NOTE

Comparison of PCR methods for the detection of genetic variants of carp edema virus

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ABSTRACT: The infection of common carp and its ornamental variety, koi, with the carp edema virus (CEV) is often associated with the occurrence of a clinical disease called 'koi sleepy disease'. The disease may lead to high mortality in both koi and common carp populations. To prevent further spread of the infection and the disease, a reliable detection method for this virus is required. However, the high genetic variability of the CEV *p4a* gene used for PCR-based diagnostics could be a serious obstacle for successful and reliable detection of virus infection in field samples. By analysing 39 field samples from different geographical origins obtained from koi and farmed carp and from all 3 genogroups of CEV, using several recently available PCR protocols, we investigated which of the protocols would allow the detection of CEV from all known genogroups present in samples from Central European carp or koi populations. The comparison of 5 different PCR protocols showed that the PCR assays (both end-point and quantitative) developed in the Centre for Environment, Fisheries and Aquaculture Science exhibited the highest analytical inclusivity and diagnostic sensitivity. Currently, this makes them the most suitable protocols for detecting viruses from all known CEV genogroups.

KEY WORDS: Carp edema virus · Koi sleepy disease · PCR · Common carp · Koi

INTRODUCTION

The infection of common carp and its ornamental variety, the koi, with the carp edema virus (CEV) is often associated with the occurrence of a clinical disease called 'koi sleepy disease' (KSD) (Way & Stone 2013). The virus mainly affects the gills of infected fish, where it causes epithelial lesions and oedema (Miyazaki et al. 2005). The disease is associated with lethargic behaviour, with affected individuals characteristically lying at the bottom of the tank. The disease may lead to a high mortality in both koi and common carp populations (Way & Stone 2013, Bachmann & Keilholz 2016). The number of countries in which infections with CEV and outbreaks of KSD were detected in carp or koi has been rising rapidly over the last several years. Currently, the virus has been recorded in several European countries (Way & Stone 2013, Jung-Schroers et al. 2015, Lewisch et al. 2015, Matras et al. 2017), the USA (Hedrick et al. 1997), and from at least 3 countries in Asia: Japan (Oyamatsu et al. 1997), India (Swaminathan et al. 2016) and China (Zhang et al. 2017).

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Information on CEV is scarce. Several electron microscope images have documented poxvirus-like particles in the infected gill epithelium (Miyazaki et al. 2005). However, despite many attempts, the cultivation of CEV *in vitro* has not been successful employing the fish cell lines presently available (Jung-Schroers et al. 2015, Lewisch et al. 2015, Swaminathan et al. 2016). Genomic data of the virus is limited to the fragment of the DNA sequence encoding the core protein P4a (Oyamatsu 1996, Matras et al. 2017), but a comparison of CEV sequences retrieved from diseased fish from various locations in Europe and Asia (see Table 1, and Fig. S1 in the Supplement at www.int-res.com/articles/suppl/d126p075 _supp.pdf) revealed a 6 to 10% degree of genetic diversity depending on the fragment of the *p4a* gene. The analyses allowed recognition of up to 3 different genetic lineages or genogroups (Matras et al. 2017). During a large epidemiological study conducted in Germany, we collected and sequenced over 150 CEV-positive samples (M. Heling et al. unpubl. data). With an increasing number of sequences available, the significant diversity of CEV, which already was described by Matras et al. (2017), became more apparent.

Because of its wide distribution and potential virulence, CEV is considered a potential risk for the koi trade and for global carp aquaculture. In order to prevent further spread of the infection and the disease, a reliable detection method for this virus is required. Because *in vitro* cultivation of CEV is not feasible with currently available fish cell lines, a CEV infection cannot be detected by re-isolation in cells, but relies on the detection of CEV-specific DNA sequences by PCR or finding virus particles by means of electron microscopy. For a long time only one end-point PCR protocol (designed by Oyamatsu et al. 1997) was available, based on virus-specific sequences obtained from infected koi in Japan. Recently, additional end-point and quantitative PCR protocols have been published, including assays developed at the Centre for Environment, Fisheries and Aquaculture Science (CEFAS) based on CEV sequences in samples obtained from koi and common carp in the UK (Matras et al. 2017), and a qPCR assay developed at the University of Veterinary Medicine in Hannover (TiHo) based on CEV sequences from koi in Germany (Adamek et al. 2016).

The high genetic variability of the CEV *p4a* gene used for designing all the PCR assays could be a serious obstacle for successful and reliable detection of virus infection in field samples, when an assay employing a double label probe is used. This became evident when samples from common carp with clinical KSD produced negative results during analysis by means of the qPCR assay based on a dual labelled probe published by Adamek et al. (2016) and the end-point PCR by Oyamatsu et al. (1997). These PCR protocols were developed on *p4a* sequence information of CEV from koi (CEV genogroup IIa) and apparently did not allow the detection of the infection in samples from KSD-affected farmed common carp (CEV genogroup I). In farmed carp, a CEV infection was confirmed using the PCR assay reported by Matras et al. (2017). Therefore, we decided to evaluate which of the available PCR assays would be able to detect all the virus variants present in the field. By analysing various field samples from different origins with several PCR protocols, we investigated which of the protocols would allow the detection of CEV from all known genogroups present in samples from Central European carp or koi populations as a step towards the determination of the analytical specificity of these tests.

MATERIALS AND METHODS

Samples

To determine if samples containing CEV from different carp varieties or virus genogroups could be detected by the different PCR assays available, 39 field samples were selected from a total of 139 samples (Table 1). These 139 field samples had been found positive for CEV by analysis using at least one of the compared PCR methods, and after sequencing products from the CEFAS end-point PCR, were found infected with 39 CEV variants which differ in their DNA sequences encoding for a P4a core protein fragment. The samples were collected from carp or koi in Poland (Matras et al. 2017) and Germany between 2011 and 2016. In some cases, fish were sampled directly after import from Japan (Table 1). If more than one fish was infected with a certain CEV variant, a sample representing this variant was selected randomly. The DNA concentration of all samples was measured with a Nanodrop ND1000 (PeqLab) photometer diluted with PCR-grade water (Thermo-Fisher Scientific) to a working concentration of 50 ng μl^{-1}. As a part of the currently presented comparison, the PCR products obtained using the CEFAS (nested) end-point PCR were re-sequenced to confirm that they carried unique CEV *p4a* sequences. Using both forward and reverse primers, the overlapping 357 bp sequence of the CEV-specific DNA fragment was

Table 1. Samples, sequence variants, their genogroup, country of origin and other countries (D: Germany; PL: Poland; UK: United Kingdom; J: Japan), number of fish individuals and fish species or variety in which carp edema virus with the selected sequences was detected. '*' indicates that the sequence was obtained from some fish imported from Japan

Sample no./ Sequence variant	Sequence GenBank ID	Geno-group	Country of origin; other countries	No. of fish with sequence	Species or variety
1	KX254012	I	PL	1	Common carp
2	KY550409	I	D	2	Common carp
3	KY550410	I	D; PL	2	Common carp
4	KY550411	I	D; PL	10	Common carp
5	KY550412	I	D	3	Common carp
6	KX253998	I	PL	1	Common carp
7	KY550413	I	D	2	Common carp
8	KY550414	I	D	1	Common carp
9	KY550415	I	D	2	Common carp
10	KY550416	I	D	1	Common carp
11	KY550417	I	D; PL, UK	34	Common carp, pike-perch
12	KX254007	I	PL	1	Common carp
13	KY550418	I	D; PL	2	Common carp
14	KX254010	I	PL	1	Common carp
15	KX254008	I	PL	1	Common carp
16	KX254005	IIb	PL	3	Common carp
17	KY550419	IIb	D	2	Koi
18	KY550420	IIa	D	1	Koi
19	KX254004	IIa	PL	1	Koi
20	KY550421	IIa	D	1	Koi
21	KY550422	IIa	D	2	Koi
22	KY550423	IIa	D	1	Koi
23	KY550424	IIa	D; UK	14	Koi
24	KY550425	IIa	D	2	Koi
25	KY550426	IIa	D	1	Koi
26	KY550427	IIa	D	2	Koi
27	KY550428	IIa	D	7	Koi
28	KY550429	IIa	D	2	Koi
29	KY550430	IIa	D	1	Koi
30	KY550431	IIa	D	1	Koi
31	KY550432	IIa	D	1	Koi
32	KY550433	IIa	D; J*	14	Koi
33	KY550434	IIa	D	1	Koi
34	KY550435	IIa	D	1	Koi
35	KY550436	IIa	D	1	Koi
36	KY550437	IIa	D	9	Koi, common carp
37	KY550438	IIa	D	1	Koi
38	KX253997	IIa	PL	1	Common carp
39	KY550439	IIa	D	5	Koi

obtained by means of Sanger sequencing, performed by LGC Genomics (Berlin).

Phylogeny

The overlapping sequences obtained from the samples were trimmed to 357 bp (location indicated in Fig. S1 in the Supplement) and were analysed with the tools available at www.phylogeny.fr (Dereeper et al. 2008). Sequences were aligned with MUSCLE, and curated with Gblocks. Phylogenetic analyses (based on maximum likelihood) were performed with PhyML. Finally, a phylogenetic tree was rendered with TreeDyn.

CEV detection

Five different PCR protocols were used for detection of CEV-specific DNA: (1) the end-point PCR designed by Oyamatsu et al. (1997), (2) the CEFAS end-point PCR assay developed by CEFAS and published by Matras et al. (2017), (3) the CEFAS quantitative (probe) PCR assay published by Matras et al. (2017), (4) the TiHo quantitative (probe) PCR assay published by Adamek et al. (2016), and (5) a TiHo SYBRGreen quantitative PCR assay designed by Adamek et al. (2017). Primer sequences are presented in Table 2 and the location of the primers within the *p4a* gene fragment is indicated in Fig. S1.

All PCRs were run at the same time with a template which had not experienced repeated freezing–thawing cycles between the runs of the different PCR assays. Apart from CEV-positive samples, non-template controls (NTCs) and 2 CEV-negative samples were included from specific pathogen free (SPF) koi and SPF common carp to insure the lack of intra-laboratory contaminations. The end-point assays were performed using the KAPA2G Robust Hot Start PCR kit (Peqlab) according to the manufacturer's instructions. The reaction mix was prepared as follows: 1× KAPA2G buffer A, 0.2 µM of each primer, 0.2 mM of each dNTP, 1 U of KAPA2G Robust Hot Start Polymerase, 5.0 µl of DNA (50 ng µl^{-1}) and nuclease-free water to a final volume of 20 µl. The amplification program included an initial denaturation at 95°C for 5 min, followed by 45 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 30 s. A final elongation step for 7 min at 72°C was performed at the end of each run. If needed, a nested reaction was per-

Table 2. Sequences of PCR primers and probes compared in this study

PCR name	Oligo name	Sequence (5′–3′)
CEFAS end-point	CEFAS_F	ATG GAG TAT CCA AAG TAC TTA G
	CEFAS_R	CTC TTC ACT ATT GTG ACT TTG
CEFAS nested end-point	CEFAS_nF	GTT ATC AAT GAA ATT TGT GTA TTG
	CEFAS_nR	TAG CAA AGT ACT ACC TCA TCC
CEFAS probe qPCR	CEFAS_qF	AGT TTT GTA KAT TGT AGC ATT TCC
	CEFAS_qR	GAT TCC TCA AGG AGT TDC AGT AAA
	CEFAS_q_Probe	[FAM] AGA GTT TGT TTC TTG CCA TAC AAA CT [BHQ1]
TiHo probe qPCR	TiHo_qF	TTT AGG AGG ACA AGT AAA GTT ACC A
	TiHo_qR	GCA AGT TAT TTC GAT GCC AAC C
	TiHo_q_Probe	[FAM] CCA GCT CCT ACA AGG AAA GCA ATT GA [BHQ1]
TiHo SYBRGreen qPCR	TiHo_Sybr_qF	CAT TTC CTA GTT TGT ATG GCA AG
	TiHo_Sybr_qR	TGA TGA TTG GAA TAA GAT GTC TGT C
Oyamatsu end-point	Oyamatsu_F	GCT GTT GCA ACC ATT TGA GA
	Oyamatsu_R	TGC AGG TTG CTC CTA ATC CT
Oyamatsu nested end-point	Oyamatsu_nF	GCT GCT GCA CTT TTA GGA GG
	Oyamatsu_nR	TGC AAG TTA TTT CGA TGC CA

formed (with nested primers and the same reaction setup) for samples which were negative in the first round of reactions. As a template for nested PCR, 5 µl of 100× diluted PCR mix from the first round of reaction was used. All end-point PCR assays were performed in duplicate for each sample using a Sensoquest LabCycler (Sensoquest). The results were visualised by 302 nm UV light screening after electrophoretic separation of the amplicons in a 1% agarose gel containing 1× Gel Red (Biotium) fluorescent DNA stain.

The reaction mix for the TiHo probe qPCR assay described by Adamek et al. (2016) contained 1× Maxima Probe qPCR Mastermix (Thermo Fisher Scientific), 900 nM of each primer, 300 nM of the probe, 5 µl of template DNA (50 ng µl^{-1}) and nuclease-free water to a final volume of 20 µl. The reaction mix for the qPCR assay developed from CEFAS (taken from Matras et al. 2017) contained 1× Maxima Probe qPCR Mastermix (Thermo Fisher Scientific), 500 nM of each primer, 200 nM of the probe, 5 µl of template DNA and nuclease-free water to a final volume of 20 µl. The amplification program for both assays included an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 30 s and annealing at 60°C for 30 s.

The quantitative PCR with TiHo SYBRGreen based detection of the amplicon was prepared as follows: 1× Maxima SYBR Green mastermix (with 10 nM of ROX), 0.2 µM of each primer, 5.0 µl of DNA (50 ng µl^{-1}) and nuclease-free water to a final volume of 20 µl. The amplification program included an initial denaturation at 95°C for 10 min, followed by 40 cycles

of denaturation at 95°C for 30 s, annealing at 55°C for 30 s and elongation at 72°C for 30 s. A dissociation curve was performed at the end of each run; results with a single melting peak over 75°C were considered positive.

All qPCR assays were performed in duplicates for each sample using a Stratagene Mx3005P cycler (Agilent). The probe based qPCRs were run simultaneously on the same plate. For all qPCR assays the cycle threshold (C_T) values were read at a threshold of 0.05 of normalised fluorescence change (dRn). Information on the analytical sensitivity of the TiHo probe quantitative PCR and the TiHo SYBRGreen PCR is given in Adamek et al. (2016, 2017). Currently, all quantitative PCRs (including CEFAS) are routinely run with supercoiled plasmid standards ranging from 10^7 to 10^1 copies of CEV *p4a* gene (see Fig. S2 in the Supplement) and are able to detect samples below 10 copies of the *p4a* gene.

RESULTS AND DISCUSSION

All field samples that produced CEV-positive results by Oyamatsu's end-point PCR, the real-time PCR assay published by Adamek et al. (2016) (TiHo probe qPCR in Fig. 1) or the TiHo SYBRGreen PCR assay were also positive when analysed by the CEFAS end-point or qPCRs (Fig. 1). In addition, the PCR assays designed by CEFAS detected CEV-specific DNA in further samples, which were negative or gave multiple bands when analysed by means of Oyamatsu's PCR assay or the TiHo probe qPCR assay
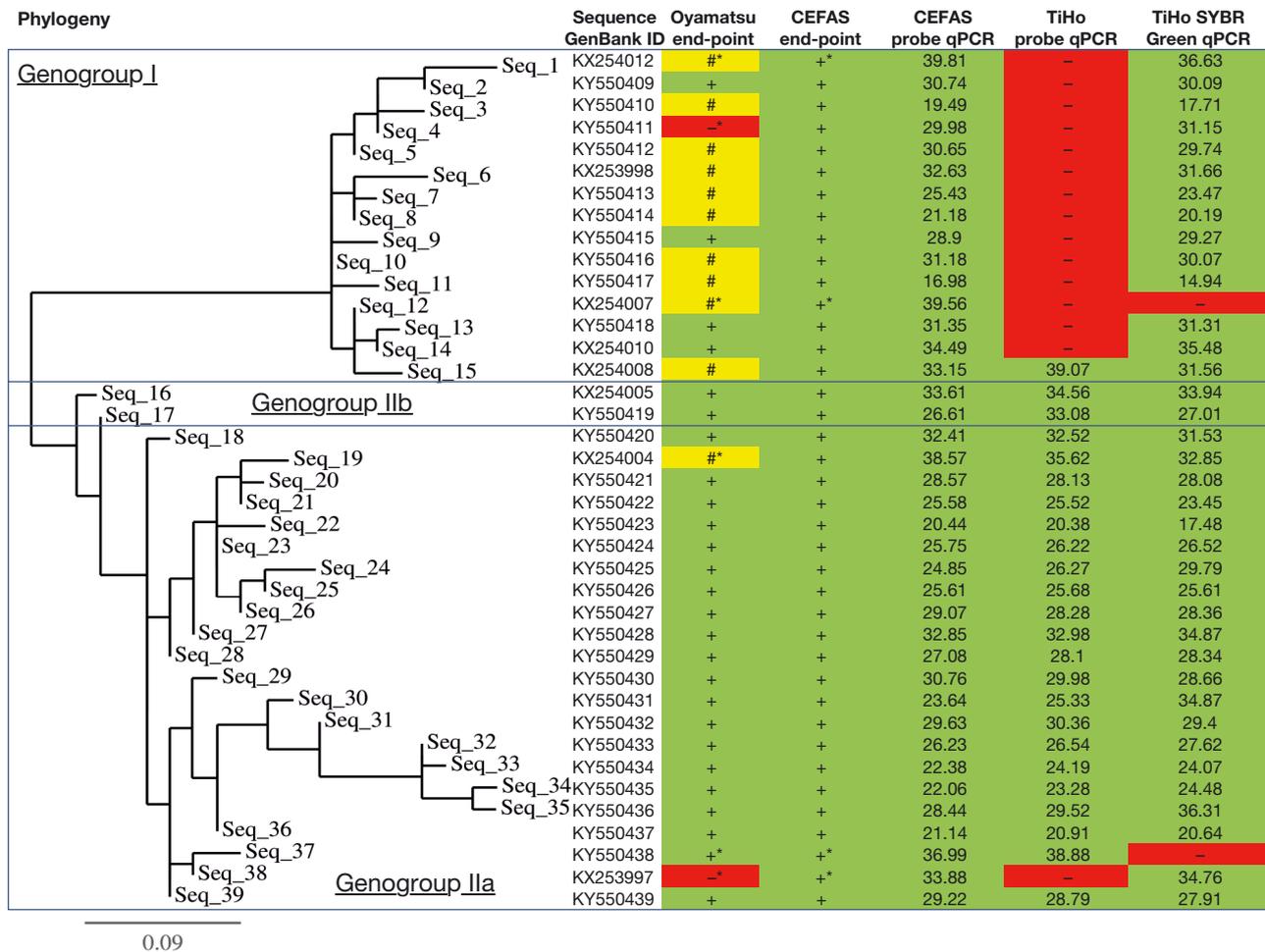
Phylogeny

Fig. 1 phylogenetic tree with genogroup labels (Genogroup I, Genogroup IIb, Genogroup IIa) and Seq_1 through Seq_39, scale bar 0.09.

Phylogeny (Seq)	Sequence GenBank ID	Oyamatsu end-point	CEFAS end-point	CEFAS probe qPCR	TiHo probe qPCR	TiHo SYBR Green qPCR
Seq_1	KX254012	#*	+*	39.81	–	36.63
Seq_2	KY550409	+	+	30.74	–	30.09
Seq_3	KY550410	#	+	19.49	–	17.71
Seq_4	KY550411	–*	+	29.98	–	31.15
Seq_5	KY550412	#	+	30.65	–	29.74
Seq_6	KX253998	#	+	32.63	–	31.66
Seq_7	KY550413	#	+	25.43	–	23.47
Seq_8	KY550414	#	+	21.18	–	20.19
Seq_9	KY550415	+	+	28.9	–	29.27
Seq_10	KY550416	#	+	31.18	–	30.07
Seq_11	KY550417	#	+	16.98	–	14.94
Seq_12	KX254007	#*	+*	39.56	–	–
Seq_13	KY550418	+	+	31.35	–	31.31
Seq_14	KX254010	+	+	34.49	–	35.48
Seq_15	KX254008	#	+	33.15	39.07	31.56
Seq_16	KX254005	+	+	33.61	34.56	33.94
Seq_17	KY550419	+	+	26.61	33.08	27.01
Seq_18	KY550420	+	+	32.41	32.52	31.53
Seq_19	KX254004	#*	+	38.57	35.62	32.85
Seq_20	KY550421	+	+	28.57	28.13	28.08
Seq_21	KY550422	+	+	25.58	25.52	23.45
Seq_22	KY550423	+	+	20.44	20.38	17.48
Seq_23	KY550424	+	+	25.75	26.22	26.52
Seq_24	KY550425	+	+	24.85	26.27	29.79
Seq_25	KY550426	+	+	25.61	25.68	25.61
Seq_26	KY550427	+	+	29.07	28.28	28.36
Seq_27	KY550428	+	+	32.85	32.98	34.87
Seq_28	KY550429	+	+	27.08	28.1	28.34
Seq_29	KY550430	+	+	30.76	29.98	28.66
Seq_30	KY550431	+	+	23.64	25.33	34.87
Seq_31	KY550432	+	+	29.63	30.36	29.4
Seq_32	KY550433	+	+	26.23	26.54	27.62
Seq_33	KY550434	+	+	22.38	24.19	24.07
Seq_34	KY550435	+	+	22.06	23.28	24.48
Seq_35	KY550436	+	+	28.44	29.52	36.31
Seq_36	KY550437	+	+	21.14	20.91	20.64
Seq_37	KY550438	+*	+*	36.99	38.88	–
Seq_38	KX253997	–*	+*	33.88	–	34.76
Seq_39	KY550439	+	+	29.22	28.79	27.91

Genogroups: Genogroup I, Genogroup IIb, Genogroup IIa. Scale bar: 0.09

Fig. 1. Phylogenic (PhyML) analysis of the 357 bp nucleotide sequence encoding the carp edema virus (CEV) P4a core protein. This analysis presents a subset of the sequences obtained during an epidemiological study performed in Germany (this study), Poland and the UK (Matras et al. 2017) into 3 genogroups. The branch length is proportional to the number of substitutions per site. Given are (1) C_T values based on delta fluorescence (dR) data obtained in the quantitative PCR protocols tested on CEV-positive samples and (2) a comparison of the specificity and sensitivity of the qPCRs to the end-point PCR protocols. '+' and green colour: positive signal in the PCR; '−' and red colour: negative PCR result. '*' indicates the use of nested end-point PCR was needed. '#' and yellow colour indicate the presence of multiple bands in an end-point PCR

(Fig. 1). The CEFAS end-point PCR assay did not produce any additional non-specific band which could hamper the interpretation of the result or sequencing of the amplicon. No PCR assays produced an amplicon in NTCs or in negative control samples.

When the amplicons received from the CEFAS end-point PCR assay for all 39 samples were re-sequenced, each was confirmed as bearing a unique sequence of a 357 bp long DNA fragment, which then was used for phylogenetic analysis. The phylogenetic analysis (Fig. 1) indicated that 15 samples constitute one clade (genogroup I), and all these samples were collected from farmed common carp. In one case the same sequence was also obtained from samples of a pike-perch *Sander lucioperca* which did

not show any symptoms of KSD and which was collected from a pond with a massive outbreak of clinical KSD in common carp (Table 1). We suspect that virus shed from carp suffering from this KSD outbreak most likely resulted in a biological contamination of the gills of the pike-perch. The sequences from genogroup I were clearly separated from 22 sequences forming genogroup IIa, which were mostly (but not exclusively) obtained from koi samples (Fig. 1, Table 1). Between these 2 genogroups, 2 sequences were assigned to a new genogroup (genogroup IIb). One of these sequences was obtained from 3 common carp from Poland; the second was recorded from 2 koi from Germany (Table 1). The findings of our phylogenetic analysis support the genetic distribu-

tion of the samples as proposed by Matras et al. (2017), with separation into genogroups I, IIa and IIb.

This phylogenetic analysis was also used to determine which genogroups of CEV would be detected by the protocols applied. As with the CEFAS endpoint PCR assay, CEV was detected in all samples by means of the qPCR assay designed by CEFAS, and when the virus load was quantified, the C_T values were in the same range as those obtained using any of the other quantitative assays applied (Fig. 1). Only in one sample did the CEFAS qPCR assay resulted in a C_T value (38.57) that was more than 5 cycles higher than when this sample was analysed with the TiHo SYBRGreen quantitative assay.

Oyamatsu's end-point PCR assay (Oyamatsu et al. 1997) worked well with samples positive for CEV from genogroups IIa and IIb. However, multiple bands were present in samples positive for CEV from genogroup I, which made interpretation of these results difficult. This, however unlikely, could be related to an off-target attachment of the primers to the common carp genome. This phenomenon did not occur in the negative controls employed. Our findings confirm earlier results (Adamek et al. 2016), which already indicated that this PCR does not reliably amplify DNA of CEV from genogroup I. Furthermore, with 2 samples containing CEV DNA, this PCR gave a negative result.

The probe-based TiHo qPCR assay provided 14 false-negative results from carp infected with CEV from genogroup I and from 1 carp infected with CEV from genogroup IIa. This was the same sample for which Oyamatsu's end-point PCR assay also failed to provide a positive signal. Therefore, the diagnostic usability of the TiHo qPCR assay is limited to the detection of CEV from genogroups IIa and IIb. The relatively narrow analytical specificity of the TiHo qPCR is related to a point mutation which seems to be characteristic of most of the samples from genogroup I, which is present at the FAM-labelled 5′ end of the probe (see Fig. S1 in the Supplement) and which provides a semi-genotyping ability. Together with Oyamatsu's end-point, both of these PCR assays amplify the region of the *p4a* gene that is more divergent (up to 10% dissimilarities) when genogroups I and IIa are compared (Fig. S1).

The TiHo SYBRGreen qPCR assay had a significantly higher analytical inclusivity and diagnostic sensitivity in samples infected with CEV from genogroup I but also from genogroups IIa and IIb. With this assay, only 1 sample from genogroup I was negative, and that sample had an extremely low virus load when analysed with the CEFAS qPCR assay. The same situation was found with a sample from genogroup IIa, which was also negative using the TiHo SYBRGreen assay but positive using the CEFAS qPCR assay. For 3 samples from this genogroup, the TiHo SYBRGreen PCR also gave a C_T value significantly higher compared to results from the other qPCR assays. This suggests that the TiHo SYBRGreen PCR is suitable for the detection of CEV from all genogroups in most of the samples, but might have a higher detection limit, which may indicate decreased sensitivity in marginally positive samples, compared to the qPCRs based on fluorescence-labelled probes. In our comparison this was the case with a sample bearing sequence #37, where the TiHo SYBRGreen qPCR was not able to detect CEV DNA (Fig. 1). Furthermore, due to the use of an intercalating dye, interpretation of the results might be more difficult because careful analysis of melting curves is needed. The melting peaks of the products from CEV genogroups I and IIa occurred at 75.6 and 76.2°C. These slight differences in melting temperatures were not useful for distinguishing between the genogroups.

In recent years the epidemiological situation involving CEV has developed dynamically, in particular with records from populations of farmed carp in various geographic locations of Europe. The sequence data of CEV from various places of origin indicate a high degree of genetic diversity and good separation of sequences from genogroups I and IIa (Fig. 1), which might suggest that the virus from genogroup I has been present in the European common carp population for a prolonged period of time, while virus from genogroup IIa could have been repeatedly introduced to European koi populations by the ornamental fish trade from Japan. Indeed, numerous batches of koi imported from Japan were found to be positive for CEV from genogroup IIa (Adamek et al. 2016). However, a model of genetic evolution should be employed to confirm this hypothesis. Furthermore, there is still a lack of knowledge on how many virus variants are present in the fish population. In addition, nothing is known about the virulence of particular genetic variants or how likely they might be as a cause of mortality in the infected population. Recent observations in our laboratory during cohabitation experiments suggest differences in virulence between viruses from different genogroups (Adamek et al. 2017). This underlines the need for a reliable detection method of CEV in carp populations at risk of infection. The determination of virus loads and genogroup provides a further assessment of the clinical relevance of the infection and the likelihood of development of disease.

CONCLUSIONS

The comparison of different PCR protocols for the detection of CEV showed that the CEFAS PCRs published by Matras et al. (2017) are the most suitable protocols for detecting viruses from all known CEV genogroups. These assays present high diagnostic sensitivity, and furthermore, they exhibit the highest analytical inclusivity, as the primers were placed in a less divergent fragment of the *p4a* gene. This makes them candidates for an inclusive screening assay. The genetic diversity of CEV has not been fully explored yet, and our results suggest that there is an urgent need for further characterisation of CEV sequences to identify an amplification target with less genetic variability. Before this is completed, we suggest using both CEFAS protocols, the end-point and the quantitative PCR assays, for routine diagnostics. By applying 2 sets of primers (or even 3 when the nested end-point PCR is used), it is less likely that false negative result will be obtained when novel mutations occur in the *p4a* target sequence currently used for CEV detection.

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