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Development and validation of a real-time PCR assay for the detection of anguillid herpesvirus 1

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Abstract

Anguillid herpesvirus 1 (AngHV1) causes a haemorrhagic disease with increased mortality in wild and farmed European eel, *Anguilla anguilla* (L.) and Japanese eel *Anguilla japonica*, Temminck & Schlegel). Detection of AngHV1 is currently based on virus isolation in cell culture, antibody-based typing assays or conventional PCR. We developed, optimized and concisely validated a diagnostic TaqMan probe based real-time PCR assay for the detection of AngHV1. The primers and probe target AngHV1 open reading frame 57, encoding the capsid protease and scaffold protein. Compared to conventional PCR, the developed real-time PCR is faster, less labour-intensive and has a reduced risk of cross-contamination. The real-time PCR assay was shown to be analytically sensitive and specific and has a high repeatability, efficiency and r^2-value. The diagnostic performance of the assay was determined by testing 10% w/v organ suspensions and virus cultures from wild and farmed European eels from the Netherlands by conventional and real-time PCR. The developed real-time PCR assay is a useful tool for the rapid and sensitive detection of AngHV1 in 10% w/v organ suspensions from wild and farmed European eels.

Keywords: AngHV1, *Anguillid herpesvirus 1*, eel herpesvirus, herpesvirus anguillae, rapid detection, real-time PCR.

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Introduction

Anguillid herpesvirus 1 (AngHV1) causes a haemorrhagic disease in Japanese eel *Anguilla japonica*, Temminck & Schlegel and European eel, *Anguilla anguilla*, (L.) (Sano, Fukuda & Sano 1990; Davidse *et al.* 1999). AngHV1 has been suggested to play a contributory role in the decline of the wild European eel stocks (Haenen *et al.* 2012) and is regularly detected in wild European eels in Europe (Van Ginneken *et al.* 2004; Jakob *et al.* 2009; Haenen *et al.* 2010; Van Beurden *et al.* 2012; Armitage *et al.* 2014; Bandin *et al.* 2014). AngHV1 is also widespread at commercial eel farms, where mortality rates caused by this virus can be as high as 30% (Sano *et al.* 1990; Chang *et al.* 2002; Haenen *et al.* 2002). Experiences from AngHV1 outbreaks in high-density intensive recirculation production systems in the Netherlands suggested that the disease is stress-induced and often part of a double infection with another virus (Van Beurden *et al.* 2012). Results from experimental infection studies were suggestive for the capability of AngHV1 to cause a latent infection (Van Nieuwstadt, Dijkstra & Haenen 2001). Taxonomically, AngHV1 belongs to the genus *Cyprinivirus* of the family *Alloherpesviridae* of the order *Herpesvirales* (Van Beurden *et al.* 2010).

Detection of AngHV1 is important in wild caught glass eels and elvers intended for farming purposes, in farmed yellow eels intended for restocking purposes, and in studies monitoring the health status of the wild European eel population

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(Haenen *et al.* 2010). At fish farms, virus outbreaks can be controlled by changing the water temperature to a non-permissive temperature for the virus. In case of an AngHV1 outbreak at an eel farm, lowering the water temperature to below 22 °C results in reduced losses (Haenen *et al.* 2002).

Diagnosis of AngHV1 cannot be based on clinical signs and gross pathology alone, as two other pathogenic eel viruses, namely the aquabirnavirus *Eel virus European* (EVE) and the rhabdovirus *Eel virus European X* (EVEX), may cause a similar haemorrhagic disease (Van Beurden *et al.* 2012). Hence, several diagnostic assays have been developed for AngHV1, including an immune peroxidase monolayer assay (IPMA) (Davidse *et al.* 1999), an indirect fluorescence antibody test (IFAT) (Varvarigos *et al.* 2011), an *in situ* hybridization assay (Shih, Hu & Wang 2003), and two conventional PCR assays targeting the DNA polymerase gene (Shih 2004; Rijsewijk *et al.* 2005). Real-time PCR uses a fluorescent intercalating dye or probe to detect amplicon formation during the thermal cyclic amplification (Mackay, Arden & Nitsche 2002; Kubista *et al.* 2006), and this methodology is increasingly used in viral detection (Watzinger, Ebner & Lion 2006). Real-time PCR overcomes the need for post-amplification gel electrophoresis, which makes this approach quicker, less labour-intensive and reduces the risk of cross-contamination.

The aim of this study was to develop, optimize and concisely validate a real-time PCR assay for the detection of AngHV1 in European eel. The assay characteristics analytical specificity, analytical sensitivity, repeatability and efficiency were determined for this assay and compared with those of the conventional PCR developed by Rijsewijk *et al.* (2005). The diagnostic performance was assessed by testing various 10% w/v organ suspensions and virus-infected cell cultures from wild and farmed European eels. Overall, the developed assay was shown to be applicable as a rapid and sensitive test method for the detection of AngHV1 in European eel.

Materials and methods

Reference viruses

For AngHV1, the analytical reference strain CVI NL-500138 (Van Beurden *et al.* 2010) and the Japanese isolate C3P2 (kindly provided by H. Fukuda) were used. For EVEX Galinier *et al.* (2012), isolate CVI NL-108778 was used. For EVE, the genetically closely related *Infectious pancreatic necrosis virus* (IPNV) strain Ab (Blake *et al.* 2001; Zhang & Suzuki 2004) was used (kindly provided by P.E.V. Jørgensen), as no AngHV1-free EVE isolate was available. Furthermore, other fish herpesviruses used were *Cyprinid herpesvirus 1* (CyHV1, strain G364, kindly provided by K. Way), *Cyprinid herpesvirus 3* (CyHV3, strain C250, kindly provided by K. Way) and *Ictalurid herpesvirus 1* (IcHV1, strain Auburn-1 clone A, ATCC VR-665).

DNA extraction

Total DNA was extracted from 200 μL of 10% w/v organ suspension or cytopathic effect (cpe) positive eel kidney (EK-1) cell cultures (Chen, Ueno & Kou 1982), freeze–thawed at −80 °C, using the QIAamp DNA Blood mini kit according to manufacturer's protocol (Qiagen, Hilden, Germany). DNA was finally dissolved in 200 μL of buffer AE and stored at −20 °C.

Sequencing

To design primers and probe in such a way that Japanese AngHV1 isolates could be detected as well, the DNA sequence of the conserved AngHV1 open reading frame (ORF) 57, encoding the capsid protease and scaffolding protein (Van Beurden *et al.* 2011b), was determined for AngHV1 C3P2 (Japan). A forward and a reverse primer spanning the entire ORF were designed based on the NCBI reference genome sequence of AngHV1 CVI NL-500138 (RefSeq ID: NC_013668), using the Primer3 Express software (Table 1), and ordered from Eurogentec (Seraing, Belgium). Conventional PCR was carried out using the Taq DNA Polymerase kit from Invitrogen (Life Technologies, Carlsbad, CA, USA), according to Van Beurden *et al.* (2011a). The amplicon was directly sequenced from both ends with eight internal primers (Table 1) on a 3130 DNA Analyzer (Applied Biosystems, Life Technologies, Carlsbad, CA, USA), using the Big Dye Terminator v1.1 sequencing kit. The 2148 bp long sequence was 99.8% homologous to the reference sequence and submitted to the NCBI genetic sequence database (GenBank ID: JQ905264).

Table 1 ORF spanning primer set and internal sequence primers used to determine the sequence of the Japanese AngHV1 isolate C3P2 ORF57, and diagnostic primer set and probe targeting AngHV1 ORF57

Primer name	Sequence 5′ → 3′	Primer use
AngHV1.ORF57.seq.F01	TCC CAT AGC GAG CTA CAC CT	ORF57 spanning forward primer
AngHV1.ORF57.seq.R01	CCC TGG AAG CAG TGA AGA AC	ORF57 spanning reverse primer
AngHV1.ORF57.seq.F04	CCC TTG ACT TTG GGT ACC TG	Internal sequence forward primer
AngHV1.ORF57.seq.F05	TAG TAG CGG TCG GTT CTG GT	Internal sequence forward primer
AngHV1.ORF57.seq.F06	CAG AGG AGC GTG ACC AAC AC	Internal sequence forward primer
AngHV1.ORF57.seq.F07	GCT CAG GCG AGT CAT CAT CT	Internal sequence forward primer
AngHV1.ORF57.seq.R04	TAA CCC GCT GGA TAC TTT GG	Internal sequence reverse primer
AngHV1.ORF57.seq.R05	GCA TAA AAA GTC TGT GCC TTT G	Internal sequence reverse primer
AngHV1.ORF57.seq.R06	GAA CAG GAG GCA AAG ACC AA	Internal sequence reverse primer
AngHV1.ORF57.seq.R07	CCT TCA GCC TTT CAT CGA AC	Internal sequence reverse primer
AngHV1.CapProt.F06	TGC TCT TGG AGT CGG TTG ATG	Diagnostic forward primer
AngHV1.CapProt.R06	CCG TGT GGG AAA AGA CTA TTT GA	Diagnostic reverse primer
AngHV1.CapProt.p06	6FAM-TCT GAA AAC CCG CTC GCC CTG A-BHQ1	Diagnostic probe

Primer and probe design

Real-time PCR primers and probe were designed based on an alignment of the available sequences of AngHV1 CVI NL-500138 ORF57 (RefSeq ID: NC_013668) and AngHV1 C3P2 (Japan) ORF57 (GenBank ID: JQ905264) using the PrimerExpress 3.0 software (Applied Biosystems). Forward primer AngHV1.CapProt.F06 and reverse primer AngHV1.CapProt.R06 resulting in an expected amplicon size of 69 bp, and probe AngHV1.CapProt.p06, were ordered from Eurogentec (Table 1).

Real-time PCR

The real-time PCR reaction mix consisted of 10 μL TaqMan Fast Universal PCR Master Mix (2×; Applied Biosystems), 0.8 μL forward primer AngHV1.CapProt.F06 (10 μM), 0.8 μL reverse primer AngHV1.CapProt.R06 (10 μM), 0.6 μL probe AngHV1.CapProt.p06 (5 μM), 0.25 μL Uracil-DNA glycosylase (New England Biolabs, Ipswich, MA, USA), molecular grade water up to a final volume of 15 μL and 5 μL template. For optimization of primer concentrations, real-time PCR reaction mixes were prepared using 10 μL SYBR Green PCR mix (Applied Biosystems) without probe and with variable primer concentrations instead. The real-time PCR reaction was carried out in a 7500 Fast Real-Time PCR system (Applied Biosystems). TaqMan real-time PCRs were run under Fast 7500 conditions, starting with 10 min at 37 °C and 10 min at 95 °C, followed by 40 cycles of 3 s at 95 °C and 30 s at 60 °C. SYBR Green real-time PCRs were run under Standard 7500 conditions, starting with 10 min at 37 °C and 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C, and ending with a dissociation stage. In each run, a positive control and a negative control were included. Data were analysed using the Sequence Detection Software version 1.4 program (Applied Biosystems) with the Auto baseline function and the threshold manually set at 0.20.

Preparation of standard curves

For assay optimization, and to determine the analytical sensitivity, repeatability and efficiency, standard 10-fold dilution series of an analytical AngHV1 reference sample were prepared. The titre of AngHV1 CVI NL-500138 was determined by 10-fold titration at 26 °C according to Van Beurden *et al.* (2011c). Briefly, 10-fold serial dilutions of virus suspension were added in 12-fold to ~80% confluent EK-1 monolayers in 96-well plates and incubated for 7 days at 26 °C. The monolayers were scored for typical cpe using an inverted light microscope. The titre was expressed in $TCID_{50}$ per ml using the Spearman–Kärber method for calculation.

Hundred and fifty microlitres of virus suspension was used for DNA extraction. Separate 10-fold serial dilution series were prepared in distilled water. Standard curves were analysed using the Microsoft Excel 2003 program (Microsoft, Redmond, WA, USA). Intra-assay and interassay variability were expressed in the mean coefficient of variation and the degree of linear correlation (r^2) within the dynamic range from three series.

Efficiency was calculated from the mean of three series using the formula: efficiency = $10^{-1/\text{slope}} - 1$.

Field samples

The field samples used in this study were taken from wild and farmed European eels at the Central Veterinary Institute (CVI), part of Wageningen UR, Lelystad, the Netherlands. Live eels and elvers were collected by fisherman or fish farmers for diagnostic purposes and transported alive to CVI. In brief, live eels were anesthetized and killed, and 10% w/v organ suspensions were prepared from the gills, and a pool of spleen, kidney and liver, according to Haenen *et al.* (2002). In case of glass eels or elvers, 10% w/v suspensions were prepared from pools of up to 10 whole glass eels or elvers. Organ suspensions were inoculated on monolayers of EK-1 cells (Chen *et al.* 1982) in a 5% CO_2 incubator at 15, 20 and 26 °C (Haenen *et al.* 2002). If cpe developed, the causative virus was identified by different typing assays. For AngHV1, a conventional PCR was used according to Rijsewijk *et al.* (2005); for EVE, an IPMA was used according to Haenen *et al.* (2010); and for EVEX, an IFAT or a real-time RT-PCR assay was used according to Van Beurden *et al.* (2011c). Other viruses were characterized by electron microscopy. The sample was considered virus negative if no cpe developed after two blind passages of 7–10 days. Virus isolates, organ suspensions and infected cell cultures were stored at −80 °C. Positive virus isolation followed by AngHV1 identification by conventional PCR was considered the gold standard in the determination of the diagnostic performance of the real-time PCR assay. All field samples tested with the real-time PCR assay were also tested with the conventional PCR assay developed by Rijsewijk *et al.* (2005), and the agreement between the two methods was expressed in Cohen's kappa coefficient: κ = (observed agreement − chance agreement)/(maximum agreement − chance agreement) (Landis & Koch 1977).

Results

Optimization

To optimize the assay's performance, different primer and probe concentrations and annealing temperatures were tested with a low and a high dilution of AngHV1 reference strain CVI500138. Primer concentrations of 100, 200, 400 and 800 nM were tested in a checkerboard pattern using SYBR Green. The primers generated a single melting curve in the SYBR Green assay. The optimal primer concentration was determined to be 400 nM for both the forward and the reverse primer (data not shown). The performance of the probe was tested in concentrations of 50, 100, 150, 200, 250 and 500 nM. With the exception of the higher Ct-value for the lowest probe concentration of 50 nM, all concentrations resulted in comparable Ct-values within a one cycle range. The optimal probe concentration based on robustness and economics of use was determined to be 150 nM. The optimal primer annealing temperature was tested at 58, 60 and 62 °C and determined to be 60 °C. No aspecific PCR products were formed in non-template controls. Control of the amplified product by gel electrophoreses revealed a single PCR product with an approximate size of 69 bp.

Repeatability and efficiency

To determine assay repeatability, standard 10-fold dilution series were prepared in triplicate and tested in the same assay (intra-assay repeatability), and on successive days by the same person (inter-assay repeatability). The real-time PCR assay detected the AngHV1 analytical reference strain CVI NL-500138 constantly over a 6-log range from undiluted to a 10^{-6} dilution (Fig. 1). In the dynamic range, the mean coefficient of variation was 0.43% for the intra-assay test and 1.40% for the interassay test. The linear correlation expressed as the r^2-value was 0.998 for the intra-assay test and 0.999 for the interassay test. The efficiency, as calculated from the mean slope, was 90.8% and 89.4% for the intra- and interassay tests, respectively.

Analytical specificity

The analytical specificity of the real-time PCR assay was determined by testing the AngHV1 analytical reference strain CVI NL-500138 and isolate C3P2 (Japan), other fish herpesviruses CyHV1, CyHV3 and IcHV1, and two other commonly observed European eel virus reference strains EVEX CVI NL-108778 and IPNV Ab.

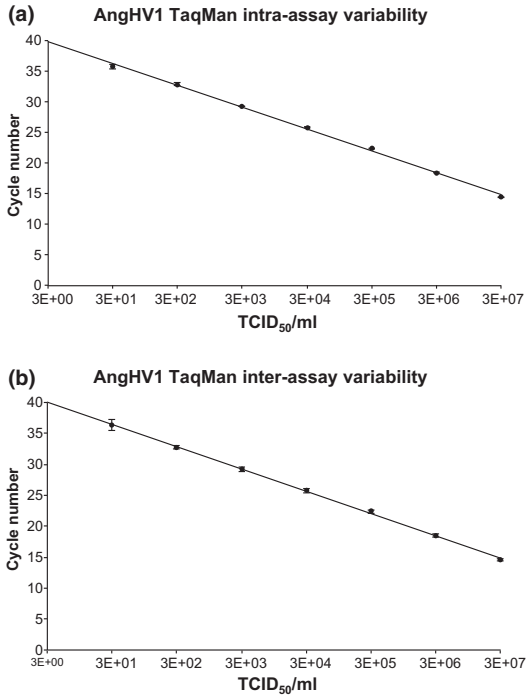

Figure 1 Standard curves of the AngHV1 real-time RT-PCR assay in the dynamic range from 3.0×10^1 to 3.0×10^7 $TCID_{50}$ per ml. (a) AngHV1 TaqMan intra-assay variability. (b) AngHV1 TaqMan interassay variability.

The results are shown in Table 2. The real-time PCR assay constantly detected AngHV1 only and not any of the other European eel viruses or other fish herpesviruses.

Analytical sensitivity

The titre of the AngHV1 analytical reference strain CVI NL-500138 was found to be $10^{7.6}$ or 3.0×10^7 $TCID_{50}$ per ml. In the inter- and intra-assay variability tests, the 10^{-6} dilution was constantly detected, the 10^{-7} dilution in 67% of the runs. Hence, the analytical sensitivity or lowest detectable virus titre was calculated to be between 3.0 and 30 $TCID_{50}$ per ml.

Diagnostic performance

A total of 72 field samples and virus cultures were tested, derived from a total of 26 batches of eels (Table 3). Positive virus isolation followed by AngHV1 identification by conventional PCR was considered the gold standard. If one 10% w/v organ suspension of a batch of eels was tested positive in virus isolation and subsequent conventional AngHV1 PCR, the sample was considered positive. A total of 11 batches of eels were tested positive for AngHV1 by the gold standard, and a total of 16 batches of eels were tested negative by the gold standard (Table 4). Diagnostic sensitivity was defined as the proportion of gold standard positive batches of eels that tested positive in the real-time PCR assay (OIE 2009). All 11 virus culture and conventional AngHV1 positive samples were tested positive by the real-time PCR assay, and hence, the diagnostic sensitivity was determined to be 100%. None of the gold standard positive samples were tested negative by the real-time PCR assay, and hence, the percentage of false negatives was 0%. Diagnostic specificity was defined as the proportion of gold standard negative batches of eels that tested negative in the real-time PCR assay (OIE 2009). Twelve of the 16 gold standard negative batches of eels were tested negative by the real-time PCR assay, resulting in a diagnostic specificity of 73%.

From the total of 72 organ suspensions and virus cultures, 44 samples generated a detectable signal in the real-time PCR assay, of which 42 samples were also tested positive in the

Table 2 Analytical specificity of the AngHV1 real-time PCR assay tested with AngHV1 reference strains, other European eel viruses, and other fish herpesviruses

	Virus	Isolate	Origin	Titre[a]	Real-time PCR
AngHV1 reference strains	AngHV1	CVI NL-500138	CVI, The Netherlands	$10^{7.6}$	+
	AngHV1	C3P2 (Japan)	Tokyo University of Fisheries, Japan	$10^{7.4}$	+
Other European eel viruses	EVEX	CVI NL-108778	CVI, The Netherlands	$10^{7.3}$	–
	IPNV	Ab	EURL for Fish Diseases, Denmark	$10^{8.1}$	–
Other fish herpesviruses	CyHV1	G364	CEFAS, UK	n.d.	–
	CyHV3	C250	CEFAS, UK	n.d.	–
	IcHV1	Auburn-1	ATCC	n.d.	–
Negative control	–	–		n.d.	–

[a]Titre expressed in $TCID_{50}$ per ml. n.d. = not done.

Table 3 Diagnostic performance of the AngHV1 real-time PCR assay tested with cpe positive and negative field samples from European eel

ID number[a]CVI NL	Sample[b]	Cpe[c]	AngHV1[d]	EVE[e]	EVEX[f]	Real-time PCR[g]
Batches positive in virus isolation						
05010366a	10% glass eels	n.a.	+	n.d.	n.d.	17.77
05010366a	Glass eels on EK-1 (20 °C)	+	+	–	–	17.81
05010366b	10% elvers	n.a.	+	+	–	20.14
05010366b	Elvers on EK-1 (20 °C)	+	+	+	–	18.81
06000725	10% gills	n.a.	+	n.d.	n.d.	33.61
06000725	10% organs	n.a.	+	n.d.	n.d.	33.58
06000725	Gills on EK-1 (26 °C)	+	+	–	–	14.18
06001456	10% glass eels	n.a.	+	n.d.	n.d.	16.33
06001456	Glass eels on EK-1 (15 °C)	+	+	–	–	19.90
06001456	Glass eels on EK-1 (20 °C)	+	+	–	–	16.42
06001456	Glass eels on EK-1 (26 °C)	+	+	–	–	15.29
09009703	10% gills	n.a.	–	n.d.	n.d.	*38.30*
09009703	10% organs	n.a.	+	n.d.	n.d.	35.89
09009703	Gills on EK-1 (15 °C)	+	–	–	+	–
09009703	Gills on EK-1 (20 °C)	+	+	+	+	36.98
09009703	Organs on EK-1 (15 °C)	+	–	+	+	–
09015163	10% gills	n.a.	+	n.d.	n.d.	31.85
09015163	10% organs	n.a.	+	n.d.	n.d.	35.81
09015163	Gills on EK-1 (26 °C)	+	+	–	–	14.39
10013402	10% gills	n.a.	+	n.d.	n.d.	20.18
10013402	10% organs	n.a.	+	n.d.	n.d.	27.96
10013402	Gills on EK-1 (26 °C)	+	+	–	–	13.77
10013402	Organs on EK-1 (15 °C)	+	+	–	+	17.12
10013402	Organs on EK-1 (20 °C)	+	+	–	+	14.80
10019981	10% gills	n.a.	+	n.d.	n.d.	14.72
	10% organs	n.a.	+	n.d.	n.d.	21.78
10019981	Gills on EK-1 (20 °C)	+	+	–	–	11.53
10019981	Organs on EK-1 (26 °C)	+	+	–	–	11.28
11003032	10% glass eels	n.a.	–	n.d.	n.d.	–
11003032	Glass eels on EK-1 (15 °C)	+	–	–	+	–
11003032	Glass eels on EK-1 (20 °C)	+	–	–	+	–
11003032	Glass eels on EK-1 (26 °C)	+	–	–	+	–
11004270	10% gills	n.a.	+	n.d.	n.d.	24.07
11004270	10% organs	n.a.	+	n.d.	n.d.	29.28
11004270	Gills on EK-1 (20 °C)	+	+	+	–	15.61
11004270	Gills on EK-1 (26 °C)	+	+	+	–	14.28
11007812	10% gills and organs (pool 2)	n.a.	+	n.d.	n.d.	21.84
11007812	Gills and organs on EK-1 (20 °C)	+	+	–	–	16.12

Table 3 *Continued*

ID number[a]CVI NL	Sample[b]	Cpe[c]	AngHV1[d]	EVE[e]	EVEX[f]	Real-time PCR[g]
11007812	Gills and organs on EK-1 (26 °C)	+	+	–	–	15.23
11017717	10% gills	n.a.	+	n.d.	n.d.	30.81
11017717	10% organs	n.a.	+	n.d.	n.d.	28.19
11017717	Gills on EK-1 (26 °C)	+	+	–	–	13.64
11017717	Organs on EK-1 (26 °C)	+	+	–	–	13.88
11019050	10% gills	n.a.	–	n.d.	n.d.	–
11019050	10% organs	n.a.	–	n.d.	n.d.	–
11019050	Gills on EK-1 (15 °C)	+[h]	–	–	–	–
11019050	Organs on EK-1 (20 °C)	+[h]	–	–	–	–
11019050	Organs on EK-1 (26 °C)	+[h]	–	–	–	–
Batches negative in virus isolation						
04002799	10% glass eels	n.a.	–	n.d.	n.d.	–
04020072	10% gills	n.a.	–	n.d.	n.d.	–
04020072	10% organs	n.a.	–	n.d.	n.d.	–
04020410	10% gills	n.a.	–	n.d.	n.d.	–
04020410	10% organs	n.a.	–	n.d.	n.d.	–
04021616	10% gills	n.a.	–	n.d.	n.d.	*38.26*
04021616	10% organs	n.a.	–	n.d.	n.d.	–
04022420	10% gills	n.a.	–	n.d.	n.d.	–
04022420	10% organs	n.a.	–	n.d.	n.d.	–
05007685	10% gills	n.a.	+	n.d.	n.d.	35.79
05007685	10% organs	n.a.	+	n.d.	n.d.	33.86
05009254	10% glass eels	n.a.	–	n.d.	n.d.	–
05020305	10% gills	n.a.	–	n.d.	n.d.	–
05020305	10% organs	n.a.	–	n.d.	n.d.	–
05020752	10% gills	n.a.	–	n.d.	n.d.	–
05020752	10% organs	n.a.	–	n.d.	n.d.	–
10009106-2	10% gills	n.a.	+	n.d.	n.d.	34.44
10009106-2	10% organs	n.a.	+	n.d.	n.d.	33.66
10012853	10% gills	n.a.	–	n.d.	n.d.	–
10012853	10% organs	n.a.	–	n.d.	n.d.	–
10014077	10% gills	n.a.	+	n.d.	n.d.	29.64
10014077	10% organs	n.a.	+	n.d.	n.d.	31.66
10016086	10% gills	n.a.	–	n.d.	n.d.	–
10016086	10% organs	n.a.	–	n.d.	n.d.	–

[a]Field samples are divided into positive and negative in virus isolation and listed on basis of their diagnostic identification (ID) number. Field samples 10013402 and 10016086 represent pools of 11 and 10 yellow eels, respectively.
[b]10% Gills = 10% w/v suspension of gills; 10% organs = 10% w/v suspension of the internal organs liver, spleen and kidney; 10% Glass eels or 10% Elvers = 10% w/v suspensions of ground whole glass eels or elvers; Gills on EK-1 = 10% w/v gill suspension inoculated on EK-1 cells; Organs on EK-1 = 10% w/v internal organ suspension inoculated on EK-1 cells; Glass eels or Elvers on EK-1 = 10% w/v suspensions of ground whole glass eels or elvers inoculated on EK-1 cells. Only cpe positive virus isolations were tested and shown.
[c]Cpe = cytopathic effect in the respective 10% w/v suspension inoculations on EK-1 cells; results in cell culture were not extrapolated to the 10% w/v suspensions. + = cpe; – = no cpe; n.a. = not applicable.
[d]Results of AngHV1 detection in 10% w/v suspensions and virus isolations by the conventional PCR, developed by Rijsewijk *et al.* (2005).
[e]An IPMA was used for EVE detection in cpe positive virus isolations; 10% w/v organ suspensions were not tested and hence marked as n.d. = not done.
[f]The IFAT and real-time PCR developed by Van Beurden *et al.* (2011c) was used for EVEX detection in cpe positive virus isolations; 10% w/v organ suspensions were not tested and hence marked as n.d. = not done.
[g]The developed real-time PCR was used for AngHV1 detection in 10% w/v suspensions and virus isolations. Ct-values are shown, with values of samples tested negative by conventional PCR, and above 38 shown in italics.
[h]Both 10% w/v gill and internal organ suspensions tested of batch CVI NL-11019050 were positive in virus isolation; AngHV1, EVE and EVEX were tested negative, but a reovirus-like agent was observed by electron microscopy.

conventional PCR (Table 5). If the virus cultures tested positive for AngHV1 by real-time or conventional PCR, the respective 10% w/v organ suspensions always tested positive by PCR as well. The two samples tested positive by real-time PCR, but negative by conventional PCR, resulted

Table 4 Overview of the diagnostic performance of the AngHV1 real-time PCR assay tested with cpe positive and negative Dutch field samples from European eel[a]

	Gold standard[b]		
	Positive	Negative	Total
Real-time PCR			
Positive	11	4	15
Negative	0	11	11
Total	11	15	26

[a]Results from a total of 26 batches of eels were compared; if one 10% w/v organ suspension was tested positive, the whole eel batch was considered positive.
[b]Gold standard = positive virus isolation followed by positive virus identification by conventional PCR (Rijsewijk *et al.* 2005).

Table 5 Comparison of the results of the AngHV1 conventional PCR assay (Rijsewijk *et al.* 2005) and the AngHV1 real-time PCR assay (this study)[a]

	Conventional PCR		
	Positive	Negative	Total
Real-time PCR			
Positive	42	2	44
Negative	0	28	28
Total	42	30	72

[a]Results from a total of 72 field samples and virus cultures, derived from a total of 26 batches of eels.

in a signal with a Ct-value >38, whereas all other positive tested samples generated Ct-values <38. None of the samples tested negative by the real-time PCR were tested positive by the conventional PCR, and hence, the observed agreement was 97.2%. With a calculated chance agreement of 51.8%, Cohen's kappa measure of agreement was $\kappa = 0.942$.

Discussion

The three most commonly observed pathogenic viruses in wild and farmed European eel are AngHV1, EVE and EVEX (Van Beurden *et al.* 2012). These three viruses may all cause a non-pathognomonic haemorrhagic disease in European and Japanese eel. Detection of these pathogens in farmed eels is crucial for control of disease outbreaks, and helpful in sustainable restoration of the wild European eel stocks by restocking healthy farmed eels. Monitoring the prevalence of these pathogens among wild European eel populations will support general health assessment and help clarifying the potential role of these viruses in the decline of the wild European eel stocks (Haenen *et al.* 2010). Detection of pathogenic eel viruses is currently based on virus isolation in cell culture, which is laborious, time-consuming and expensive. We developed a real-time PCR assay for the fast and sensitive detection of AngHV1 in 10% w/v organ suspensions.

The primers and probe for the real-time PCR assay were based on sequences of AngHV1 ORF57 encoding the conserved capsid protease and scaffold protein. Primer and probe concentrations and annealing temperature were optimized. The assay had a high repeatability, with a constant detection of the analytical AngHV1 reference sample in triplicate 10-fold dilution series over a 6-log range. The mean coefficient of variation was <2%, the r^2-value was higher than 0.998, and the efficiency was about 90%. The analytical specificity of the assay was high, as neither EVEX, IPNV Ab nor other tested fish herpesvirus reference samples resulted in a detectable product. The analytical sensitivity of the assay for the AngHV1 reference isolate was determined to be between 3.0 and 30 $TCID_{50}$ per ml, which was comparable to the analytical sensitivity of the conventional PCR developed by Rijsewijk *et al.* (2005).

The diagnostic performance of the real-time PCR assay was determined using the results from the tested field isolates. Positive virus isolation followed by positive AngHV1 conventional PCR was considered the gold standard. The sensitivity of the real-time PCR assay was determined to be 100%, which was expected. The relatively low diagnostic specificity of 73% can be explained by the fact that PCR is generally more sensitive than cell culture based virus isolation, because of the exponential amplification of specific genetic material. Interpretation of the real-time PCR results is then based on the assumption that a positive signal equals the presence of the target species (Hiney 2001). The real-time PCR positive/virus isolation negative samples generally resulted in high Ct-values. It is therefore likely that these samples are actually target species positive, but below the detection limit of virus isolation, or that virus viability has been affected by for example preservation conditions. This view is supported by the high analytical specificity of the real-time PCR assay as determined with reference virus strains.

Although the 72 tested field samples were derived from 26 batches of eels and hence not

independent, the real-time PCR assay and the conventional PCR assay developed by Rijsewijk *et al.* (2005) showed almost perfect agreement with a Cohen's kappa measure of agreement of $\kappa = 0.942$ (Cohen 1960). Depending on the intended use of the real-time PCR assay (e.g. for AngHV1 confirmation on an eel farm, or for certifying freedom of AngHV1 in a restocking program), a cut-off Ct-value can be applied for this assay to minimize misclassification (see Caraguel *et al.* 2011). With the field data from this study, a receiver operating characteristic (ROC) analysis can be carried out (data not shown). The Ct-value that yields the best combination of diagnostic sensitivity and diagnostic specificity was calculated to be Ct 37.6. Hence, in our hands, samples with a Ct-value of $\leq$37.6 can be considered as positive. In general, compared to cell culture based virus isolation, PCR is less laborious, quicker and cheaper. Real-time PCR additionally overcomes the need for gel electrophoreses, which reduces the risk of cross-contamination.

Recently, significant progress has been made with regard to the non-culture based detection of pathogenic European eel viruses. Real-time PCR assays have been developed for the detection of EVEX (Van Beurden *et al.* 2011c), AngHV1 (this paper) and EVE (in progress) in 10% w/v suspensions of gills and internal organs. We still recommend to inoculate these suspensions on a permissive cell line to demonstrate the presence of an actual virus infection and to be able to detect less common pathogenic eel viruses as well. With the developed real-time PCR assays, the presence of the most common pathogenic European eel viruses can now be demonstrated within a day.

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References

Armitage J., Hewlett N.R., Twigg M., Lewin N.C., Reading A.J., Williams C.F., Aprahamian M., Way K., Feist S.W. & Peeler E.J. (2014) Detection of Herpesvirus anguillae during two mortality investigations of wild European eel in England: implications for fishery management. *Fisheries Management and Ecology* **21**, 1–12.

Bandin I., Souto S., Cutrin J.M., Lopez-Vazquez C., Olveira J.G., Esteve C., Alcaide E. & Dopazo C.P. (2014) Presence of viruses in wild eels *Anguilla anguilla* L, from the Albufera Lake (Spain). *Journal of Fish Diseases* **37**, 597–607.

Blake S., Ma J.Y., Caporale D.A., Jairath S. & Nicholson B.L. (2001) Phylogenetic relationships of aquatic birnaviruses based on deduced amino acid sequences of genome segment A cDNA. *Diseases of Aquatic Organisms* **45**, 89–102.

Caraguel C.G.B., Stryhn H., Gagné N., Dohoo I.R. & Hammell K.L. (2011) Selection of a cutoff value for real-time polymerase chain reaction results to fit a diagnostic purpose: analytical and epidemiologic approaches. *Journal of Veterinary Diagnostic Investigation* **23**, 2–15.

Chang P.H., Pan Y.H., Wu C.M., Kuo S.T. & Chung H.Y. (2002) Isolation and molecular characterization of herpesvirus from cultured European eels *Anguilla anguilla* in Taiwan. *Diseases of Aquatic Organisms* **50**, 111–118.

Chen S.N., Ueno Y. & Kou G.H. (1982) A cell line derived from Japanese eel (*Anguilla japonica*) kidney. *Proceedings of the National Science Council, Republic of China. Part B, Life sciences* **6**, 93–100.

Davidse A., Haenen O., Dijkstra S., Van Nieuwstadt A., Van Der Vorst T., Wagenaar F. & Wellenberg G. (1999) First isolation of herpesvirus of eel (Herpesvirus anguillae) in diseased European eel *(Anguilla anguilla* L.) in Europe. *Bulletin of the European Association of Fish Pathologists* **19**, 137–141.

Galinier R., Van Beurden S., Amilhat E., Castric J., Schoehn G., Verneau O., Fazio G., Allienne J.-F., Engelsma M., Sasal P. & Faliex E. (2012) Complete genomic sequence and taxonomic position of Eel virus European X (EVEX), a rhabdovirus of European eel. *Virus Research* **166**, 1–12.

Haenen O.L.M., Dijkstra S.G., Tulden P.W., Davidse A., Van Nieuwstadt A.P., Wagenaar F. & Wellenberg G.J. (2002) Herpesvirus anguillae (HVA) isolations from disease outbreaks in cultured European eel, *Anguilla anguilla* in the Netherlands since 1996. *Bulletin of the European Association of Fish Pathologists* **22**, 247–257.

Haenen O.L.M., Lehmann J., Engelsma M.Y., Stürenberg F.-J., Roozenburg I., Kerkhoff S. & Klein Breteler J. (2010) The health status of European silver eels, *Anguilla anguilla*, in the Dutch River Rhine Watershed and Lake IJsselmeer. *Aquaculture* **309**, 15–24.

Haenen O.L.M., Mladineo I., Konecny R., Yoshimizu M., Groman D., Muñoz P., Saraiva A., Bergmann S.M. & Van Beurden S.J. (2012) Diseases of eels in an international perspective: Workshop on Eel Diseases at the 15th International Conference on Diseases of Fish and Shellfish, Split, Croatia, 2011. *Bulletin of the European Association of Fish Pathologists* **32**, 109–115.

Hiney M. (2001) Validation of non-culture-based pathogen detection systems: theoretical problems and practical considerations. In: *Risk Analysis in Aquatic Animal Health* (ed. by C.J. Rodgers), pp. 259–264. World organisation for Animal Health, Paris, France.

Jakob E., Neuhaus H., Steinhagen D., Luckhardt B. & Hanel R. (2009) Monitoring of Herpesvirus anguillae (HVA) infections in European eel, *Anguilla anguilla* (L.), in northern Germany. *Journal of Fish Diseases* **32**, 557–561.

Kubista M., Andrade J.M., Bengtsson M., Forootan A., Jonak J., Lind K., Sindelka R., Sjoback R., Sjogreen B., Strombom L., Stahlberg A. & Zoric N. (2006) The real-time polymerase chain reaction. *Molecular Aspects of Medicine* **27**, 95–125.

Landis J.R. & Koch G.G. (1977) The measurement of observer agreement for categorical data. *Biometrics* **33**, 159–174.

Mackay I.M., Arden K.E. & Nitsche A. (2002) Real-time PCR in virology. *Nucleic Acids Research* **30**, 1292–1305.

OIE (2009) *Manual of Diagnostic Tests for Aquatic Animals*. World Organization for Animal Health, Paris.

Rijsewijk F., Pritz-Verschuren S., Kerkhoff S., Botter A., Willemsen M., Van Nieuwstadt T. & Haenen O. (2005) Development of a polymerase chain reaction for the detection of Anguillid herpesvirus DNA in eels based on the herpesvirus DNA polymerase gene. *Journal of Virological Methods* **124**, 87–94.

Sano M., Fukuda H. & Sano T. (1990) Isolation and characterization of a new herpesvirus from eel. In: *Pathology in Marine Sciences* (eds by F.O. Perkins & T.C. Cheng), pp. 15–31. Academic Press, San Diego.

Shih H.H. (2004) A polymerase chain reaction for detecting Herpesvirus anguillae in asymptomatic eels. *Taiwania* **49**, 1–6.

Shih H.H., Hu C.W. & Wang C.S. (2003) Detection of Herpesvirus anguillae infection in eel using in situ hybridization. *Journal of Applied Ichthyology* **19**, 99–103.

Van Beurden S.J., Bossers A., Voorbergen-Laarman M.H., Haenen O.L.M., Peters S., Abma-Henkens M.H., Peeters B.P., Rottier P.J. & Engelsma M.Y. (2010) Complete genome sequence and taxonomic position of anguillid herpesvirus 1. *Journal of General Virology* **91**, 880–887.

Van Beurden S.J., Forlenza M., Westphal A.H., Wiegertjes G.F., Haenen O.L.M. & Engelsma M.Y. (2011a) The alloherpesviral counterparts of interleukin 10 in European eel and common carp. *Fish and Shellfish Immunology* **31**, 1211–1217.

Van Beurden S.J., Leroy B., Wattiez R., Haenen O.L.M., Boeren S., Vervoort J.J.M., Peeters B.P.H., Rottier P.J.M., Engelsma M.Y. & Vanderplasschen A.F. (2011b) Identification and localization of structural proteins of anguillid herpesvirus 1 by mass spectrometry. *Veterinary Research* **42**, 105.

Van Beurden S.J., Voorbergen-Laarman M.A., Roozenburg I., Boerlage A.S., Haenen O.L.M. & Engelsma M.Y. (2011c) Development and validation of a two-step real-time RT-PCR for the detection of eel virus European X in European eel, *Anguilla anguilla*. *Journal of Virological Methods* **171**, 352–359.

Van Beurden S.J., Engelsma M.Y., Roozenburg I., Voorbergen-Laarman M.A., Van Tulden P.W., Kerkhoff S., Van Nieuwstadt A.P., Davidse A. & Haenen O.L. (2012) Viral diseases of wild and farmed European eel *Anguilla anguilla* with particular reference to the Netherlands. *Diseases of Aquatic Organisms* **101**, 69–86.

Van Ginneken V., Haenen O., Coldenhoff K., Willemze R., Antonissen E., Van Tulden P., Dijkstra S., Wagenaar F. & Van Thillart Den G. (2004) Presence of eel viruses in eel species from various geographic regions. *Bulletin of the European Association of Fish Pathologists* **24**, 268–272.

Van Nieuwstadt A., Dijkstra S. & Haenen O. (2001) Persistence of herpesvirus of eel Herpesvirus anguillae in farmed European eel *Anguilla anguilla*. *Diseases of Aquatic Organisms* **45**, 103–107.

Varvarigos P., Vendramin N., Cappellozza E. & Bovo G. (2011) First confirmation of Herpes Virus Anguillae (HVA) and Infectious Pancreatic Necrosis (IPN) virus infecting European eels *Anguilla anguilla* farmed in Greece. *Bulletin of the European Association of Fish Pathologists* **31**, 101–111.

Watzinger F., Ebner K. & Lion T. (2006) Detection and monitoring of virus infections by real-time PCR. *Molecular Aspects of Medicine* **27**, 254–298.

Zhang C.X. & Suzuki S. (2004) Aquabirnaviruses isolated from marine organisms form a distinct genogroup from other aquabirnaviruses. *Journal of Fish Diseases* **27**, 633–643.

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