Assessment of genetic tools for detection of carp edema virus (CEV) by a laboratory comparison test in Germany

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Abstract

Carp edema virus (CEV) was initially detected in Japan in the 1970s as the causative agent of koi sleepy disease (KSD) in koi and common carp (*Cyprinus carpio* L.) populations. CEV has spread all over the world mainly by trading of asymptomatic carrier fish. In Germany KSD was detected for the first time in spring 2009 from samples collected from imported Japanese koi at water temperatures below 10°C. In 2014 large outbreaks of KSD in common carp and koi became evident in Germany. Several unsuccessful investigations were carried out to isolate the agent in permanently growing fish derived cell cultures. For diagnosis, the clinical signs of KSD were difficult to differentiate from the clinical signs related with koi herpesvirus disease (KHVD), except during the advanced stage of

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disease, when the fish started to lie down ("sleep") on the bottom of the tank. The only successful and reliable diagnostic methods were PCRs and qPCR. However, virus sequences obtained from diseased specimens demonstrated high geographical diversity. Therefore, the original primer pairs, published from Japan in the 1990s, gave negative or uncertain results in detection of CEV in samples from carp and koi in Europe, and new primers and probes had to be designed for PCR and nested PCR as well as for TaqMan and SYBR green based qPCRs. While some of them recognised CEV obtained from koi samples only, a decision was made to use only PCR and qPCR assays that detect all variants of CEV for diagnostic testing. Three molecular assays were chosen to conduct a laboratory comparison test with 10 Animal Health Laboratories all over Germany. The aim was to evaluate and standardise CEV detection with defined samples, thereby making the results directly comparable. The results show that even though all laboratories used different PCR kits and cycler systems, they were able to identify CEV in samples from koi and common carp with given primers and a probe. Only in samples diluted above 1:1000, were the results negative by PCR and qPCR in a few laboratories. Some participating laboratories also identified CEV from koi and carp samples by sequence analysis. These results confirm that accurate and reproducible CEV diagnostic results can be achieved resulting in both an improved control strategy, and the implementation of more effective biosecurity practices, for koi and carp farms. Additionally, it was shown that primers and the probe designed for PCR, nested PCR and qPCR are very sensitive and specific to CEV. The results were reproducible across multiple laboratories that utilised different manufacturers of both PCR kits and machines as well as varying assay conditions.

Introduction

With approximately 4.56 million tonnes of production, common carp (*Cyprinus carpio* L.) is one of the most frequently aquacultured fish species worldwide (FAO, 2016). This makes common carp an important fish for table production with a high economical value in aquaculture, and with potential for increasing production and consumption further (FAO, 2016). The coloured variety, the koi, represents an expensive animal kept for ornamental purposes mainly in garden ponds (Rakus et al., 2013). For the last 30 years production and stocking were limited by different diseases inducing enormous economic losses in both varieties world-wide. Koi herpesvirus disease (KHVD) (Haenen et al., 2004), spring viremia of carp (SVC) (Fijan et al., 1971), and over the last 10 years the carp edema virus (CEV) also termed koi sleepy disease virus (KSDV) inducing koi sleepy disease (KSD) (Way et al., 2017) have been threats to the carp and koi industry. Similar to KHV, the most important way of spreading CEV appears to be the trade of asymptomatic individuals. CEV was initially detected in Japan by PCR (Oyamatsu et al., 1997) and also by electron microscopy (Miyazaki et al., 2005). The PCR protocol designed by Oyamatsu et al. (1997) is based on the only known fragment of the nucleotide sequence of the CEV genome, a fragment of the gene encoding the P4a protein, resembling a gene encoding for a capsid core protein. Due to the diversity in the nucleotide sequences of this fragment in isolates from Europe, from 2010 to 2012 this PCR did not give reliable results for samples taken from koi and common carp in Europe. Therefore, new primers for an end-point PCR as well as primers and a probe for qPCR were established by the Centre for Environment, Fisheries and Aquaculture Science (CEFAS; Weymouth, England) covering all known genetic variants of CEV present in Europe and in Asia in common carp and koi (Matras et al., 2017; Dr. D. Stone, CEFAS, pers. comm.). By using these PCR tests up to three genogroups, with one clearly European (I) and one Asian or Japanese genogroup (IIa),

were described (Matras et al., 2017). The detection problems using the PCR, previously designed by Oyamatsu et al. (1997), seem to be limited to European samples, because all samples from China (Ouyang et al., 2018) and India (Swaminathan et al., 2016) were tested positive for CEV using this method.

However, the current study was undertaken to evaluate the outcome of CEV detection among Animal Health Laboratories in Germany using a standardised array of samples which included both CEV genogroups, originating from both common carp and koi at various concentrations. This allowed the reliability of CEV detection using the PCR protocols designed by CEFAS but when using different chemistry and instrumentation to be evaluated across multiple diagnostic laboratories. The results of the inter-laboratory comparison show that using the proposed molecular methods, even on different and locally preferred conditions such as various PCR kits and equipment, it was possible to consistently detect CEV accurately in the samples tested.

Materials and methods

Samples and protocols

DNA samples (n = 12) were prepared using Qiamp DNA Mini Kit (Qiagen) from gill tissues of two clinically affected common carp (infected with CEV from genogroup I) and two clinically affected koi (infected with CEV from genogroup IIa), respectively. In addition, KHV positive samples as well as CEV negative controls were included into the array of samples to test assay specificity. A serial dilution from each CEV positive sample was prepared and tested by CEV qPCR in the reference laboratory (Table 1). These DNA samples were aliquoted and sent frozen to the participating laboratories together with the protocols for the comparison test.

Table 1. Preparation of standardised DNA samples obtained from four CEV positive fish (two common carp, two koi) and one CEV negative but KHV positive koi

Sample	Content	Dilution	Original Cq values	Correct assessment
1	koi 1	undiluted	24	CEV positive
2		1:10	27	
3	KHV 08-5-192*	-	no cq	negative
4	koi 1	1:100	30	CEV positive
5	koi 2	1:1,000	33	
6	KHV 08-5-192*	-	no cq	negative
7	koi 2	1:10,000	37	questionable
8	carp 1	undiluted	25	CEV positive
9		1:10	28	
10		1:100	31	
11	carp 2	1:1,000	35	
12		1:10,000	38	suspicious

* negative test controls

Methods for laboratory comparison

The methods used in the comparison were the conventional PCR and nested PCR protocols as previously published by Matras et al. (2017), with all +ve samples confirmed as CEV by DNA sequence analysis. Simultaneously a CEV qPCR, described by Adamek et al. (2017) was also included. All assays based on procedures designed by CEFAS in 2016. All laboratories must use the primers and the protocols provided but with their own routine, kits and equipment (Table 2).

Table 2. PCR kits and PCR cyclers used for the CEV comparison test in the 10 diagnostic laboratories

PCR kits	PCR cycler
Hotstar TaqPolymerase (Qiagen) GoTaq® G2 Flexi DNA-Polymerase (Promega) Kapa Taq PCR-Kit (Sigma-Aldrich) Faststart High Fidelity PCR System (Sigma-Aldrich)	Biometra T3 (Biometra) T-Personal (Biometra) Mastercycler Pro S (Eppendorf)

qPCR kits	qPCR cycler
TaqMan Universal PCR Mastermix (Applied Biosystems) Quantitect Multiplex PCR-Kit (Qiagen) QuantiTect Multiplex PCR NoRox-Kit (Qiagen) Kapa Probe QPCR Mastermix (PeqLab)	MX3005P (Stratagene/BioRad) 7500 RealTime PCR System (Applied Biosystems) LightCycler 480 II (Roche)

Results

All laboratories (10/10) performed the CEV qPCR and were able to detect CEV from at least 7/10 CEV positive samples, but there were visible differences in virus genome quantification between four to nine Cq values. While all laboratories recognised the controls (KHV samples) as CEV negative, in CEV sample 7 (koi 2, 1:10,000), 11 (carp 2, 1:1,000) and 12 (carp 2, 1:10,000) some of the laboratories were able to detect the low level of CEV DNA. Only one laboratory detected CEV DNA from sample 7 and from samples 11 and 12 only three or two laboratories, respectively, achieved a positive signal by PCR or qPCR (Table 3).

One laboratory provided details of five test replicates for each sample (Table 4). The CEV qPCR was always positive with koi and common carp CEV samples with original Cq value lower than 36 but negative with the KHV samples (samples 3 and 6). This laboratory had a negative result for sample 7 but positive results using samples 11 and 12. Due to a higher percentage of positive reactions, this laboratory confirmed samples 11 and 12 to be CEV positive.

Conventional PCR and nested PCR

Only 6 of 10 laboratories carried out the conventional (end-point) PCR and nested PCR. Additionally, in one laboratory two different PCR kits were used (Table 2).

The results show that the CEV negative samples and most of the CEV positive samples were identified correctly. The exception again were samples 7, 11 and 12 (Table 5).

It was shown again that in samples with low virus loads (samples 5, 7, 11 and 12) the PCR can fail and lead to a false-negative result. The detection limit for conventional PCR ap-

Table 3. Results from CEV qPCR

CEV sample	Original Cq value*	Mean Cq values when comparing test results for all laboratories (n=10)	Min-max Cq values
1	24	24.45	23 – 27.80
2	27	28.23	20.24 – 30.10
3	-	-	-
4	30	31.97	30 – 34.40
5	33	36.87	33 – 39.60
6	-	-	-
7	37	-	-
8	25	26.94	24 – 32.50
9	28	30.68	28 - 36
10	31	33.84	31 – 39.90
11	35	37.45	35 - 39
12	38	>37**	>37**

* before sending to the laboratories
** one laboratory only with a positive signal

Table 4. Assessment of the CEV qPCR (5 replicates) in one laboratory

CEV sample	Mean value	% positives	Assessments
1	22.5	100%	CEV
2	26.9	100%	CEV
3	no cq	0%	negative
4	30	100%	CEV
5	37	100%	CEV
6	no cq	0%	negative
7*	no cq	0%	negative
8	25.5	100%	CEV
9	29.4	100%	CEV
10	31.8	100%	CEV
11	36.9	80%	CEV
12	37.9	60%	CEV

* CEV positive sample in a high dilution

Table 5. Results of the conventional PCR and nested PCR

CEV sample	original result*	Positive result per laboratories (n=6)
1	+	6 / 6
2	+	6 / 6
3	-	0 / 6
4	+	5 / 6
5	+	4 / 6
6	-	0 / 6
7	+	1 / 6
8	+	6 / 6
9	+	6 / 6
10	+	6 / 6
11	+	3 / 6
12	+	1 / 6

* before sending to the laboratories

peared to be at an amount of virus specific genome copies equivalent to a Cq value of ≤ 33 when measured by CEV qPCR. For sample 4, one PCR kit failed in detecting CEV specific DNA sequences in the laboratory that used two different kits. The other kit generated a positive signal using DNA from this sample.

Sequence analysis

The six laboratories using conventional PCR or nested PCR provided up to eight sequences for CEV from the carp and koi samples. The carp CEV sequences were 99 – 100 % identical between the laboratories and the koi CEV sequences showed a similarity of 98 – 100%. A clear differentiation between the two genogroups was achieved, with 23 to 25 mismatches over the 400 bp sequence between carp CEV (genogroup I) koi CEV (genogroup IIa) (Figure 1). In the phylogenetic tree the carp CEV sequences clustered very close as well as the koi CEV sequences and both clusters were clearly differentiated (Figure 2). Koi CEV sequences were taxonomically grouped into the genogroup IIa, carp CEV sequences into genogroup I.

Discussion

Ten German laboratories participated in a comparison exercise for detection of CEV. The aim of the comparison was to determine the analytic sensitivity and specificity of published PCR methods with standardised samples from common carp and koi as well as standardised protocols but using available PCR kits and PCR/ real-time PCR equipment in each laboratory. Additionally, all PCR products generated from conventional PCR and / or nested PCR were sequenced and compared.

CEV qPCR (Adamek et al., 2017) was performed in all participating laboratories. In six laboratories the conventional CEV PCR and the nested PCR (Matras et al., 2017) were performed and subsequently the PCR products were sequenced. CEV was reliably detected by all participating laboratories. The negative control samples containing KHV DNA were correctly recognised as CEV negative by all 10 laboratories. Due to practicalities when pre-

Figure 1. Distribution of mismatches (red bars) of carp from koi CEV (428 bp)

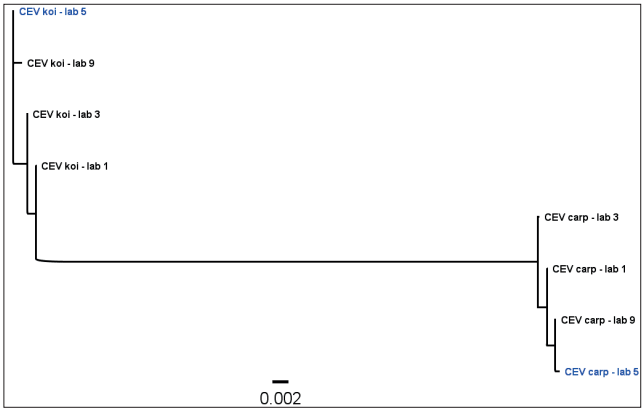

Figure 2. Phylogenetic tree of carp and koi CEV (P4a gene) from NCBI database

paring the CEV infected material, all samples were frozen and thawed at least twice which can lead to a decrease of detectable specific CEV DNA. This was visible when results from the original laboratory were compared to those of the receiving laboratories. Importantly, the differences in the equipment for qPCR, in laboratory practice, and in evaluation of the results (fluorescence detection method, threshold setup) did not influence significantly the successful detection of CEV.

Very low virus loads were detected using the CEV qPCR in samples 7 and 12, which contained CEV-specific DNA at a dilution of 1:10,000. The results from all laboratories show that the detection limit seems to be at Cq > 37. Therefore, in samples 7 and 12 a positive signal was found only by one laboratory. This laboratory documented that it is possible to detect CEV DNA in samples with a low virus load if the entire sample volume was used in several PCRs. Unfortunately, this laboratory did not confirm the positive reactions by sequence analysis.

Currently it is still not possible to quantify the absolute amount of specific CEV DNA within a sample. Additionally, it is also not possible to quantify relatively due to a lack of a suitable internal control.

A comparison of the sequences submitted by the laboratories showed that all laboratories achieved almost identical sequences for both koi and common carp CEV with only a few single-point mismatches. This shows altogether a very high diagnostic standard within the laboratories. Further plans are progressing to conduct an additional validation of the chosen methods in all laboratories using accessory positive and negative samples and an inclusion of internal controls

In conclusion, all 10 participating German Animal Health laboratories successfully took part in the comparison exercise. Conventional PCR, nested PCR and qPCR designed by CEFAS in 2016 and first published by Matras et al. (2017) are sufficiently sensitive for monitoring and surveillance of CEV in carp or koi populations and gave comparable results when used with different chemistry and PCR instrumentation. The qPCR was excellently suited for monitoring the presence of CEV at a farm level. For investigation of single carp or koi or single samples the conventional PCR, and perhaps including the nested PCR, with a sequence analysis is strongly recommended.

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