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Development of a real-time duplex PCR assay for simultaneously diagnosis of KHV and CEV in common carp and koi

Pengembangan *real-time* PCR dupleks untuk diagnosis KHV dan CEV secara simultan pada ikan mas dan koi

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ABSTRACT

Koi herpesvirus (KHV) and carp edema virus (CEV) are potential risks for the koi trade and for global common carp aquaculture. Due to the severe impact of both viruses, a robust diagnostic tool was required, capable of detecting and distinguishing both infections with high accuracy and precision. The aim of this study was to describe a real-time duplex TaqMan PCR assay for simultaneous detection of KHV and CEV in common carp and koi. Two pairs of primers with two TaqMan probes were used to amplify specific and conserved regions of KHV and CEV DNA. This assay was confirmed to be sensitive and specific. Limit of detections of the assay were 15 DNA copies/µL for KHV and 150 DNA copies/µL for CEV, respectively. This assay was able to identify and distinguish CEV and KHV, but was unable to identify other pathogens and sample matrices. For clinical validation, 18 KHV and CEV positive samples each, as well as 12 negative samples were tested with three different test methods, i.e., real-time duplex PCR, real-time simplex PCR, and conventional PCR. All three tests provide optimal conformity of results. The results showed that real-time duplex PCR was able to detect the presence and distinguish each pathogen in infected fish. This real-time duplex PCR assay is a rapid, sensitive, and specific test for detecting KHV and CEV in carp fish, thus it can be considered a valid alternative assay in aquaculture clinical laboratories.

Keywords: *Cyprinus carpio*, CEV, diagnostic, KHV, real-time duplex PCR

ABSTRAK

Koi herpesvirus (KHV) dan carp edema virus (CEV) merupakan risiko potensial untuk perdagangan koi dan budidaya ikan mas global. Karena dampak dari keduanya yang parah, maka diperlukan perangkat diagnostik yang tangguh, yang mampu mendeteksi dan membedakan keduanya dengan akurasi dan presisi yang tinggi. Tujuan dari penelitian ini adalah menjabarkan pengujian *real time* TaqMan PCR dupleks untuk mendeteksi KHV dan CEV pada ikan mas dan koi secara simultan dalam satu reaksi. Dua pasang primer dengan dua TaqMan probe digunakan untuk mengamplifikasi wilayah spesifik dan lestari dari DNA KHV dan CEV. Pengujian ini terbukti sensitif dan spesifik. Sensitivitas dari pengujian ini adalah 15 kopi DNA/µL untuk KHV dan 150 kopi DNA/µL untuk CEV. Pengujian ini mampu mengidentifikasi dan membedakan CEV dan KHV, tetapi tidak dapat mengidentifikasi patogen lain dan matriks sampel. Untuk validasi klinis, masing-masing 18 sampel positif KHV dan CEV, serta 12 sampel negatif diuji dengan tiga metode pengujian yang berbeda, yaitu *real-time* PCR dupleks, *real-time* PCR tunggal dan PCR konvensional. Ketiga pengujian tersebut memberikan kesesuaian hasil yang optimal. Hasil penelitian menunjukkan bahwa *real-time* PCR dupleks mampu mendeteksi keberadaan dan membedakan setiap patogen pada ikan yang terinfeksi. Uji *real-time* PCR dupleks ini merupakan pengujian yang cepat, sensitif, dan spesifik untuk mendeteksi KHV dan CEV pada ikan koi dan mas, sehingga dapat dipertimbangkan sebagai pengujian alternatif yang valid di laboratorium klinis akuakultur.

Kata kunci: *Cyprinus carpio*, CEV, diagnostik, KHV, r*eal-time* PCR dupleks

INTRODUCTION

International and domestic trade in koi (*Cyprinus rubrofuscus*) has led to the global spread of fish pathogens in cyprinid fish, mainly koi and common carp (*Cyprinus carpio carpio*) (Gotesman *et al*., 2013; Haenen *et al*., 2016; Way *et al*., 2017). Koi herpesvirus disease (KHVD) and carp edema virus disease (CEVD) are two contagious viral pathogens in both varieties of cyprinid (Adamek *et al*., 2022; Dong *et al*., 2013; Gibson-Reinemer *et al*., 2017; Lovy *et al*., 2018; Thresher *et al*., 2018; Xu *et al*., 2013). The KHV outbreaks have caused mass mortalities of up to 80-95% in koi and common carp populations, causing severe economic loss and significant social impact. Epidemiological observations indicated that the first outbreak occurred from an imported koi that has just been transported from Surabaya to Blitar, as the disease transmitted from koi to common carp, the spread of the disease to new areas was expeditious (Sunarto *et al*., 2005). Meanwhile, CEVD or known as koi sleepy disease (KSD) has never been reported in Indonesia, but the international trade in koi has likely led to the global spread of CEVD/KSD with outbreaks documented in imported koi and common carp in several countries worldwide, since the disease was first characterized from Japanese koi in 1970s (Adamek *et al*., 2022; Haenen *et al*., 2013; Jung-Shoroers *et al*., 2015; Kim *et al*., 2018; Lewisch *et al*., 2015; Stevens *et al*., 2018; Swaminathan *et al*., 2016; Vesely *et al*., 2015; Way & Stone, 2013; Way *et al*., 2017).

Both diseases have similar clinical signs including lethargy, pale and swollen gills, sunken eyes and skin changes (Gilad *et al*., 2004; Miyazaki *et al*., 2005; Way & Stone, 2013). Co-infection between KHV and CEV has been reported during mortality events of culture and wild common carp in USA and Iraq (Padhi *et al*., 2019; Toffan *et al*., 2019). Multiple diagnostic methods such as, inoculation virus in cell cultures, histological examination, and electron microscopy are required to detect and differentiate these two viral pathogens (Eckart *et al*., 2019; Miwa *et al*., 2015; Miyazaki *et al*., 2005; Miyazaki *et al*., 2008; Ouyang *et al*., 2018; Wang *et al*., 2013). However, these methods are time-consuming and labor-intensive. Molecular assays, such as PCR and real-time PCR methods, have been used for rapid and sensitive detection of these viruses separately (Adamek *et al*., 2016; Gilad *et al*., 2004; Gray *et al*., 2002; Matras *et al*., 2017; Monaghan *et al*., 2015; Oyamatsu *et al*., 1997).

Real-time PCR is preferred over conventional PCR in clinical laboratories because there is no need for a post-amplification process, leading to faster analysis and reduced risk of amplicon contamination (Engstrom-Melnyk *et al*., 2015). Real-time PCR can also provide an estimate of viral pathogen titer in the tissue (Adamek *et al*., 2016; Gilad *et al*., 2004). At present, the challenges in this field are the development of protocols to enable multiplex detection and identification of several pathogens in a single assay, and furthermore, the application of techniques based on DNA analysis in aquaculture facilities as routine diagnostic methods. The aim of this study was to describe a real-time duplex TaqMan PCR assay for simultaneous detection of KHV and CEV in common carp and koi.

MATERIALS AND METHODS

Clinical samples, plasmids, and bacterial strains

Fifteen samples of common carp and koi gill tissue obtained from farm monitoring results in Indonesia during 2020 to 2022 that were detected positive for KHV by routine PCR tests, used as clinical tissue samples of KHV-infected fish. Fifteen gill tissue samples of imported koi from Japan during 2021 to 2022 that were detected positive for CEV by routine PCR tests, also used as clinical tissue samples of CEV-infected fish. All CEV-infected koi from Japan have been taken action by competent authorities in accordance with the Indonesian government's fish quarantine regulations. All of KHV- and CEV positive tissue were stored separately at -80 °C in 70% alcohol until being analysed. Three mixtures of KHV-infected koi gill tissue samples from Indonesian farm and CEV-infected imported koi gill tissue samples from Japan were used as clinical tissue samples of mixed infection of KHV and CEV.

Twelve tissue samples of KHV- and CEV-non-infected koi and common carp were obtained from common carp and koi from Indonesian farms, Japan and China imported koi. Positive controls consisted of recombinant plasmids containing specific and conserved genes of these two viruses (KHV-pGEMT-38, CEV-pGEMT-38), these two plasmids were cloned using specific primers (Table 1). Negative controls consist of nuclease-free water, and two bacterial isolates that are likely to be found in the koi and common carp,

Aeromonas salmonicida and *Yersinia ruckeri*. The clinical tissue sample, plasmids, and bacterial isolates were tested to assess the specificity and sensitivity of the real-time duplex PCR.

DNA extraction

DNA extraction of tissue samples and bacterial isolates was carried out using DTAB-CTAB (Genereach, Taiwan) following the supplier's recommendations. The DNA was eluted in 100 µL TE buffer and stored at -80 °C before testing. The concentration and purity of DNA were assessed using a NanoDrop 1000 spectrophotometer (Thermo Fischer Scientific, Inc., USA).

Oligonucleotide primers and DNA probes

Detection of each viral pathogen was achieved by targeting specific genes. Detection of KHV was carried out by targeting a gene encoding a conserved protein of unknown function using a pair of primers and a TaqMan probe that had been developed (Gilad *et al*., 2004). Detection of CEV was carried out by core protein gene using a pair of primers and primer-probe that had been developed (Matras *et al*., 2017), and modification was made for the fluorescence label-dye of TaqMan probe. Both pairs of primers and TaqMan DNA probes are listed in Table 1. All primers and probes were synthesized by IDT, USA.

Real-time duplex PCR assay

Real-time duplex PCR assays were performed in a 20 µl final reaction volume consisting of SensiFAST™ Probe No-ROX Kit (Bioline, Meridian Bioscience, USA) according to the manufacturer's instructions, 0.4 µM for each primer of KHV and CEV and 0.2 µM for each probe of KHV and CEV, and 2 µL of DNA samples. The PCR amplification consisted of 2 min at 95 °C, followed by 40 cycles of 10 s at 95 °C and 50 s at 60 °C (acquiring to green and yellow). Amplification, detection, and data analysis were performed by Rotor-Gene Q MDx 5plex HRM (Qiagen, Germany).

Analytical sensitivity and specificity

The analytical sensitivity of the method was assessed by determining the limit of detection (LoD) as the lowest viral titre that can be detected by real-time duplex TaqMan PCR. The sensitivity of real-time duplex PCR was determined using seven serial dilutions of each specific plasmid (KHV-pGEMT-38, CEV-pGEMT-38). Since the presence of other oligonucleotides and the fluorescent probe could alter the efficiency of PCR amplification, each set of primers and probe was tested in an individual format as well as in a duplex format, to find out the systematic deviations and efficiencies in the amplification curve when comparing between real-time duplex PCR test results with real-time individual PCR test results. The specificity of the method was assessed by testing the DNA of clinically KHV-infected common carp and CEV-infected koi (proved by positive results of routine PCR testing and were sequenced, and sequence data were analyzed using BioEdit 7.2 software and compared with the corresponding sequence data in GenBank), mixtures of both KHV-infected common carp with CEV-infected koi sample extracts, and for negative controls, DNA of *Aeromonas salmonicida, Yersinia ruckeri*, KHV- and CEV-non infected common carp and koi, and distilled water was included.

Intra-assay repeatability and inter-assay reproducibility

In order to determine the intra- and inter-assay variability, various mixed concentrations of KHV-pGEMT-38 dan CEV-pGEMT-38 plasmids (10^5 & 10^2 copies of each; 10^3 & 10^4 copies of each; and 10^1 & 10^6 copies of each) were tested

Table 1. Primer dan TaqMan probe.

Assay parameter	Primer & probe	Sequence (5' – 3')	Product size	Reference
KHV	KHV-86f	5' - GACGCCGGAGACCTTGTG - 3'	78 bp	Gilad *et al*., 2004
	KHV-163r	5' - CGGGTTCTTATTTTTGTCCTTGTT - 3'		
	KHV-109p	5' - FAM - CTTCCTCTGCTCGGCGAGCACG – BHQ1 - 3'		
CEV	CEV qFor1	5'- AGTTTTGTAKATTGTAGCATTTCC - 3'	76 bp	Matras *et al*., 2017
	CEV qRev1	5'- GATTCCTCAAGGAGTTDCAGTAAA - 3'		
	CEV Probe1	5'- HEX - AGAGTTTGTTTCTTGCCATACAA – ACT - BHQ1 - 3'		

using real-time duplex PCR. The assays were performed in triplicate within the same run (intra-assay), on three different days (inter-assay) by two different operators. The coefficient of variations (CV) was calculated according to the EPA (2004).

Detection of clinical samples

DNA from 18 clinical samples each of KHV and CEV that were known to be positive by routine conventional PCR were also subjected to real-time duplex PCR. Clinical samples were tested using three different methods, i.e., real-time duplex PCR, real-time individual PCR, and routine conventional PCR for each virus. All real-time individual PCR and routine conventional PCR parameters were determined as follows (Gilad *et al*., 2004; Gray *et al*., 2002; Matras *et al*., 2017; Oyamatsu *et al*., 1997). The test results of the three methods for each virus were compared.

RESULTS AND DISCUSSION

Result

Limit of detections, amplification efficiencies, and standard curve linearities

The sensitivity of the real-time duplex PCR assay was 15 copies/µL for KHV and 150 copies/µL for CEV template, respectively (Figure 1), and the sensitivities were forty and ten times higher than those of the routine PCRs. Furthermore, the detection limits for the duplex and individual assay formats were nearly identical. The efficiencies and linearities of the reactions were determined by generating standard curves in which serial dilutions of plasmids were tested. No difference in amplification efficiencies was observed between the individual and duplex formats, as measured by the slopes of amplification curves during the exponential phase and the cycle threshold (CT) values obtained with individual samples.

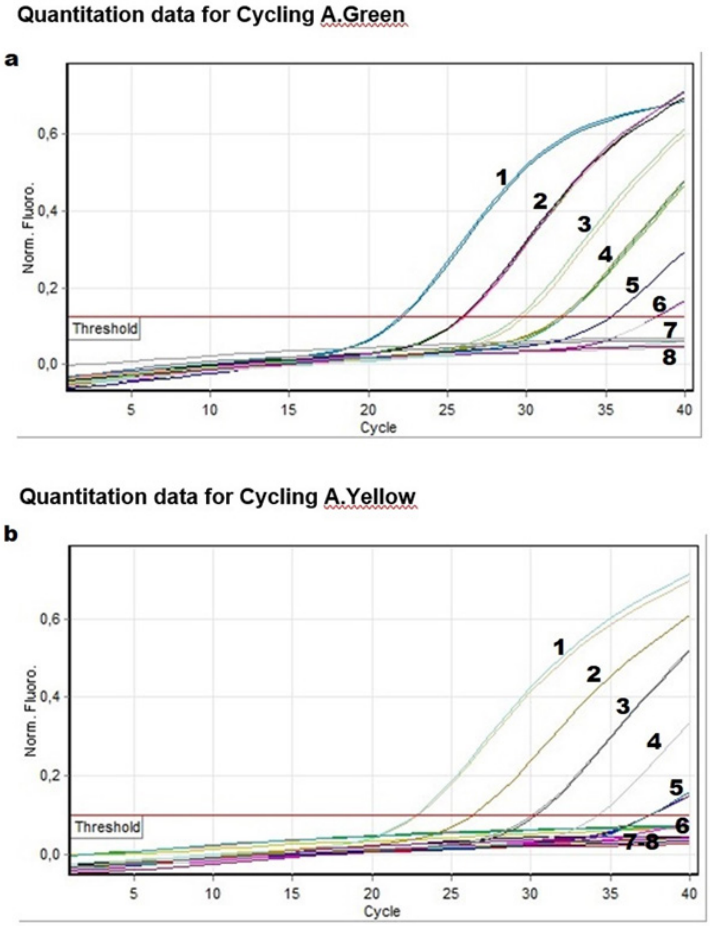

Figure 1. Analytical sensitivity of real-time duplex PCR. Amplification curves of KHV a. 1) 1.5×10^{6} copies/µl; 2) 1.5×10^{5} copies/µl; 3) 1.5×10^{4} copies/µl; 4) 1.5×10^{3} copies/µl; 5) 1.5×10^{2} copies/µl; 6) 1.5×10^{1} copies/µl; 7) 1.5×10^{0} copies/µl; 8) negative control. Amplification curves of CEV b. 1) 1.5×10^{6} copies/µl; 2) 1.5×10^{5} copies/µl; 3) 1.5×10^{4} copies/µl; 4) 1.5×10^{3} copies/µl; 5) 1.5×10^{2} copies/µl; 6) 1.5×10^{1} copies/µl; 7) 1.5×10^{0} copies/µl; 8) negative control.

The standard curve efficiencies of real-time duplex PCR were 1.05 and 0.87 for KHV and CEV, respectively, with coefficients of determination (R^2) were 0.99375 and 0.99930 (Figure 2). According to Broeders *et al*. (2014), the slope of the regression curve should be between -3.9 and -2.9 corresponding to PCR efficiencies ranging from 80% to 120% with R^2 for each target should be ≥0.98 for real-time multiplex PCR format. The result of these experiments indicates that there was no systematic deviation in the amplification curves when comparing the duplex assay with the single-target assays.

Analytical specificity

The real-time duplex PCR is capable of detecting KHV and CEV. No positive results were observed when *Aeromonas salmonicida*, *Yersinia ruckeri,* KHV- and CEV-non infected samples, and water were tested. A mixture of KHV and CEV sample extracts with different concentrations could still be identified by this assay, which implies that the real-time duplex PCR assay can be used for the simultaneous detection of infection with both viruses. The real-time duplex PCR results of different samples showed that one specific amplification curve was displayed when fish was infected by only one of these two viral pathogens, whereas two specific amplification curves were displayed when fish were infected by two viral pathogens, and no amplification curves were displayed for samples containing *Aeromonas salmonicida*, *Yersinia ruckeri*, KHV- and CEV-non infected samples, and water (Figure 3). The results indicate that real-time duplex PCR was able to detect and differentiate the presence of each pathogen in clinically infected fish.

Intra- and inter-assay variabilities

The coefficient of variation within runs and between days were calculated as the percentage of the ratio of standard deviation and the average of the curve threshold (CT) values obtained. Different concentrations of KHV-pGEMT-38 and CEV-pGEMT-38 plasmids, when mixed, can still be identified by this test, and demonstrated good repeatability and reproducibility (Table 2). The intra-assay CV ranged from 0.08 to 0.157% and the inter-assay CV ranged from 0.203 to 0.336% for KHV. The intra-assay CV ranged from 0.052 to 1.357% and the inter-assay CV ranged from 0.897 to 2.355% for CEV. These results showed that this real-time duplex PCR had high precision and can be used for simultaneous infection detection of both viruses.

Detection of clinical samples

In total, 45 fish samples were tested for KHV and CEV using three distinct analytical methods, and the results were compared. Real-time duplex PCR test results showed that 15 samples tested positive only for KHV, 15 samples tested positive only for CEV, three samples tested positive for both of viruses, and 12 samples tested negative for both of viruses. The results obtained were the same as those obtained by routine conventional PCR and real-time simplex PCR. There were no significant differences between the CT values

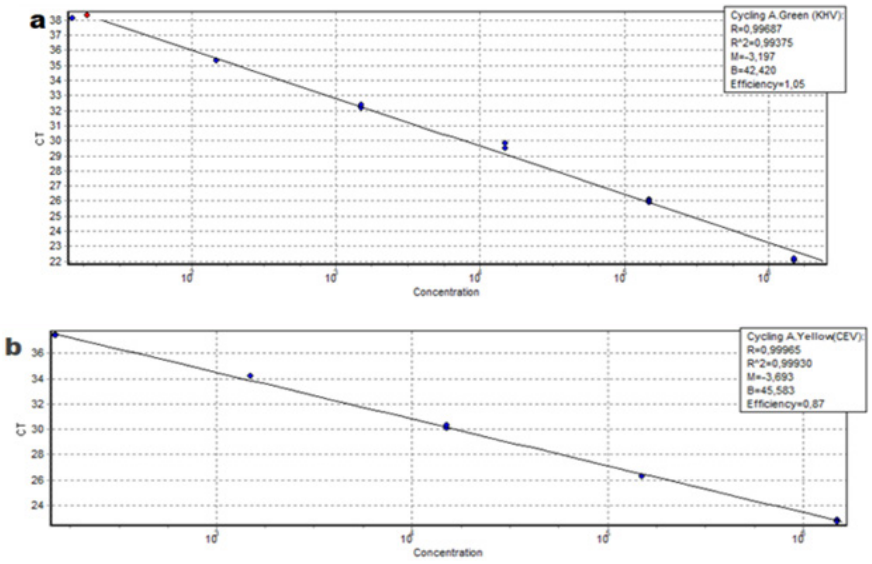

Figure 2. Standard curve of KHV (a); standard curve of CEV(b).

obtained by real-time duplex PCR and real-time simplex PCR of each sample detected positive for KHV or CEV.

Indeed, the concordance between the three tests was optimal, according to the Landis and Koch scale, and significant, with an overall Cohen's kappa statistic of 1 and p<0.001. These results confirm that the real-time duplex PCR assay has a good performance. Since the mean CT value corresponding to the LoDs is ≤39, the diagnostic ''cut-off'' limits were set at 38 CT for both viruses. Detailed results of all clinical samples and bacterial isolates being tested are listed in Table 3.

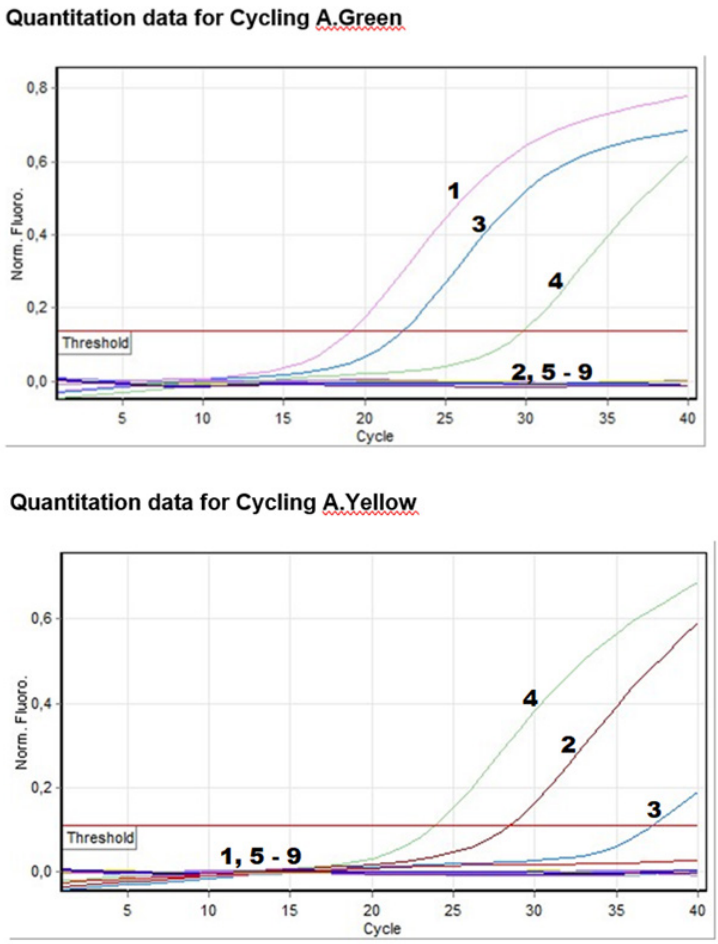

Figure 3. Analytical specificity of KHV a. analytical specificity of CEV; b. 1) DNA of KHV-infected koi; 2) DNA of CEV-infected common carp; 3) mixture of KHV-infected koi and CEV-infected common carp DNA extracts (concentration of KHV DNA higher than CEV DNA); 4) mixture of KHV-infected koi and CEV-infected common carp DNA extracts (concentration of CEV DNA higher than KHV DNA); 5) DNA of *Aeromonas salmonicida*; 6) DNA of *Yersinia ruckeri*; 7) DNA of KHV- and CEV- noninfected koi; 8) DNA of KHV- and CEV- noninfected common carp; 9) negative control.

Table 2. Intra-assay repeatability and inter-assay reproducibility.

Assay parameter	Viral concentration	Intra-assay variability (CV%)	Inter-assay variability (%CV)
KHV	10^5 DNA copies/µl	0.080 ≤ CV ≤ 0.117	0.272 ≤ CV ≤ 0.273
	10^3 DNA copies/µl	0.082 ≤ CV ≤ 0.129	0.335 ≤ CV ≤ 0.337
	10^1 DNA copies/µl	0.120 ≤ CV ≤ 0.157	0.203 ≤ CV ≤ 0.204
CEV	10^6 DNA copies/µl	1.083 ≤ CV ≤ 1.357	2.279 ≤ CV ≤ 2.355
	10^4 DNA copies/µl	0.554 ≤ CV ≤ 1.063	1.883 ≤ CV ≤ 1.935
	10^2 DNA copies/µl	0.052 ≤ CV ≤ 0.931	0.897 ≤ CV ≤ 0.909

Table 3. Results of real-time duplex PCR of clinical samples.

Host fish	Pathogen	Matrix	Date of acquisition	Results					
				Real-time duplex PCR		Real-time single PCR		Conventional PCR	
				KHV	CEV	KHV	CEV	KHV	CEV
Koi	KHV	Gills	11 Apr 2020	+ (29,36)	-	+ (29,52)	-	+	-
Koi	KHV	Gills	11 Apr 2020	+ (29,28)	-	+ (28,75)	-	+	-
Koi	KHV	Gills	11 Apr 2020	+ (32,27)	-	+ (32,31)	-	+	-
Common carp	KHV	Gills	20 Oct 2020	+ (28,76)	-	+ (28,52)	-	+	-
Common carp	KHV	Gills	20 Oct 2020	+ (30,16)	-	+ (29,54)	-	+	-
Koi	CEV	Gills	20 May 2021	-	+ (22,90)	-	+ (22,37)	-	+
Koi	CEV	Gills	20 May 2021	-	+ (22,57)	-	+ (22,25)	-	+
Koi	CEV	Gills	24 May 2021	-	+ (27,63)	-	+ (25,98)	-	+
Koi	CEV	Gills	24 May 2021	-	+ (19,83)	-	+ (19,61)	-	+
Koi	KHV	Gills	27 May 2021	+ (33,65)	-	+ (32,98)	-	+	-
Koi	CEV	Gills	31 May 2021	-	+ (21,23)	-	+ (20,86)	-	+
Koi	CEV	Gills	31 May 2021	-	+ (23,41)	-	+ (23,29)	-	+
Koi	KHV	Gills	02 Jun 2021	+ (31,49)	-	+ (31,05)	-	+	-
Common carp	KHV	Gills	02 Jun 2021	+ (30,71)	-	+ (30,56)	-	+	-
Koi	CEV	Gills	14 Jun 2021	-	+ (34,84)	-	+ (33,98)	-	+
Koi	CEV	Gills	14 Jun 2021	-	+ (33,97)	-	+ (33,68)	-	+
Koi	CEV	Gills	21 Jun 2021	-	+ (30,18)	-	+ (29,14)	-	+
Koi	KHV / CEV	Gills	28 Oct 2021/ 24 May 2021	+ (29,83)	+ (26,74)	+ (29,78)	+ (26,53)	+	+
Koi	KHV / CEV	Gills	28 Oct 2021 / 24 May 2021	+ (28,97)	+ (27,48)	+ (28,69)	+ (27,36)	+	+
Koi	KHV / CEV	Gills	28 Oct 2021 / 24 May 2021	+ (30,16)	+ (26,35)	+ (30,07)	+ (26,17)	+	+
Koi	CEV	Gills	03 Nov 2021	-	+ (35,22)	-	+ (35,27)	-	+
Koi	CEV	Gills	03 Nov 2021	-	+ (33,58)	-	+ (34,03)	-	+

Table 3. (Continue).

Common carp	KHV	Gills	30 Nov 2021	+ (33,87)	-	+ (32,96)	-	+	-
Koi	CEV	Gills	29 Des 2021	-	+ (28,12)	-	+ (28,20)	-	+
Koi	-	Gills	04 Jan 2022	-	-	-	-	-	-
Common carp	-	Gills	06 Jan 2022	-	-	-	-	-	-
Common carp	-	Gills	24 Jan 2022	-	-	-	-	-	-
Koi	KHV	Gills	27 Jan 2022	+ (33,22)	-	+ (32,98)	-	+	-
Koi	-	Gills	3 Feb 2022	-	-	-	-	-	-
Koi	-	Gills	7 Feb 2022	-	-	-	-	-	-
Koi	-	Gills	17 Feb 2022	-	-	-	-	-	-
Common carp	-	Gills	22 Feb 2022	-	-	-	-	-	-
Koi	KHV	Gills	01 Apr 2022	+ (35,80)	-	+ (35,60)	-	+	-
Koi	-	Gills	11 Mar 2022	-	-	-	-	-	-
Koi	-	Gills	12 Apr 2022	-	-	-	-	-	-
Common carp	KHV	Gills	6 Aug 2022	+ (29,54)	-	+ (29,53)	-	+	-
Common carp	KHV	Gills	26 Aug 2022	+ (36,30)	-	+ (35,80)	-	+	-
Common carp	KHV	Gills	20 Sep 2022	+ (26,51)	-	+ (26,78)	-	+	-
Koi	KHV	Gills	04 Nov 2022	+ (30,74)	-	+ (28,95)	-	+	-
Koi	CEV	Gills	14 Nov 2022	-	+ (34,17)	-	+ (34,23)	-	+
Koi	CEV	Gills	14 Nov 2022	-	+ (34,57)	-	+ (33,96)	-	+
Common carp	CEV	Gills	30 Nov 2022	-	+ (29,17)	-	+ (29,27)	-	+
ATCC 29473	*Yersinia ruckeri*	Isolate	2020	-	-	-	-	-	-
ATCC 33658	*Aeromonas salmonicida*	Isolate	2020	-	-	-	-	-	-
Distilled water	-	-	-	-	-	-	-	-	-

Discussion

KHV and CEV are two of the most serious viral pathogens of cyprinid fish, causing significant impact to cyprinid trade and the aquaculture industry, especially koi and common carp (Padhi *et al*., 2019; Sunarto *et al*., 2005). Both viruses have almost the same clinical signs, and mixed infection of both viruses can occur (Padhi *et al*., 2019; Toffan *et al*., 2019). The presence of KHV and CEV in common and koi carp with no clinical signs, which can act as reservoirs of the diseases, can lead to the spread of the diseases. The availability of rapid, specific, and sensitive diagnostic tools to correctly identify and distinguish the two viral pathogens simultaneously is therefore necessary.

Many PCR-based diagnostic tools have been developed to promptly diagnose KHV and CEV diseases separately, particularly conventional PCR and real-time PCR (Adamek *et al*., 2016; Gilad *et al*., 2004; Matras *et al*., 2017; Gray *et al*., 2002; Oyamatsu *et al*., 1997). Real-time PCR is preferred over conventional PCR in clinical laboratories. Conventional PCR is known to be time-consuming and contamination prone. Real-time PCR avoids these disadvantages, since no post-amplification process is required. In addition, in Taq-Man-based real-time PCR, the specificity of the test is improved by using specific probes instead of fluorescent intercalants as in SYBR Green-based real-time PCR. Detection of the two viral pathogens independently using real-time TaqMan PCR still remains time-consuming and costly, therefore simultaneous testing of the two viruses was developed in this study to be able to reduce these disadvantages.

In this study, a real-time duplex PCR assay was developed using two pairs of primers and two TaqMan probes designed from the conserved and specific regions of the KHV and CEV viruses, allowing this method provides results with high accuracy and precision in detecting and distinguishing the two viral infections. One main advantage of this assay compared to other available tests is that it is a duplex. By using this approach, it is possible to identify all two pathogens in the same reaction vessel. The simultaneous detection of KHV and CEV is especially useful, because these viruses commonly cause mixed infection in *Cyprinus* varieties.

This assay is also able to save costs, as it reduces the amount of PCR reagent consumption, where two viruses share the enzymes polymerase, dNTP, and buffer in one reaction. In addition, this assay is also able to reduce testing time because it is carried out simultaneously at the same time, and does not require post-amplification handling, so it can reduce contamination due to the electrophoresis process. In addition, this real-time duplex PCR assay does not require the unique expertise involved, such as in morphology-based tests, and can be performed in any laboratory with adequate infrastructure for real-time PCR testing. Therefore, this real-time duplex PCR assay can be extremely useful as a rapid and sensitive complement to the existing diagnostic method.

The good performance in terms of sensitivity and repeatability associated with the optimal efficiency and linearity of the reaction makes this real-time duplex PCR assay can be applied in aquaculture clinical laboratories as a routine diagnostic method and this assay is suitable for quantitative assay, both in culture and wild carps. However, the duplex feature of this assay is optional; if so preferred, the two components can be utilized as single-targeting assays or combined into duplex assays without impacting the quality of the results. This makes this assay adaptable to circumstances that may not require the simultaneous detection of all two for a diagnostic decision.

CONCLUSION

This study has shown that the real-time duplex PCR assay could be used as a reliable and specific diagnostic tool to detect KHV and CEV simultaneously in carp and koi aquaculture farms. This technique could be used to speed time of diagnosis in order to anticipate devastating outbreaks. The method can be easily performed when it is compared to methods such as cell cultures, histological examination, electron microscopy, and conventional PCR.

Conflict of Interest

The authors declare not to have any conflict of interest related to the content of this article.

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