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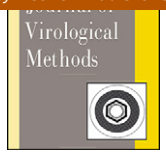

Protocol

Development and validation of a two-step real-time RT-PCR for the detection of eel virus European X in European eel, *Anguilla anguilla*

Steven J. van Beurden[a,b], Michal A. Voorbergen-Laarman[a], Ineke Roozenburg[a], Annette S. Boerlage[a,c], Olga L.M. Haenen[a], Marc Y. Engelsma[a,*]

[a] *Laboratory for Fish and Shellfish Diseases, Central Veterinary Institute, part of Wageningen UR, P.O. Box 65, 8200 AB Lelystad, The Netherlands*
[b] *Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, P.O. Box 80.165, 3508 TD Utrecht, The Netherlands*
[c] *Aquaculture and Fisheries Group, Wageningen University, part of Wageningen UR, P.O. Box 338, 6700 AH Wageningen, The Netherlands*

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A B S T R A C T

Eel virus European X (EVEX) is one of the most common pathogenic viruses in farmed and wild European eel (*Anguilla anguilla*) in the Netherlands. The virus causes a hemorrhagic disease resulting in increased mortality rates. Cell culture and antibody-based detection of EVEX are laborious and time consuming. Therefore, a two-step real-time reverse transcriptase (RT-)PCR assay was developed for rapid detection of EVEX. Primers and probe for the assay were designed based on a sequence of the RNA polymerase or L gene of EVEX. The real-time RT-PCR assay was validated both for use with SYBR Green chemistry and for use with a TaqMan probe. The assay is sensitive, specific, repeatable, efficient and has a high r^2-value. The real-time RT-PCR assay was further evaluated by testing field samples of European eels from the Netherlands, which were positive or negative for EVEX by virus isolation followed by an indirect fluorescent antibody test. The real-time RT-PCR assay allows rapid, sensitive and specific laboratory detection of EVEX in RNA extracts from 10% eel organ suspensions and cell cultures with cytopathic effects, and is a valuable contribution to the diagnosis of viral diseases of eel.

1. Introduction

Production of eel (*Anguilla* spp.) for consumption is based entirely on wild catches of glass eels or elvers, as artificial reproduction of eel in culture is not possible currently on a commercial scale. This leads to the constant introduction of disease agents in the aquaculture producing systems. European eel (*Anguilla anguilla*) is produced generally in intensive recirculation systems with a regulated water temperature. The high stocking densities make detection and control of diseases vital for sustainable farming. The three viral agents that are observed regularly in farmed European eel in the Netherlands are: *Anguillid herpesvirus 1* (AngHV-1), member of the genus *Cyprinivirus*, family *Alloherpesviridae*, order *Herpesvirales* (Sano et al., 1990; van Beurden et al., 2010); *Infectious pancreatic necrosis virus* of eel, also known as eel virus European (EVE), member of the genus *Aquabirnavirus*, family *Birnaviridae* (Sano, 1976; Sano et al., 1981); and eel virus European X (EVEX), a rhabdovirus from eel.

The first rhabdovirus from eel was diagnosed in Japan in young American eel (*Anguilla rostrata*) by Sano (1976) and designated eel virus American (EVA). A related rhabdovirus, EVEX, was isolated later from European elvers (Sano et al., 1977). EVA and EVEX are highly similar in morphological (Nishimura et al., 1981), serological (Hill et al., 1980; Nishimura et al., 1981) and physicochemical characteristics (Hill et al., 1980), and were proposed to be two strains of a single virus species (Hill et al., 1980). Preliminary molecular comparison of partial sequences from the RNA polymerase or L gene from EVEX and EVA reference isolates confirms the existence of two lineages with 91.5% sequence identity over a 2040 bp fragment (M.Y. Engelsma, unpublished results). The virus has been tentatively placed in the genus *Vesiculovirus*, family *Rhabdovirus*, order *Mononegavirales* (Castric et al., 1984; Hill et al., 1980).

Other eel rhabdoviruses have been described since and include five rhabdovirus isolates found in elvers of the Loire estuary (Castric et al., 1984). Three isolates were serologically similar or closely related to EVEX, the two other isolates were classified as lyssaviruses. In 1992, a rhabdoviral disease characterized by cutaneous erosion and ulceration was observed in Japanese eel (*Anguilla japonica*) and designated rhabdoviral dermatitis (Kobayashi and Miyazaki, 1996). In serological tests the virus was similar to EVEX and EVA. Neither EVA nor rhabdoviral dermatitis has ever been reported in eels in Europe, nor has the susceptibility of European eels for these viruses been determined experimentally.

* Corresponding author. Tel.: +31 320 238729; fax: +31 320 238153.
E-mail address: marc.engelsma@wur.nl (M.Y. Engelsma).

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Table 1
Virus isolates used for validation of the EVEX real-time RT-PCR assay.

	Virus	Isolate	Origin	Date of intake[a]
EVEX reference strains	EVEX	153311	CVI[b]	October 1992
	EVEX	J6BF4	B.J. Hill	November 1986
EVA reference strains	EVA	Y.H.	N. Okamoto	December 1999
	EVA	J6BF4	B.J. Hill	November 1986
Eel virus reference strains	AngHV-1	500138	CVI	May 1998
	EVE	421218	CVI	April 1997
Fish vesiculovirus reference strains	SVCV	Unknown	P. de Kinkelin	July 1987
	PFRV	Unknown	B.J. Hill	March 1998
	Perch rhabdovirus	Unknown	L. Bigarre	July 2010
Dutch EVEX isolates	EVEX	108778	CVI	June 1990
	EVEX + AngHV-1	396702	CVI	August 1996
	EVEX	459083	CVI	September 1997
	EVEX	574597	CVI	January 2001
Virus positive Dutch eel samples	EVEX	313006	CVI	December 1994
	EVEX + AngHV-1	08003252	CVI	February 2008
	EVEX + EVE	09009703	CVI	June 2009
	AngHV-1	08009225	CVI	April 2008
	EVE	05007688	CVI	April 2005
	AngHV-1 + EVE	06029463-1	CVI	October 2006
Negative Dutch eel samples	–	05017527-1	CVI	September 2005
	–	05018319-1	CVI	October 2005
	–	05018401-1	CVI	October 2005
	–	05020305-1	CVI	November 2005
	–	05020752-1	CVI	November 2005
	–	05021787-1	CVI	December 2005

[a] Date of arrival of the sample or virus reference isolate at the Central Veterinary Institute (CVI), part of Wageningen UR, in Lelystad, the Netherlands.
[b] Virus isolated or eel organ suspension prepared at the CVI from farmed or wild European eels originating from the Netherlands.

EVEX is widespread in the European eel population, as it has been detected in eels originating from Germany (Ahne and Thomsen, 1985; Shchelkunov et al., 1989), France (Castric and Chastel, 1980; Castric et al., 1984; Jørgensen et al., 1994), Denmark, the United Kingdom, Sweden (Jørgensen et al., 1994) and the Netherlands and Italy (van Ginneken et al., 2004). Infection with EVEX is not always accompanied by clinical signs (Castric and Chastel, 1980; Castric et al., 1984; Jørgensen et al., 1994; Sano et al., 1977; van Ginneken et al., 2004), but may result in a severe hemorrhagic disease with significant mortality (Shchelkunov et al., 1989; van Ginneken et al., 2005).

The gross pathology of viral diseases in eel is non-pathognomonic and there is a possibility of double infections with two viruses (Ahne and Thomsen, 1985; Haenen et al., 2002; Jørgensen et al., 1994; van Ginneken et al., 2004), which necessitates specific diagnostic testing for identification of the pathogen. A limited number of antibody-based assays have been described to identify EVEX, such as the enzyme linked immuno-sorbent assay by Dixon and Hill (1984). Only after identification of the causative agent can adequate quarantine and water temperature regulation measures be implemented to control the virus outbreak in farmed European eel. During an EVEX outbreak, raising the water temperature to a non-permissive temperature for the virus has proven successful in reducing clinical signs and eel losses (O.L.M. Haenen, personal communication).

Compared with antibody based tests used to detect EVEX, a real-time reverse transcriptase (RT-)PCR has several major advantages. Real-time PCR is a rapid and sensitive detection method, with a reduced risk of cross-contamination compared to conventional PCR (Mackay et al., 2002). RNA extraction and real-time RT-PCR are relatively easy to perform, and the presence of a virus can be demonstrated within a day. The aim of this study was to develop a real-time RT-PCR assay for the detection of EVEX in European eel. The sensitivity and specificity of the assay were tested with several virus isolates. The real-time RT-PCR assay was shown to be an effective diagnostic tool for the detection of EVEX in RNA extractions from 10% eel organ suspensions or EVEX-infected cell cultures showing a cytopathic effect (cpe).

2. Materials and methods

2.1. Virus isolates

EVEX strain 153311 was isolated at the Central Veterinary Institute (CVI), part of Wageningen UR, in Lelystad, the Netherlands, from farmed eel in 1992. The genome of this virus isolate was sequenced completely (R. Galinier, unpublished results), enabling primer and probe design for the real-time PCR assay. EVEX isolates 153311 and J6BF4, and EVA isolates Y.H. and J6BF4, were used as reference virus strains (Table 1). Four Dutch eel virus isolates, which were positive for EVEX by an indirect fluorescent antibody test (IFAT), and the Dutch reference strains AngHV-1 500138 and EVE 421218 were used to determine the specificity with regard to European eel viruses. *Spring viremia of carp virus* (SVCV), pike fry rhabdovirus (PFRV) and perch rhabdovirus were used to determine the specificity with regard to other fish vesiculoviruses. Eel kidney cell line material was used as a negative control. Virus isolation followed by an IFAT was used as the gold standard test for the detection and identification of EVEX.

2.2. Cell and virus culture

The eel kidney cell line EK-1 (Chen et al., 1982) was used to propagate EVEX. Cells were cultured in monolayers in sterile plastic culture flasks (Falcon, BD Biosciences, Bedford, MA, USA) with cell culture medium, consisting of Leibovitz's L-15 medium (Gibco, Invitrogen, Carlsbad, CA, USA), supplemented with 5% (v/v) fetal bovine serum (FBS, Bodinco, Alkmaar, The Netherlands), 0.075% (w/v) sodium bicarbonate ($NaHCO_3$, Gibco), 2 mM L-glutamine (Gibco), 0.005% (w/v) gentamicin (Gibco) and

40 μM 2-mercaptoethanol (Gibco). For cell culture on 6-well and 96-well plates (Cellstar, Greiner bio-one, Frickenhausen, Germany) 0.26% (w/v) $NaHCO_3$ was used instead of 0.075% (w/v). Cells were incubated at 26 °C in a 5% CO_2-incubator (Nuaire, Plymouth, MN, USA).

EVEX and the other eel viruses were propagated in 1–2 days old ~80% confluent EK-1 monolayers in sterile plastic culture flasks with virus growth medium, consisting of Leibovitz's L-15 medium with 2% (v/v) FBS, 0.075% (w/v) $NaHCO_3$, 2 mM L-glutamine and antibiotics (0.012% (w/v) kanamycin (Sigma–Aldrich, St. Louis, MO, USA) and 270 IE/ml penicillin G (Astellas Pharma, Tokyo, Japan)) in a 5% CO_2-incubator. For virus propagation on 6-well and 96-well plates 0.26% (w/v) $NaHCO_3$ was used instead of 0.075% (w/v). AngHV-1 was propagated at 26 °C, EVEX was propagated at 20 °C and EVE was propagated at 15 °C.

To test the assay's performance under practical conditions, EVEX positive and negative diagnostic field samples of European eels from the Netherlands were used, consisting of 10% organ suspensions and cpe positive cell cultures. Samples of gill tissue and pooled samples of liver, spleen and kidney tissues were ground with sterile pestle, mortar, sand and diluted with virus isolation medium, consisting of Leibovitz's L-15 medium with 2% (v/v) FBS and antibiotics, to prepare a 10% (w/v) clarified and filtered organ suspension according to Rijsewijk et al. (2005). Three hundred microlitres of filtered and unfiltered 10% organ suspensions were diluted with 2 ml of virus growth medium and inoculated onto ~80% confluent EK-1 monolayers in 6-well plates in a 5% CO_2-incubator at 15 °C, 20 °C and 26 °C. After 1 h the inoculum was replaced by 5 ml of fresh virus growth medium. The cell cultures were incubated and checked daily for the appearance of cpe using an inverted light microscope (Olympus, Beckman Coulter, Brea, CA, USA). If no cpe developed after 7–10 days, the cell cultures were frozen at −80 °C for at least 4 h, after which a passage was performed. For that 2 ml of thawed medium from the inoculated cell cultures was diluted with 1 ml of virus growth medium and inoculated onto fresh EK-1 cell monolayers at the respective temperatures for 1 h. The inoculum was then replaced with 5 ml of fresh virus growth medium, and the cell cultures were incubated for another 7–10 days. If no cpe developed after two passages, the 10% organ suspensions were considered virus negative.

2.3. Virus titration

The titre of EVEX isolate 153311 was determined by a 10-fold titration. Fifty microlitres of 10-fold serial dilutions were added in 12 replicates to ~80% confluent EK-1 monolayers in 96-wells plates and incubated for 1 h at 20 °C, after which 100 μl virus growth medium was added to each well. After 7 days the monolayers were scored for typical cpe using an inverted light microscope. The titre was expressed in the 50% tissue culture infectious dose per ml ($TCID_{50}$/ml) as calculated by the Spearman–Kärber method.

2.4. Indirect fluorescent antibody test

Fifty microlitres of a 10-fold serial dilution of the isolated virus and an EVEX reference isolate as a control were added to ~80% confluent EK-1 monolayers in 96-well plates. After incubation of the plates for 1 h at 20 °C, 100 μl virus growth medium was added per well. After further incubation for 48 h at 20 °C the medium was decanted and the plates were rinsed once with PBS pH 7.2. The cells were then fixed by drying the plates for 2 h at room temperature, freezing for 1 h at −20 °C and adding 100 μl 10% (v/v) formalin in PBS pH 7.2 to each well. After 10 min at room temperature, the formalin was decanted and the plates were rinsed once with PBS pH 7.2. Fifty microlitres of rabbit-anti-EVEX antiserum K12 Denmark 2.2.87 diluted 1:500 in PBS pH 7.2 was added per well. The plates were incubated for 1 h at 37 °C followed by rinsing twice with PBS pH 7.2. Fifty microlitres of fluorescein-isothiocyanate (FITC) conjugated swine-anti-rabbit polyclonal antibody (Dako, Glostrup, Denmark) diluted 1:50 in PBS pH 7.2 was added per well and the plates were incubated for 30 min at 37 °C. Finally, the plates were rinsed three times with PBS pH 7.2 and examined at 495 nm using a fluorescence microscope (Carl Zeiss, Oberkochen, Germany). Virus isolates were diagnosed positive for EVEX if cells showing a bright green granular fluorescence of the cytoplasm around the nucleus were observed (Fig. 1A). The number of fluorescent cells should decrease with the dilution to approve the test result. Virus isolates were diagnosed negative for EVEX if no green fluorescent cells were observed (Fig. 1B).

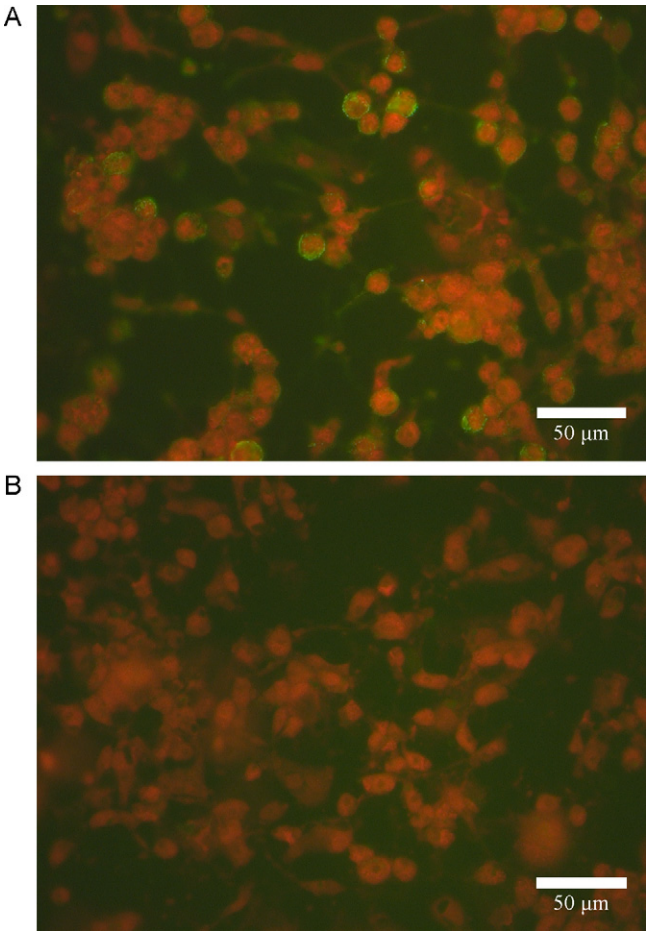

Fig. 1. Immunofluorescent staining of EVEX isolate 153311 in EK-1 cells. The IFAT was performed as described in Section 2.4. For photograph purposes the IFAT was performed on Lab-Tek Permanox Chamber Slides (Nunc, Thermo Fisher Scientific, Waltham, MA, USA). Cell nuclei were stained red with 10 μg/ml propidium iodide (Sigma–Aldrich). Cells were embedded with Glycergel Mounting Medium (Dako). Photographs were taken at 495 nm using an Axioskop 40 fluorescence microscope (Carl Zeiss) at 48 h post-infection (400×). (A) EVEX infected EK-1 cells. (B) Non-infected EK-1 cells. (For interpretation of the references to color in this figure legend and the corresponding text, the reader is referred to the web version of the article.)

2.5. RNA extraction

Total RNA was extracted from 10% organ suspensions and EK-1 cell cultures freeze–thawed at −80 °C. One hundred microlitres of cell or organ suspension was added to 600 μl buffer RTL containing 6 μl 2-mercaptoethanol (Sigma–Aldrich) and the suspension was homogenized by vortexing and pipetting. RNA was extracted using a QIAamp RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturers protocol for animal cells using spin tech-

nology. After extraction and washing the RNA was eluted in 50 μl RNase-free water (Qiagen), 1 μl RNaseOUT Ribonuclease Inhibitor (Invitrogen) was added and the RNA was stored at −80 °C.

2.6. Primer and probe design

The target sequence for the real-time PCR assay was based on the RNA polymerase or L-gene of EVEX isolate 153311 (EMBL Bank ID: FN557213). Based on this sequence, forward primer EVEX.F04 (5′-GGACAAGGAGAGGATGATTACGA-3′), reverse primer EVEX.R04 (5′-AAATTGTCCCATGACTCTGAACAC-3′) and VIC labelled probe EVEX.p04 (VIC-ACAACCATCAAAGGAAAGAGTCTAACGGCC-TAMRA) were designed using the Primer Express version 3.0 program (Applied Biosystems, Life Technologies, Carlsbad, CA, USA). The primers generated an amplicon of 120 bp. The primers were obtained from Eurogentec (Seraing, Belgium) and the probe from Applied Biosystems.

2.7. Reverse transcription

Reverse transcription (RT) was carried out using the TaqMan Reverse Transcriptase Reagent kit (Applied Biosystems). The total volume of the RT mix was 50 μl per reaction, containing 5 μl RT buffer (10×), 11 μl $MgCl_2$ (25 mM), 10 μl dNTP mixture (2.5 mM of each dNTP), 2.5 μl Random Hexamers (50 μM), 1 μl RNase Inhibitor (20 U/μl), 1.25 MultiScribe Reverse Transcriptase (50 U/μl), 14.25 μl DNase and RNase free water (Sigma–Aldrich) and 5 μl template, and was prepared on ice. The thermal profile of the RT program consisted of 10 min incubation at 25 °C, 30 min RT at 48 °C, 5 min RT inactivation at 95 °C and cooling down to 4 °C, and was performed in a 96-well GeneAmp PCR System 9700 (Applied Biosystems). A RNA sample from an EVEX reference isolate was included as a positive control in every RT reaction. The resulting complementary DNA (cDNA) was stored at −20 °C.

2.8. Preparation of a standard serial dilution series

In order to determine the detection limit, assay precision, square of the correlation coefficient (r^2) and efficiency, standard curves were generated. Four separate RNA extractions and RT reactions of EVEX isolate 153311 were performed and 10-fold serial dilution series in DNase and RNase free water were made. Standard curves were generated from three single cDNA serial dilution series prepared from separately extracted and reverse transcribed RNA, and from a single cDNA serial dilution series tested in triplicate.

2.9. SYBR Green real-time PCR

The total volume of the SYBR Green real-time PCR mix was 20 μl per reaction, containing 10 μl SYBR Green PCR Mix (Applied Biosystems), 0.8 μl forward primer EVEX.F04 (10 μM), 0.8 μl reverse primer EVEX.R04 (10 μM), 0.25 μl Uracil-DNA Glycosylase (UDG, 5 U/μl, New England Biolabs, Ipswich, MA, USA), 3.15 μl water and 5 μl template. In every real-time PCR plate a cDNA sample from an EVEX reference isolate and a water sample were included as positive and negative control, respectively. The thermal profile of the SYBR Green real-time PCR program consisted of 10 min at 37 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C, and a dissociation stage at the end of the run from 60 °C to 95 °C, and was carried out in a 7500 Fast Real-Time PCR system (Applied Biosystems) under Standard 7500 conditions.

2.10. TaqMan real-time PCR

The total volume of the TaqMan real-time PCR mix was 20 μl per reaction, containing 10 μl TaqMan Fast Universal PCR Master Mix (2×), 0.8 μl forward primer EVEX.F04 (10 μM), 0.8 μl reverse primer EVEX.R04 (10 μM), 0.8 μl probe EVEX.p04 (5 μM), 0.25 μl UDG, 2.75 μl water and 5 μl template. The thermal profile of the TaqMan real-time PCR program consisted of 10 min at 37 °C, 10 min at 95 °C and 40 cycles of 3 s at 95 °C and 30 s at 60 °C, and was carried out in a 7500 Fast Real-Time PCR system under Fast 7500 conditions. A positive and a negative control were included in every run.

2.11. Real-time PCR data analyses

The quantitation plots, melting curves and standard curves were analyzed using the Sequence Detection Software version 1.4 program (Applied Biosystems) with the Auto baseline function. After evaluation of the threshold set by the Auto Ct function, the threshold was manually set at 0.20 for the SYBR Green real-time PCR assay and at 0.05 for the TaqMan real-time PCR assay. Slope and r^2 were calculated from the standard curves using the Microsoft Excel 2003 program (Microsoft, Redmond, WA, USA). Efficiency was calculated from the slope using the formula: efficiency = $10^{-1/\text{slope}} - 1$. Assay precision was assessed as intra-assay and inter-assay variability expressed as the mean coefficient of variation and calculated from three 10-fold serial dilution series.

3. Results

3.1. Primer performance and optimization

The primer and probe concentrations for the SYBR Green and TaqMan real-time PCR assays were optimized using a checkerboard system. The specificity of the primers was analyzed in a SYBR Green setting by melting curve analysis and by gel electrophoresis. Positive samples always generated a single peak in the melting curve analysis and a single band of the expected amplicon size of 120 bp in the gel electrophoresis. Negative samples did not result in a PCR product indicating that no additional products, such as self-primers or cross-primers, were formed. The mean and the standard deviation of the melting temperatures (T_m) of the SYBR Green real-time PCR assay were calculated based on two EVEX reference strains, four Dutch virus isolates and a series of positive field samples (Tables 1 and 3), and determined to be 78.6 ± 0.5 °C.

3.2. Sensitivity

The detection limit of the SYBR Green and the TaqMan real-time RT-PCR assay for detecting EVEX was determined by testing for the lowest detectable virus titre, expressed as $TCID_{50}$/ml. The titre of EVEX isolate 153311 as determined by 10-fold titration appeared to be $10^{7.3}$ or 2.0×10^7. In the triplicate 10-fold dilution series of the viral cDNA, both the SYBR Green and the TaqMan real-time PCR assay were able to detect EVEX down to 20 $TCID_{50}$/ml.

3.3. Specificity

The specificity of the assay was determined by testing two EVEX and two EVA reference strains, four Dutch EVEX isolates (confirmed as positive by IFAT), two other European eel viruses, three related fish vesiculoviruses, and EK-1 cell line material as negative control. Both the SYBR Green and the TaqMan real-time RT-PCR assay readily detected the EVEX reference strains and the Dutch EVEX isolates (Table 2). The two EVA reference strains were detected but with higher cycle threshold (Ct-) values. No PCR products were generated from AngHV-1, EVE, SVCV, PFRV, perch rhabdovirus or the negative control (Table 2).

Table 2
Specificity of the EVEX real-time RT-PCR assay.

	Virus isolates		SYBR Green		TaqMan
	Virus	Isolate	Ct	T_m	Ct
EVEX reference strains	EVEX	153311	13.83	79.0	14.93
	EVEX	J6BF4[a]	21.26	77.0	19.28
EVA reference strains	EVA	Y.H.	29.34	78.1	32.70
	EVA	J6BF4	28.22	78.1	31.40
Eel virus reference strains	AngHV-1	500138	–	–	–
	EVE	421218	–	–	–
Fish vesiculovirus reference strains	SVCV	De Kinkelin	–	–	–
	PFRV	Hill	–	–	–
	Perch rhabdovirus	Bigarre	–	–	–
Negative control	–	–	–	–	–
Dutch EVEX isolates	EVEX	108778	14.73	78.4	15.40
	EVEX + AngHV-1	396702	14.50	78.7	15.50
	EVEX	459083	15.33	78.4	16.37
	EVEX	574597	15.84	79.0	17.00

[a] EVEX reference isolate J6BF4 was diluted 10^{-1} before RNA was extracted.

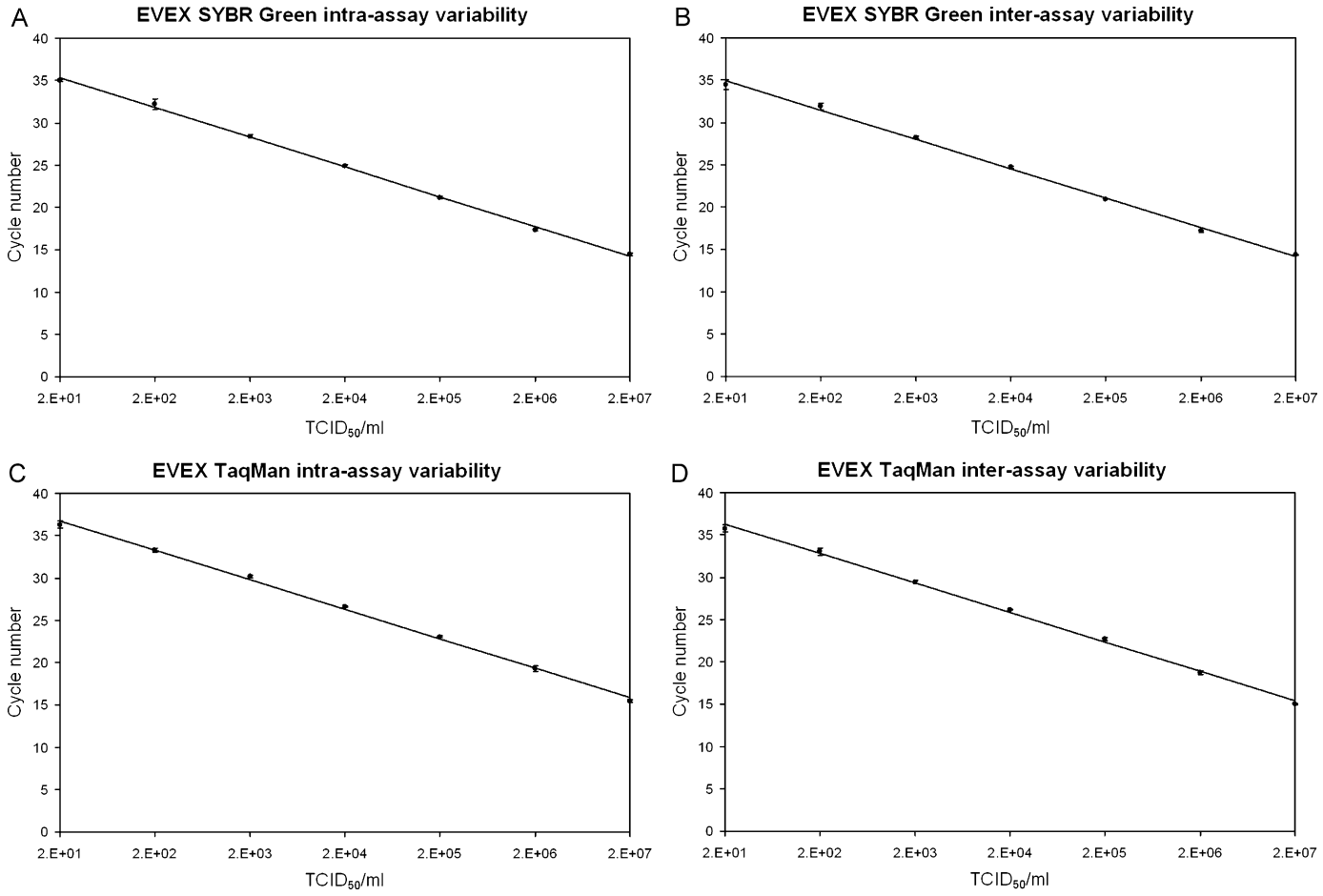

Fig. 2. Standard curves of the EVEX real-time RT-PCR assay in the dynamic range from 2.0×10^1 to 2.0×10^7 $TCID_{50}$/ml. (A) EVEX SYBR Green intra-assay variability. (B) EVEX SYBR Green inter-assay variability. (C) EVEX TaqMan intra-assay variability. (D) EVEX TaqMan inter-assay variability.

Table 3
Assay precision, efficiency and linear correlation of the EVEX real-time RT-PCR assay.

Assay	Precision	Coefficient of variation (%)	Linear correlation (r^2)	Efficiency (%)
SYBR Green	Intra-assay	0.86 ± 0.53	0.9986	92.3
	Inter-assay	0.72 ± 0.54	0.9977	94.4
TaqMan	Intra-assay	0.81 ± 0.45	0.9980	93.6
	Inter-assay	0.82 ± 0.48	0.9977	93.7

3.4. Assay precision

Standard curves for the SYBR Green and the TaqMan real-time RT-PCR assay were generated from 10-fold dilution series of viral RNA reverse transcribed into cDNA. For the intra-assay variability three separate RNA isolations and subsequent reverse transcriptions were tested once by both real-time PCR assays in three separate runs. For the inter-assay variability one RNA isolation and subsequent reverse transcription was tested by both real-time PCR assays in triplicate in a single run. Fig. 2A–D shows the respective standard curves of the SYBR Green and the TaqMan real-time RT-PCR assay in the dynamic range, ranging from a 10^0 to a 10^{-6} dilution or from 2.0×10^7 to 2.0×10^1 $TCID_{50}$/ml. Assay precision was determined based on the mean Ct-values per serial dilution, the standard deviation and the coefficient of variation (data not shown), and expressed as the mean coefficient of variation and its standard deviation. The intra- and inter-assay variability appeared to be <1% for both the SYBR Green and the TaqMan real-time RT-PCR assay (Table 3).

3.5. Linear correlation and efficiency

The linear correlation, as determined by calculating the r^2 from the standard curves, is shown in Table 3. The r^2-values for the SYBR Green and the TaqMan real-time RT-PCR assay appeared to be >0.997 in both the intra- and the inter-assay experiments. The amplification efficiency as calculated from the slope of the standard curves is also shown in Table 3. The SYBR Green real-time RT-PCR assay had an efficiency of 92.3% and 94.4% for the intra- and inter-assay standard curve, respectively, the TaqMan real-time RT-PCR assay had an efficiency of 93.6% and 93.7% for the intra- and inter-assay standard curve, respectively.

3.6. Field samples

Ten percent gill and/or 10% internal organ suspensions and respective cpe positive cell cultures of three EVEX positive field samples, either with or without a second pathogenic eel virus, were detected by both the SYBR Green and the TaqMan real-time RT-PCR assay (Table 4). Only the 10% gill suspension and not the 10% internal organ suspension of field isolate 09009703 was positive by the IFAT. A weak signal was detected by the SYBR Green assay in the 10% organ suspension of this field isolate, but not in the virus culture of the internal organs. Gill and/or internal organ suspensions and respective virus cultures of AngHV-1 and/or EVE positive field samples were tested negative by both assays. No detectable PCR products were generated in gill and internal organ suspensions of six virus negative field samples.

4. Discussion

EVEX is one of the three common pathogenic viruses in farmed and wild European eels and is responsible for a hemorrhagic disease and increased mortality rates. Fast detection of this virus is essential in order to be able to take adequate quarantine and water temperature regulation measures to control an outbreak in eel culture systems. Traditional antibody-based tests to identify EVEX, such as the IFAT described in this paper, are laborious and depend on virus isolation which is time consuming. In this study a real-time RT-PCR assay for the detection of EVEX in European eel was described.

The primer and probe concentrations for the real-time PCR assay were optimized and the primers were shown to be specific, resulting in a single PCR product with a constant melting temperature. The real-time RT-PCR assay was shown to be sensitive since it was able to detect EVEX down to 20 $TCID_{50}$/ml. The EVEX reference strains and all Dutch EVEX isolates tested positive by IFAT were detected by the assay. The assay was shown to be specific since there was no cross-reaction with other common European eel viruses or with related fish vesiculoviruses. The two EVA reference isolates resulted in detectable PCR products, but with generally higher Ct-values. This lower sensitivity for the detection of EVA, which has never been reported in European eels up to date, is most likely due to the sequence variation at the targeted area (Fig. 3).

The assay characteristics were determined using a cultured EVEX virus strain in the dynamic range 2.0×10^1 to 2.0×10^7 $TCID_{50}$/ml. The mean coefficient of variation for both the intra- and inter-assay variability of the SYBR Green and the TaqMan real-time RT-PCR assay are very low, indicating that results are highly repeatable. The standard curves resulted in high r^2-values, indicating that results in the dynamic range show a high degree of linear correlation. The efficiency of the real-time RT-PCR was determined using the slopes of the standard curves and found to be high.

The real-time RT-PCR assay was developed for use with SYBR Green chemistry or with a TaqMan probe. In SYBR Green based real-time PCR a detectable signal is generated by the binding of an intercalating fluorescent dye to the double stranded PCR fragments (Mackay et al., 2002). This real-time PCR variant is quite easy to develop, relatively inexpensive in use and suffers less from template sequence variation, however, the thermal profile is relatively long and includes a dissociation stage necessary to evaluate the generated PCR product. In TaqMan probe-based real-time PCR a detectable signal is generated by the binding and subsequent degradation of a fluorescent probe specific for the amplicon. This real-time PCR variant can be run within an hour, but is more difficult to develop, more expensive in use and sequence variation may hamper hybridization of the probe. The described real-time RT-PCR assay for the detection of EVEX in European eel using the EVEX.04 primerset was shown to be specific, precise, efficient and correlated linearly when run as SYBR Green or as TaqMan probe based assay. The SYBR Green based assay repeatedly generated Ct-values about one cycle below the TaqMan probe-based assay, indicating a somewhat higher sensitivity. However, since both assays are able to detect EVEX in a dynamic range of 6 logs down to a virus concentration of 20 $TCID_{50}$/ml, both assays can be considered equally useful in practice.

The performance of the real-time RT-PCR assay under practical conditions was evaluated by testing a series of field samples of clinically healthy and diseased European eels from the Netherlands. All organ suspensions and virus positive cell cultures that tested positive for EVEX by the IFAT also tested positive by both the SYBR Green and the TaqMan real-time RT-PCR assay, indicating a high diagnostic sensitivity. All but one of the field samples that tested negative for virus presence in cell culture or tested negative for EVEX by the IFAT also tested negative by the real-time PCR assay, indicating a high diagnostic specificity. In the SYBR Green real-time RT-PCR assay, which had a slightly lower detection limit than the TaqMan assay, a weak PCR signal with a correct T_m was generated in the 10% organ suspension of field isolate 09009703, of which only the culture of the 10% gill suspension was EVEX positive in the IFAT. This indicates that in this case the concentration of EVEX in the internal organs was below the detection limit of cell culture-based virus isolation, or that the presence of EVEX was masked by the overgrowth of AngHV-1.

The detection limit of the real-time PCR assay of 20 $TCID_{50}$/ml is comparable to the sensitivity of cell culture-based virus isolation, and was confirmed by the testing of EVEX positive and negative field isolates. It is important to remember, however, that PCR-based techniques only demonstrate the presence of specific genetic material and not the existence of infectious virus particles or an infection (Hiney, 2001). Nevertheless, the validation of the real-time PCR assay for the detection of EVEX as described in this paper shows

Table 4
Eel virus positive and negative field samples tested by the EVEX real-time RT-PCR assay.

Field samples			IFAT	SYBR Green		TaqMan
Isolate	Virus	Diagnostic material[a]		Ct	T_m	Ct
313006	EVEX	10% organs	N.D.[b]	30.95	78.4	33.11
		Organs on EK-1	Positive	14.53	78.7	15.60
08003252	EVEX + AngHV-1	10% gills	N.D.	17.06	78.7	18.55
		10% organs	N.D.	30.54	78.7	31.58
		Gills on EK-1	Positive	12.40	78.7	14.31
		Organs on EK-1	Positive	13.27	78.7	15.73
09009703	EVEX + EVE	10% gills	N.D.	25.14	79.0	26.27
		10% organs	N.D.	36.02	79.0	–
		Gills on EK-1	Positive	14.75	79.3	15.88
		Organs on EK-1	Negative	–	–	–
08009225	AngHV-1	10% gills	N.D.	–	–	–
		10% organs	N.D.	–	–	–
		Gills on EK-1	Negative	–	–	–
		Organs on EK-1	Negative	–	–	–
05007688	EVE	10% organs	N.D.	–	–	–
		Organs on EK-1	Negative	–	–	–
06029463-1	AngHV-1 + EVE	10% gills	N.D.	–	–	–
		10% organs	N.D.	–	–	–
		Gills on EK-1	Negative	–	–	–
		Organs on EK-1	Negative	–	–	–
05017527-1	–	10% gills	N.D.	–	–	–
		10% organs	N.D.	–	–	–
05018319-1	–	10% gills	N.D.	–	–	–
		10% organs	N.D.	–	–	–
05018401-1	–	10% gills	N.D.	–	–	–
		10% organs	N.D.	–	–	–
05020305-1	–	10% gills	N.D.	–	–	–
		10% organs	N.D.	–	–	–
05020752-1	–	10% gills	N.D.	–	–	–
		10% organs	N.D.	–	–	–
05021787-1	–	10% gills	N.D.	–	–	–
		10% organs	N.D.	–	–	–

[a] 10% organs: 10% suspension of internal organs liver, spleen and kidney; organs on EK-1: 10% internal organ suspension inoculated on EK-1 cells; 10% gills: 10% suspension of gills; gills on EK-1: 10% gill suspension inoculated on EK-1 cells.
[b] N.D.: not done, only cpe positive cell cultures and not 10% organ suspensions were tested for the presence of EVEX by the IFAT.

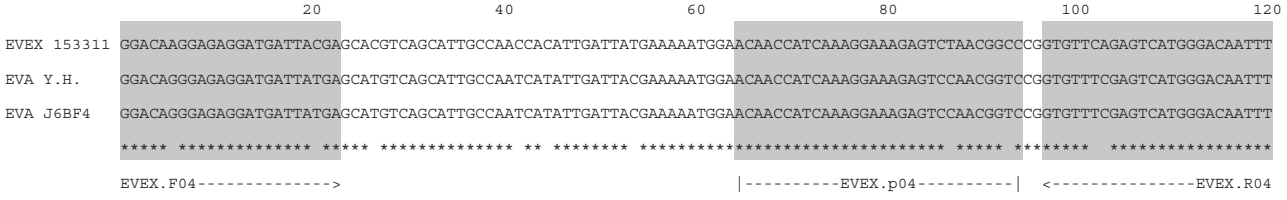

Fig. 3. Nucleotide sequence alignment of the RNA polymerase or L gene sequences targeted by the EVEX real-time RT-PCR from EVEX isolate 153311 and EVA isolates Y.H. and J6BF4. Asterisks at the bottom of the alignment indicate consensus nucleotide sequences. The primer and probe regions are boxed and indicated.

that a positive PCR signal originates from specific EVEX RNA, and supports the assumption that a positive PCR signal correlates with the presence of infectious EVEX virions.

When a disease outbreak occurs in farmed eel stocks, suddenly increased mortality rates and hemorrhagic clinical signs may indicate the presence of a pathogenic virus. For virus isolation 10% suspensions of gills and internal organs should be prepared and inoculated on a permissive cell line, preferably from eel origin. Using the conventional PCR described to detect AngHV-1 (Rijsewijk et al., 2005) and the real-time PCR assay to detect EVEX (this paper), the presence of these viruses can be demonstrated within a day, allowing adequate control measures to be taken. However, classical virus isolation remains essential in order to prove the presence of infectious virus particles and not to overlook the presence of other eel viruses. The development of (real-time RT-)PCR assays for other common eel viruses, such as EVE, would support the fast non-culture-based molecular detection of pathogenic viruses in eel.

In summary, a two-step real-time RT-PCR for the detection of EVEX in European eel was developed. The real-time RT-PCR assay was shown to be sensitive, specific, repeatable, efficient and correlated linearly when used with either SYBR Green or a TaqMan probe. The assay is a valuable diagnostic tool for the rapid detection of EVEX in European eel.

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