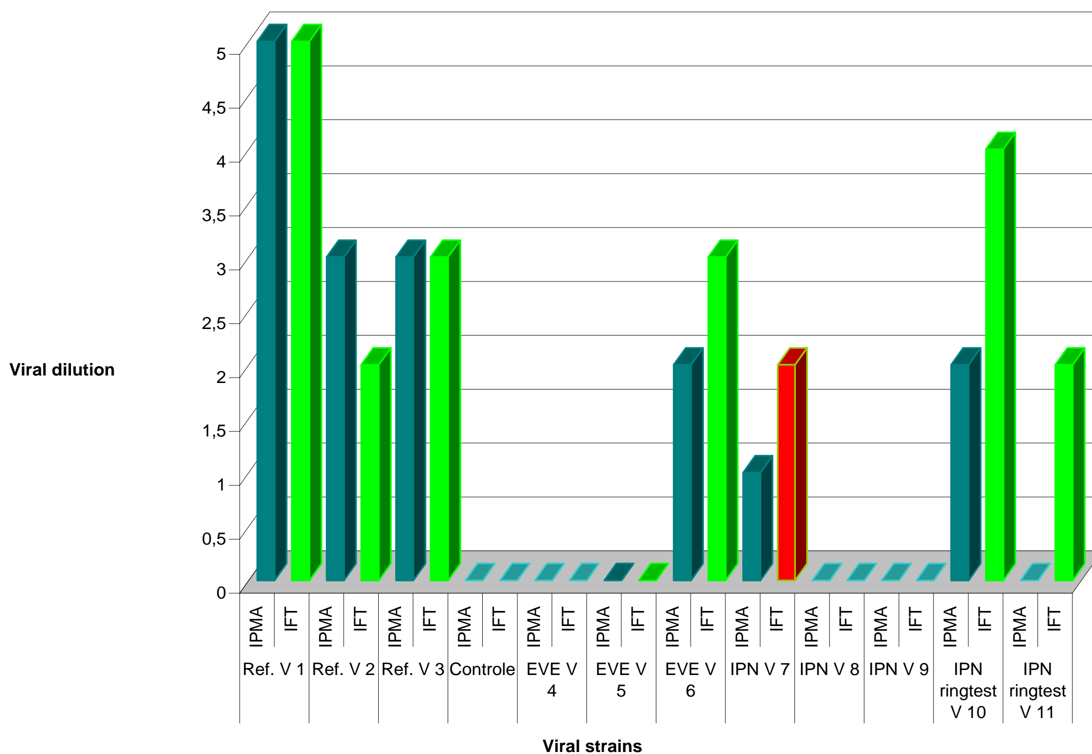


Figure 9: The results between the iIFT and the iIPMA. On the Y-axis the viral dilution is presented, whereby number 5 represents a viral dilution of 10^{-4}. The red bar is a doubtful iIFT result but was decided as being negative.

Polymerase chain reaction (PCR)

Comparison of the Taqman PCR and the SYBR® Green PCR

The SYBR® Green PCR was tested in triplo with the EVE primers and the Taqman PCR was tested in triplo with the several IPNV primers to get an indication of the performance of the PCR. The Taqman PCR was tested with three different reverse primers namely with reverse primer 1 (VP3R1), reverse primer 2 (VP3R2) and a mix of both reverse primers VP3RM. The Taqman PCR with reverse primer VP3RM had the lowest mean Ct value, the mean Ct value of the Taqman PCR with reverse primer VP3R1 was slightly higher. The mean Ct value of the SYBR® Green PCR was lower than the Taqman PCR VP3R2 but higher than the other two Taqman PCRs ad described above. The results of the SYBR® Green PCR, VP 1 and Taqman PCR, VP 3 are shown in figure 10. The Taqman PCR VP3R1 was extracted from an article which focuses on IPNV strain Sp [1]. That's explains why this PCR was very specific for IPNV Sp and less sensitive for the other strains [1]. Because the aim of this study was based on aquabirnaviruses in general this specific PCR is not further used. Both the SYBR® Green PCR and the Taqman PCRs with VP3RM were used to determine the sensitivity with a 10 fold cDNA dilution which was tested in triplo.

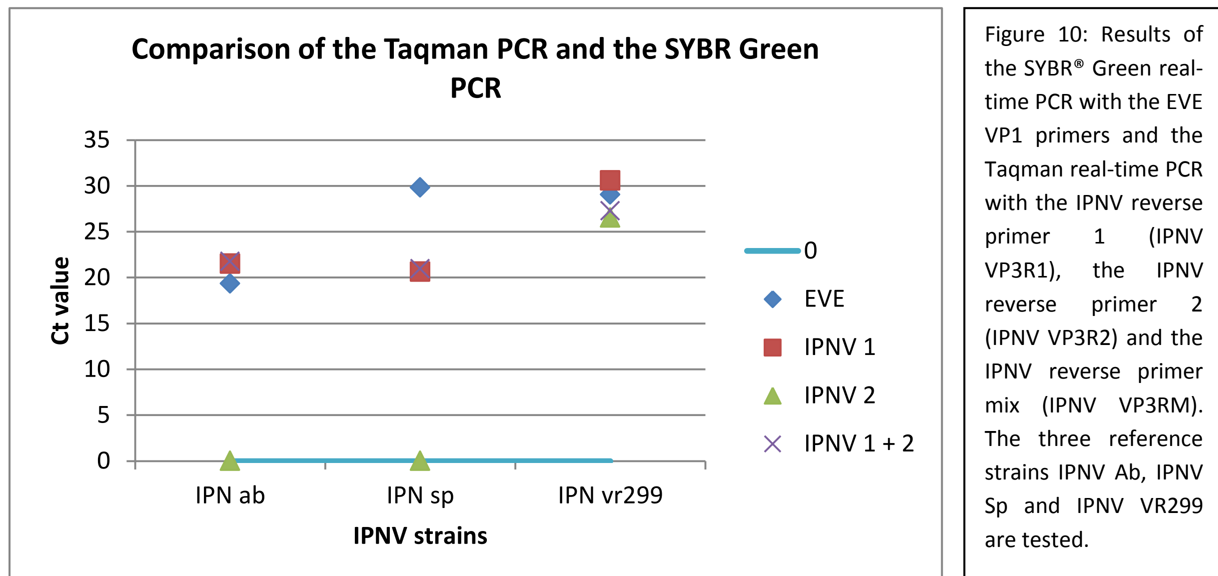


Figure 10: Results of the SYBR® Green real-time PCR with the EVE VP1 primers and the Taqman real-time PCR with the IPNV reverse primer 1 (IPNV VP3R1), the IPNV reverse primer 2 (IPNV VP3R2) and the IPNV reverse primer mix (IPNV VP3RM). The three reference strains IPNV Ab, IPNV Sp and IPNV VR299 are tested.

Sensitivity

The sensitivity of the PCR was tested by determining the lowest detection limit of the Taqman real-time PCR, VP 3 and the SYBR® Green real-time PCR, VP 1. The SYBR® Green PCR works with EVE VP1 F06 and EVE.VP1.R06 primers and had the lowest Ct values in the 10-fold dilution of the strain IPNV Ab. IPNV Ab was detected by the SYBR® Green PCR until the dilution of 10^{-3} as shown in figure 11. The Taqman real-time PCR works with the VP3 probe and VP3F and VP3RM primers detected the strain IPNV Ab until the dilution of 10^{-2} and had a higher Ct value in comparison with the SYBR® Green PCR, VP 1. The Ct value of the 10-fold dilution of the strain IPNV Sp and IPNV VR299 where higher in the SYBR® Green PCR than in the VP3RM Taqman PCR. The VP3RM Taqman PCR had the lowest Ct values and the highest detection limit in the 10-fold dilution of strain IPNV Sp and IPNV VR299 as shown in figure 12-13. The detection limit of the SYBR® Green PCR was lower and the Ct values were higher in the 10-fold dilution of strain IPNV SP and IPNV VR299. The Ct value of IPNV Ab and IPNV Sp were low in comparison with IPNV VR299 in both the Taqman PCR VP3RM and the SYBR® Green PCR VP1.

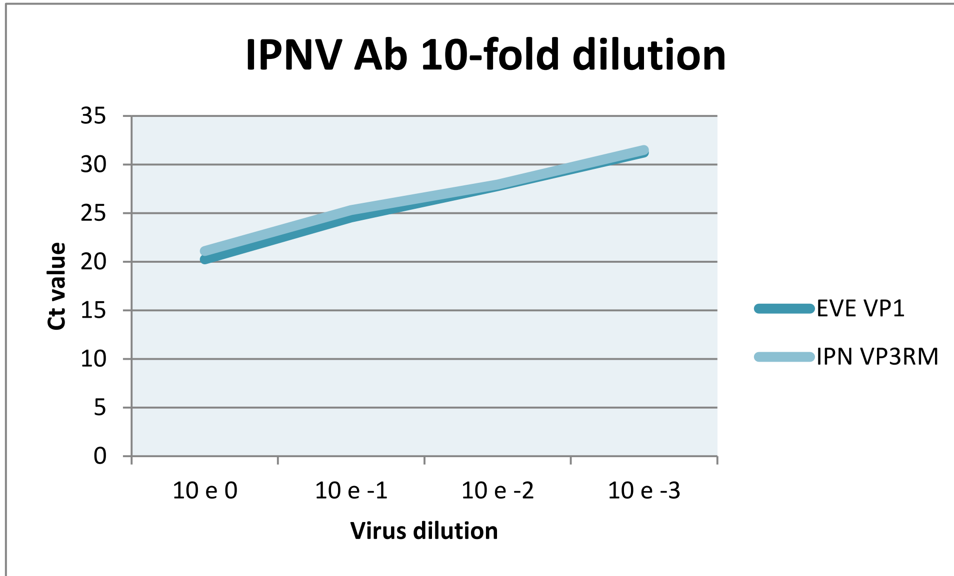


Figure 11: Ten-fold dilution of IPNV Ab tested with the SYBR® Green real-time PCR (EVE VP1 primers) and the Taqman real-time PCR (IPNV reverse primer mix (IPNV VP3RM)).

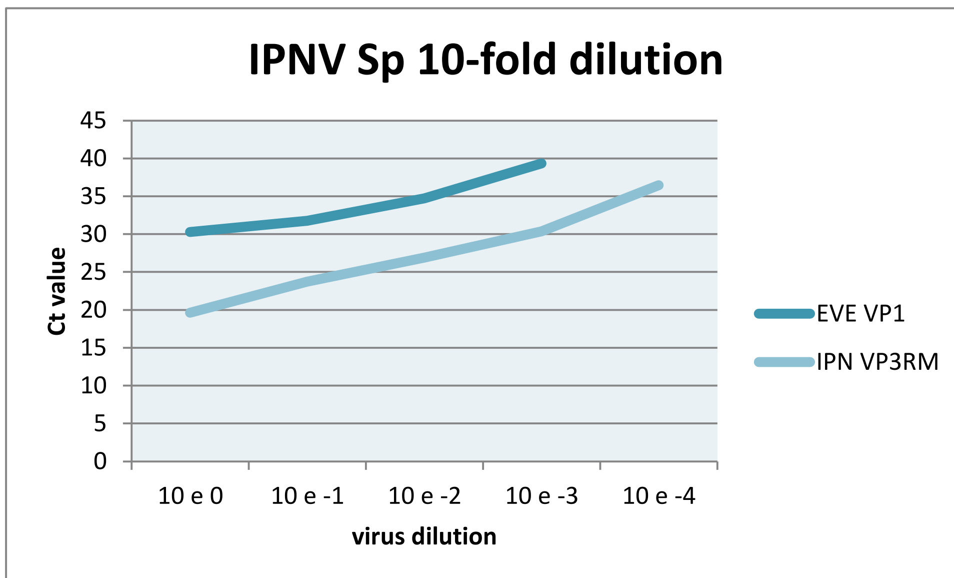


Figure 12: Ten-fold dilution of IPNV Sp tested with the SYBR® Green real-time PCR (EVE VP1 primers) and the Taqman real-time PCR (IPNV reverse primer mix (IPNV VP3RM)).

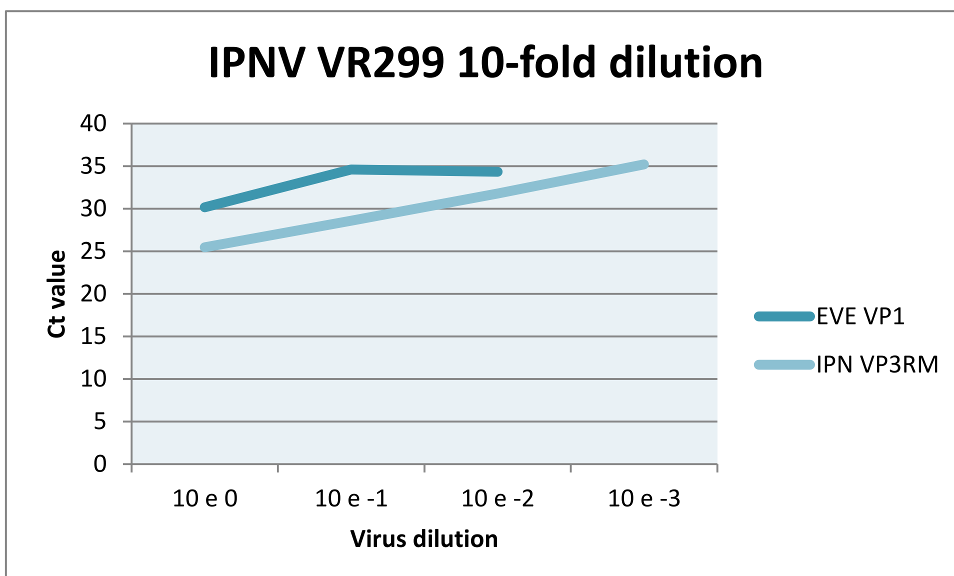


Figure 13: Ten-fold dilution of IPNV VR299 tested with the SYBR® Green real-time PCR (EVE VP1 primers) and the Taqman real-time PCR (IPNV reverse primer mix (IPNV VP3RM)).

Repeatability

The repeatability was the level of concordance between the results of a sample. The level of concordance was determined within de run (intra-assay) and between runs (inter-assay). The coefficient of variation (CV) can be calculated from the average and the standard deviation. When the coefficient of variation is below 5 it is acceptable. The CV from the intra-assay was below 4 in all PCRs whereby the CV was noted as CV %, the results are presented in figure 11-13. The inter-assay CV was calculated from the 2-fold dilution which was performed in triplo on four successive days. The CV from the inter-assay was below 4 in the 2-fold dilution. Figure 14 shows a PCR area where PCR mixes were made.

Figure 14:The PCR area were the PCR mix is made is extra clean. CVI©

Specificity

The specificity of the assay was determined by testing three IPNV strains, three EVE strains, one AngHV1 strain, one EVEX strain, one VHS strain, one IHN strain, one host DNA of eel, and one host strain of trout as negative control. The Taqman PCR tested the IPNV and EVE strains as positive. No PCR products were generated from EVEX, VHS, IHN, AngHV1 or host strains [15].

RNA purification

RNA purification with TRIzol

The RNA purification was performed 24 hours and 48 hours after the start of the incubation to generate the highest possible RNA product. The purified RNA was tested on agarose E-gel to get an indication of the RNA which was present in the samples which were purified. All the 17 viruses showed 2 bands on the agarose E-gel and a vague band at the bottom of the gel as shown in figure 16. One band appeared at 1000 base pairs (bp) and one band appeared at 3000 bp. The intensity of the bands between the samples were comparable but between day 1 and day 2 of the same sample the intensity differed. To get an indication of the contaminating RNA which was present in the eluted RNA the samples are also tested with the PCR for the presence of EVE, IPNV, HVA, EVEX and eel host. All eluted RNA samples were positive for EVE with the exception of three samples namely EVE strain V 12, V 16 and V 18. When performing the EVEX PCR all eluted RNA samples were negative with the exception of one sample namely V13. All eluted RNA samples were positive for eel host DNA, and the Ct value was comparable with the Ct of the EVE PCR as shown in figure 15. All eluted RNA samples were negative for AngHV-1 except one EVE strain V 24.

RNA purification with a sucrose gradient

Two RNA samples which were extracted from the sucrose gradient were tested on an agarose E-gel. V 20, a field strain from trout and V 24, and a field strain from eel were used. The 2 RNA sucrose samples, a 500 bp DNA ladder, 2 RNA eluted samples and a control sample were loaded on the gel. The 500 bp ladder was clearly visible and the 2 eluted RNA samples showed 2 bands, one at 1000 bp and one at 3000 bp. The control sample (Distilled Water DNase/RNase Free) and the 2 RNA sucrose samples showed no bands in the agarose E-gel as shown in figure 17.

RNA purification with TRIzol

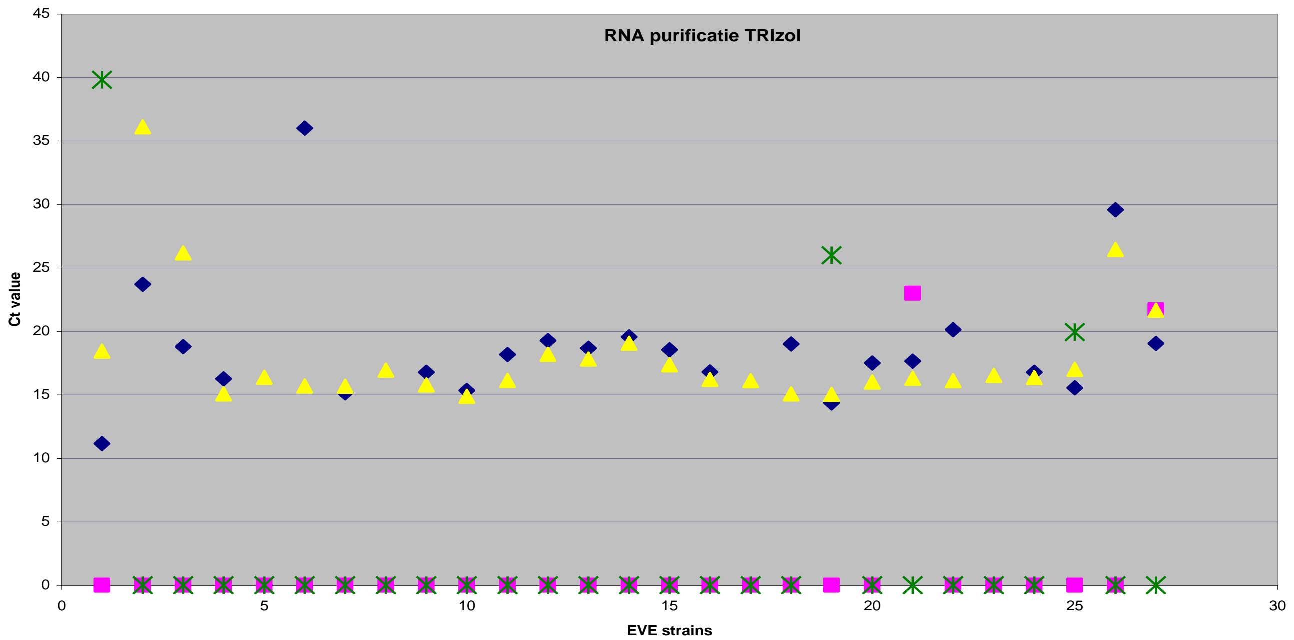
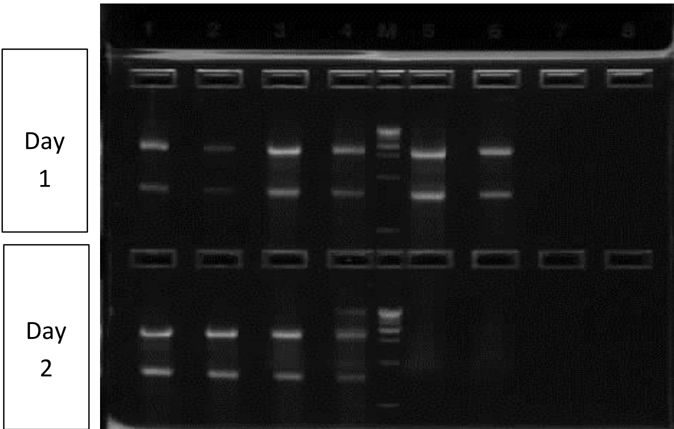


Figure 15: Results from the RNA purification with TRIzol tested for EVE, eel host, AngHV-1 and EVEX in the SYBR Green PCR. The yellow points represent the eel host, the blue points represent EVE, the pink points represent EVEX virus and the green points represent AngHV-1.

Agarose E-gel



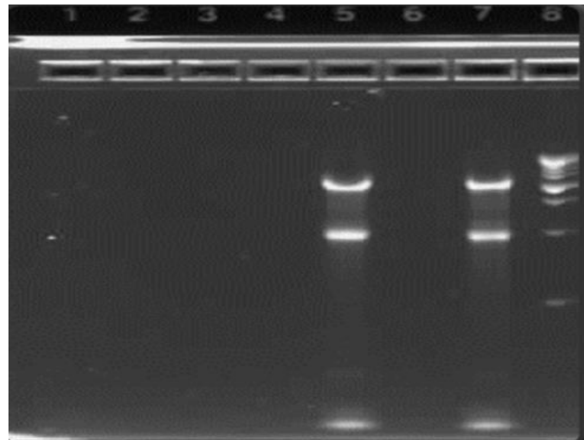


Figure 16: Agarose E-gel from the TRIzol purification which has been running for 45 minutes. The samples which are loaded on the gel are: eluted RNA TRIzol purification EVE field strain V 21 (1), eluted RNA TRIzol purification EVE field strain V 16 (2), eluted RNA TRIzol purification EVE field strain V 20 (3), eluted RNA TRIzol purification EVE field strain V 23 (4), eluted RNA TRIzol purification EVE field strain V 14 (5), eluted RNA TRIzol purification EVE field strain V 13 (6), 500 base pair DNA ladder (M).

Figure 17: Agarose E-gel from the sucrose purification which has been running for 45 minutes. The samples which are loaded on the gel are: RNA sucrose purification V20 (1), RNA sucrose purification V24 (3), water (2,4,6) eluted RNA TRIzol purification EVE field strain V 13 (5), eluted RNA TRIzol purification EVE field strain V 14 (7), 500 base pair DNA ladder (M).

Discussion

The comparison of iIPMA and the iIFT

The results of the iIFT and the iIPMA were comparable to each but were different in two samples. Different results were seen when performing the tests on field strain V7 from rainbow trout. When performing the IIPMA the undiluted well was positive for *aquabirnavirus*, the second well which was the 10^{-1} dilution was negative. The iIFT showed little green spots in the first and second well, i.e. the undiluted and the 10^{-1} dilution, figure 18 shows a positive result. During the interpretation, there was doubt as to whether this reaction was positive or negative. Because of the doubt it was decided to judge the sample as negative. It is however possible that this reaction was positive, as reading the test is a bit subjective. On the other hand, it could be due to an error in the practice of the test that the result is incorrect. There was also a disconcordance between the results of the tests when the 2011 ring test samples were tested. The result of the IFT was positive and the result of the iIPMA was negative. However when the sample was offered to the CVI the sample was positive when the iIPMA was performed. The disagreement can be explained by a titer loss in the practice or an error in the sample.

There are several hypothesis that give an explanation for the fact that the antibody tests show negative results of samples which are positive in the PCR. The field strains are tested with the PCR at the time the outbreak took place. Afterwards the samples are stored in the freezer of the CVI at -80°C and now, several years later they are used for the iIFT and iIPMA. The samples may have lost virus titer which makes it difficult to cultivate the viruses on cells. The virus is no longer present in the sample or the virus is present in such a low amount that incubation of 48 hours is insufficient. As a result, the virus is no longer detectable by the iIPMA or iIFT. Another possibility is that the sample was a mixed virus infection and that another virus has taken the upper hand making the *aquabirnavirus* no longer detectable by the antibody tests. A co-infection with *aquabirnaviruses* and AngHV1 is common. If the sample contains a high concentration of AngHV1 it will overgrow the *aquabirnavirus* under ideal conditions. The PCR will detect the *aquabirnavirus* because it's very sensitive through the amplification step and detect the smallest amount of RNA from the genetic material of the *aquabirnavirus*. The antibody tests will only detect intact virus which has replicated during the incubation period.

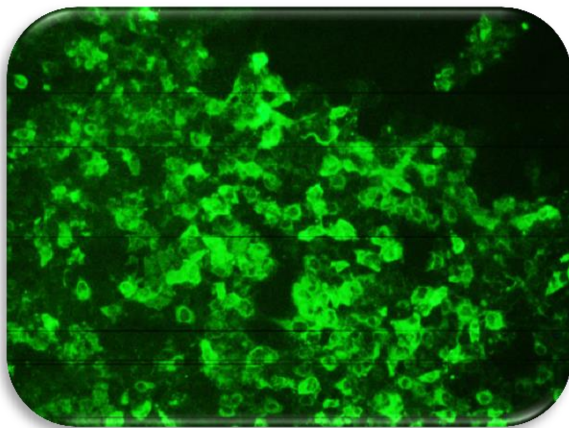


Figure 18: Positive iIFT test, the cytoplasm of the cells is green and the nucleus is black. CVI©

Further research should be performed to get a clearer overview of these two serological assays. Both between the assays themselves as in relationship with the PCR. Possibly, a PCR can be performed to test all samples that were used for the antibody tests. More clarity will be obtained about the concentration of *aquabirnaviruses*, the presence of other viruses and the relationship between the concentration of *aquabirnaviruses* and the outcome of the antibody testing. Another possibility for further research is to perform the antibody test several times with more samples, so the correlation between the test can be determined statistically.

Polymerase chain reaction (PCR)

In the beginning the SYBR® Green PCR, (VP 1, eel) and the Taqman PCR (VP3, trout) were tested to get an indication of the performance of both tests. The SYBR® Green PCR has moderate average Ct values, the test detects all three strains. The Ct value is higher than the average Ct value of the Taqman PCR VP3RM. The Taqman PCR VP3R1 has also low Ct values but higher than the Ct values of those of the Taqman PCR VP3RM. The Taqman PCR VP3R2 was tested in the beginning and was very sensitive for strain IPNV VR299, and did not detect strain IPNV Ab and strain IPNV Sp. The other Taqman PCRs were not further tested and the Taqman PCR VP3RM has the preference. The SYBR® Green PCR has a better sensitivity for the strain IPNV Ab in comparison with the Taqman PCR. The SYBR® Green PCR is specific for IPNV Ab, and can be used to detect aquabirnavirus of the eel. The Taqman PCR VP3RM has the lowest average Ct value, detects all three strains and has a good sensitivity for the strains IPNV Sp and IPNV VR299. For IPNV Ab the difference between both PCRs is small based on the Ct value, as in average 1 Ct difference is recognized. The difference between both PCRs is higher when discussed IPNV Sp and IPNV VR299. Based on the information above the Taqman PCR VP3RM is more sensitive than the SYBR® Green PCR. The Ct value for IPNV VR299 is some higher than the Ct values of the other two IPNV strains but IPNV VR299 has also high Ct value in the other PCRs. This indicates that there were low concentrations of IPNV VR299 in the sample or that both the SYBR® Green PCR and Taqman PCRs were less sensitive for strain VR299. The Taqman PCR VP3RM is specific for the strains IPNV Sp and IPNV VR299 which are aquabirnavirus strains of the trout. The Taqman PCR VP3RM can be used to detect aquabirnaviruses from the eel and the trout. The repeatability of the inter-assay and the intra-assay from the SYBR® Green PCR and the Taqman PCR VP3RM is calculated based on the performed PCRs. The correlation coefficient (CV) is below 4 which means that the PCRs are good repeatability. The Taqman PCR tested the IPNV and EVE strains as positive and no PCR products were generated from EVEX, VHS, IHN, AngHV1 or host DNA [15]. This means that the Taqman PCR VP3RM has a good specificity. Both the Taqman PCR VP3RM and the SYBR Green PCR VP1 are suitable for the diagnostic of aquabirnavirus. The Taqman PCR VP3RM diagnoses IPNV from the trout and the SYBR Green PCR diagnoses EVE from the eel.

RNA purification

RNA purification with TRIzol

The RNA purification is performed with the ultimate goal to develop high quality and pure RNA for high throughput sequencing from several field strains as shown in table 1. SDS (sodium dodecyl sulphate) and vanadyl ribonucleoside complexes are the key factors of the TRIzol purification. SDS dissolves membranes, inactivates ribonucleases and disrupts protein nucleic acid interaction. Vanadyl ribonucleoside complexes inhibits ribonucleases and is adequate for RNA isolation because pancreatic deoxyribonuclease I is not inhibited [18]. The idea of the extraction is to keep the cell nucleus intact and removed to reduce contamination with host DNA. When the RNA purification was performed there was little known about the quantity and the purity of the RNA. The agarose E-gel was performed to get an indication about value of the samples as shown in figure 19. All the samples which were purified with TRIzol showed the same bands on the agarose E-gel, one band at 1000 bp and one band at 3000 bp. Segment A from the *aquabirnavirus* consists of 3079 bp and segment B consists of 2784 bp. The band of 3000 bp which was visible in all samples in the agarose E-gel could be explained as RNA from *aquabirnavirus*. However, it is difficult to explain the band of 1000 bp which was visible by all samples on the agarose E-gel. The band of 1000 bp is too small to be EVE, IPNV, EVEX or AngHV-1. It is probable that the band is from possible ribosomal RNA's. Thereby the vague band at the bottom of the agarose E-gel is contamination of the eluted RNA sample which

Figure 19: E-gel base

may give disruption during sequencing. The quantity of some samples is higher than others which may be caused by the quantity of cytopathic effect (CPE) which was present at the start of the purification. When the CPE was high at the start of the purification, the quantity of RNA was lower. Because of this effect the purification is performed after 24 hours and 48 hours of incubation. The ideal moment to perform the purification is just prior to the onset of CPE.
Besides the agarose E-gel also the PCR was performed to get an indication about value of the samples. Three samples where negative for EVE which could be explained by several theories. Maybe no *aquabirnavirus* was present in the sample either the PCR, or the IPNV purification protocol didn't work. All samples where positive when performing the host PCR and the Ct value was high and comparable with the Ct value of the EVE PCR. The eluted RNA samples which were obtained by the IPNV purification were not used for high throughput sequencing because the quantity of the host genetic material was too high in comparison with the quantity of EVE genetic material. But it would be suitable to use the extracts for sequencing with targeted primers. Therefore the virus was worked up in a different way using sucrose gradients to compare the results.

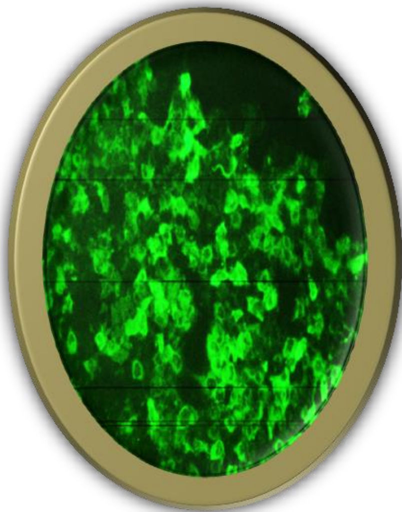
RNA purification with a sucrose gradient
The RNA purification with the sucrose gradients was performed because there was doubt about the quantity and purity of the eluted RNA samples which were obtained by performing the RNA purification with TRIzol and the two methods were compared. Two samples were used for the RNA purification with sucrose, one IPNV field strain and one EVE field strain. The fact that the samples which where purified with the sucrose gradient showed nothing on E-gel may be caused by either a pipette error, an error in the sucrose gradient or E-gel procedure or a low virus titer. For the RNA purification with TRIzol no CPE should is required and for the RNA purification with the sucrose gradient a lot of CPE is required. When performing the sucrose gradient 30%- 40% CPE was evident in the samples which may explain the results of the samples in the E-gel. The RNA samples which were obtained by the sucrose gradient purification were not used for sequencing because there were no bands presents on the agarose E-gel. The risk for sequencing other products than the product of the *aquabirnavirus* either sequencing nothing at all is too large. Therefore, sequencing was not further performed.

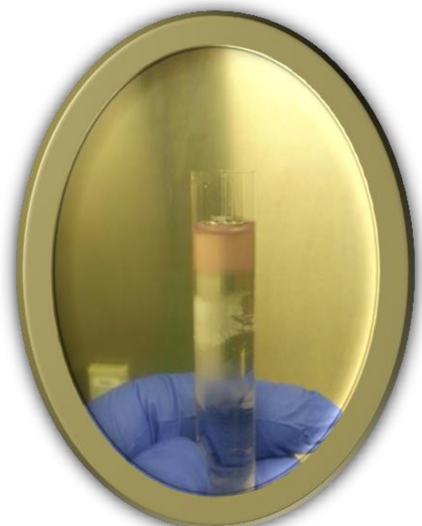
Conclusions

Based on this research, there are indications that it is possible to use the iIFT as a standard diagnostic test. Currently there are insufficient data to transfer the iIPMA to a iIFT as standard diagnostic test, as there were too many negative results for iIFT compared to the iIPMA. To use the iIFT as a standard diagnostic test more EVE and IPNV samples should be tested with both antibody tests. Thereby the antibody tests should be run more often to collect more data.

The SYBR® Green PCR VP1 is sensitive for IPNV Ab and is a specific test to diagnose EVE, *aquabirnavirus* from the eel. The Taqman PCR VP3RM is sensitive for IPNV Sp and IPNV VR299. This PCR specific diagnoses IPNV, *aquabirnavirus* from the trout but can also be used to diagnose IPNV Ab strains. The Taqman PCR VP3RM is sensitive, specific and repeatability and after further validation it will be a good diagnostic test for detecting *aquabirnavirus*.

Several EVE strains were purified to get highly quality and pure RNA for high throughput sequencing. There is doubt about the purity of the samples of the strains which were treated with TRIzol and the sucrose gradient. The RNA samples where therefore not suitable for high throughput sequencing but could be used for targeted sequencing with specific primers.





References

1. Orpetveit, I., et al., *Detection of Infectious Pancreatic Necrosis Virus in Subclinically Infected Atlantic Salmon by Virus Isolation in Cell Culture or Real-Time Reverse Transcription Polymerase Chain Reaction: Influence of Sample Preservation and Storage.* Journal of Veterinary Diagnostic Investigation, 2010. **22**(6): p. 886-895.
2. Zhang, C.X. and S. Suzuki, *Aquabirnaviruses isolated from marine organisms form a distinct genogroup from other aquabirnaviruses.* J Fish Dis, 2004. **27**(11): p. 633-43.
3. van Beurden, S.J., et al., *Viral diseases of wild and farmed European eel Anguilla anguilla with particular reference to the Netherlands.* Dis Aquat Organ, 2012. **101**(1): p. 69-86.
4. Drenner, S.M., et al., *A synthesis of tagging studies examining the behaviour and survival of anadromous salmonids in marine environments.* PLoS One, 2012. **7**(3): p. e31311.
5. Crane, M. and A. Hyatt, *Viruses of fish: an overview of significant pathogens.* Viruses, 2011. **3**(11): p. 2025-46.
6. Sano, T., *Viral diseases of cultured fishes in Japan.* Fish Pathol, 1976. **10**: p. 221-226.
7. Haenen, O., *Deel 7 - Visvirussen. Infectieuze Pancreatische Necrose Virus (IPNV) van salmoniden en Europees Virus van Paling (EVE).* Aquacultuur, 2000(6): p. 32-35.
8. Lefebvre, F., et al., *Is the continental life of the European eel Anguilla anguilla affected by the parasitic invader Anguillicoloides crassus?* Proc Biol Sci, 2013. **280**(1754): p. 20122916.
9. Bilotta, G.S., et al., *The decline of the European eel Anguilla anguilla: quantifying and managing escapement to support conservation.* J Fish Biol, 2011. **78**(1): p. 23-38.
10. Dekker, W., et al., *Worldwide decline of eel resources necessitates immediate action, Québec Declaration of Concern.* Fisheries, 2003. **28**(12): p. 28-30.
11. L.T.N. Heinsbroek, J.G.K., *Feeding and growth of glass eels, Anguilla anguilla L.: the effect of feeding stimulants on feed intake, energy metabolism and growth.* Aquaqulture and Fisheries Managment, 1992. **23**: p. 327-336.
12. Conduct, E.A.C.o., *Anguilla anguilla.* Food and Agriculture Organization of the United Nations, 2013.
13. Robert, R.J.P., M.D., *Infectious pancreatic necrosis in Atlantic salmon, Salmo salar L.* Journal of fish diseases, 2005. **28**: p. 383-390.
14. Arcuicultura, P.e., *IPNV by salmon*, I.b. salmon, Editor 2009 Marcos Godoy: Austral de Chile.
15. van Beurden, S.J., et al., *Development and validation of a two-step real-time RT-PCR for the detection of eel virus European X in European eel, Anguilla anguilla.* J Virol Methods, 2011. **171**: p. 352-359.
16. Van Beurden, S.J., et al., *Identification and localization of structural proteins of anguillid herpesvirus 1 by mass spectrometry.* Veterinary Research, 2011. **42**(105).
17. Bowers, R.M., S.E. Lapatra, and A.K. Dhar, *Detection and quantitation of infectious pancreatic necrosis virus by real-time reverse transcriptase-polymerase chain reaction using lethal and non-lethal tissue sampling.* J Virol Methods, 2008. **147**(2): p. 226-34.
18. Rio, D.C., et al., *Purification of RNA by SDS solubilization and phenol extraction.* Cold Spring Harb Protoc, 2010. **2010**(6): p. pdb prot5438.

CENTRAAL VETERINAIR INSTITUUT

WAGENINGEN UR