Vol. 112: 37–44, 2014
doi: 10.3354/dao02785

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published November 13

Detection and application of circular (concatemeric) DNA as an indicator of koi herpesvirus infection

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ABSTRACT: Herpesviruses form a long continuous DNA molecule, or head-to-tail concatemer, as a replicating intermediate in the host. In this study, we developed a DNA-specific PCR assay for detecting the infection stage of koi herpesvirus (KHV) based on the presence of this 'endless' DNA. The 295 kbp double-stranded DNA KHV genome consists of a 251 kbp unique long region and two 22 kbp direct repeats (DR_L and DR_R) at each genome terminus. We designed a new primer set (DR primer set) based on the DR region spanning the presumed circular or concatemeric junction. Using the DR primer set, a PCR product was obtained from KHV-infected common carp brain (CCB) cells, but not from the virus-infected cell culture supernatant, implying that the PCR assay could detect intracellular virus in the host. The synthesis of a presumptive circular or concatemeric genome in virus-infected CCB cells was examined in a time-course experiment together with viral mRNA of the terminase gene, copy numbers of the viral genome, and infectious viral titer. The mRNA was first detected in the cells at 6 h post-inoculation (hpi), and the copy number of viral genome in the cells started to increase at 12 hpi. Subsequently, circular or concatemeric DNA was detected in the cells at 18 hpi, and progeny virus was detected in the cell culture supernatant at 24 hpi. These findings suggest that detection of the circular or concatemeric KHV genome with the developed PCR method can be used to determine the stage of KHV infection.

KEY WORDS: Cyprinid herpesvirus 3 · Koi herpesvirus · 'Endless' DNA · Concatemer · PCR assay

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INTRODUCTION

In common carp *Cyprinus carpio* and koi carp *C. carpio koi*, infection by cyprinid herpesvirus 3 (CyHV-3; aka koi herpesvirus, KHV) is associated with acute viremia and mass mortality. This virus has a global distribution and is associated with considerable economic losses (Haenen et al. 2004). Based on morphological and genetic characteristics, KHV is classified as a member of the family *Alloherpesviridae* which causes herpesvirus infections in finfishes (Waltzek et al. 2009). Among the members of the *Alloherpesviridae*, KHV is genetically closer to carp pox virus (CyHV-1) and goldfish hematopoietic necrosis virus (CyHV-2), than other viruses such as channel catfish virus (CCV) (Waltzek et al. 2005).

KHV has a large, 295 kbp double-stranded DNA genome consisting of a 251 kbp unique long (UL) region and two 22 kbp direct repeats (DR_L and DR_R) at each genome terminus (Aoki et al. 2007). This genome structure is the same as that of human herpesvirus 6 (HHV-6), which forms a long continuous DNA molecule, or head-to-tail concatemer, containing multiple copies of the viral genome (Thomson et al. 1994, Gompels & Macaulay 1995). CCV has 18.5 kbp DR_L and DR_R regions at each of the genome termini, and the intracellular genome has an 'endless' (circular or concatemer) configuration in acutely infected brown bullhead cells (Cebrian et al. 1983). Gray et al. (1999) designed a PCR primer set spanning the DR_R-to-DR_L junction in order to detect the presence of this circular or concatemeric DNA and verified latent infection of catfish by CCV. These studies demonstrated that KHV also forms circular or concatemeric DNA during the infection stage in the host and that the

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configuration can be used as an indicator of intracellular viral infection.

Although detection of viral mRNA by reverse transcription (RT)-PCR can be used to indicate viral replication in the host, mRNA is unstable in the tissue of sacrificed fish. On the other hand, detection of genomic DNA by PCR is easier to perform than RT-PCR, but conventional PCR cannot be used to determine whether the detected viral genome is derived from virus that replicated within the specimen, or whether the specimen was simply contaminated with the virus. In the present study, a new PCR primer set was designed based on the DR region of KHV-U (GenBank accession number DQ657948), KHV-I (DQ177346), and KHV-J (AP008984) to detect circular or concatemeric DNA, which is presumed to be produced during the viral infection stage (Fig. 1). We then verified efficacy of the PCR assay for identifying virus infection of CCB cells using a time-course experiment. We also performed RT-PCR to detect viral mRNA (Yuasa et al. 2012), TaqMan real-time PCR to measure the copy numbers of viral genome (Gilad et al. 2004), and quantitative isolation of progeny viruses by determining 50% tissue culture infectious dose ($TCID_{50}$). Finally, we clarified the life cycle of KHV *in vitro*.

MATERIALS AND METHODS

Virus

A KHV isolate (NRIA0301) from a diseased wild common carp captured in Japan was used in this study. The isolate was propagated in common carp brain (CCB; Neukirch et al. 1999) cells cultured in minimum essential medium (MEM; GIBCO/BRL Life Technologies) supplemented with 2% fetal bovine serum (FBS) and antibiotic-antimycotic (GIBCO/BRL Life Technologies) at 20°C for 7 d, after which the supernatant was stored at −80°C. The viral titer of this stock was $10^{5.8}$ $TCID_{50}$ ml^{-1}.

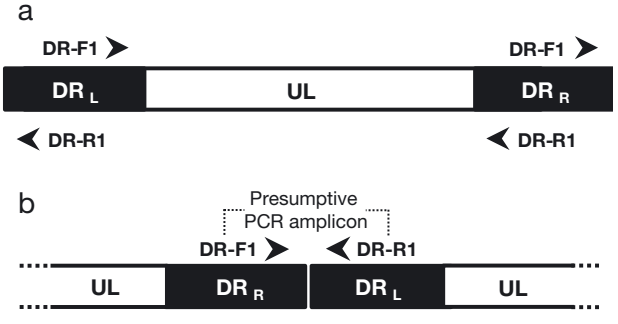

Fig. 1. Location of primers in the linear genome and the circular or concatemeric genome of koi herpesvirus (KHV). Primer annealing sites are indicated by arrowheads. The direct repeat (DR) primer set (DR-F1 and DR-R1) was designed based on the DR sequences of KHV-U (GenBank accession number DQ657948), KHV-I (DQ177346), and KHV-J (AP008984). (a) KHV genome (295 kbp) containing a 251 kbp unique long (UL) region and two 22 kbp terminal direct repeats (DR_L and DR_R). (b) Presumed DR_R-to-DR_L junction of circular or concatemeric KHV genome

Design of primers targeting circular or concatemeric DNA

A PCR primer set was newly designed to amplify the presumed DR junction (Fig. 1) according to the nucleotide sequences of KHV-U, KHV-I, and KHV-J (Aoki et al. 2007). The primer set (DR primer set), spanning the DR junction, consisted of a forward primer (KHV DR-F1: 5′AGG GTT AGG GCT CTG GTT GT′3) and a reverse primer (KHV DR-R1: 5′AGG GCT TGA GAC GGT AGG TT′3).

PCR targeting the DR junction (DR-PCR) and sequencing of DR-PCR amplicon

CCB cells cultured in a 25 cm^2 cell culture flask (Corning) containing MEM supplemented with 2% FBS were inoculated with KHV, followed by incubation at 20°C for 4 d. Total DNA was extracted from the whole CCB cells using a QIAamp DNA Mini Kit (Qiagen). PCR was performed using the DR primer set with 1 µl of the total cell DNA as template and *TaKaRa Ex Taq* Hot Start Version (TaKaRa). The thermal profile consisted of 1 denaturation cycle of 94°C for 2 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 63°C for 30 s, and extension at 72°C for 30 s, followed by a final extension step of 72°C for 7 min. To confirm the existence of the KHV genome, 2 additional PCR assays were also performed, following the *Sph* method (Yuasa et al. 2005) and the TK (thymidine kinase) method (Bercovier et al. 2005). Amplification products were analyzed by electrophoresis using 2% agarose gels and ethidium bromide staining. After electrophoresis, the PCR product in the agarose gel was excised and purified using a Wizard® SV Gel and PCR Clean-Up System (Promega) and directly sequenced using BigDye Terminator Kit v3.1 (Applied Biosystems). Sequencing reactions were purified using Agencourt CleanSEQ (Beckman Coulter) and loaded into an ABI PRISM 3130 *xl* Genetic Analyzer (Applied Biosystems).

Sensitivity and specificity of DR-PCR

KHV was prepared by 2 different methods. In one method, KHV-infected CCB cells in a 25 cm^2 cell culture flask prepared as described above were cultured at 20°C for 48 h. Whole infected CCB cells and 200 µl of the supernatant were then collected separately. Total DNA in each sample was extracted using a QIAamp DNA Mini Kit. In the other method, purified KHV was treated by DNase I as follows. KHV was propagated at 20°C for 4 d in CCB cells prepared in two 175 cm^2 cell culture flasks (Nunc), and the cell culture supernatant was filtered through a 0.45 µm membrane filter (EMD Millipore). Supernatant (90 ml) was mixed with 30 ml of polyethylene glycol solution (40% PEG 6000, 2 M NaCl), followed by incubation at 4°C for 1 h. After centrifugation (25 000 × *g*, 10 min, 4°C), the sediment was suspended in 1 ml of PBS. The concentrated virus suspension was overlaid with 10, 20, 30, 40, and 50% (w/w) CsCl gradient, and centrifuged (150 000 × *g*, 3 h, 20°C) with a SW40Ti rotor in an Optima XL-90 centrifuge (Beckman Coulter). The visible band was aspirated and dialyzed with PBS at 4°C overnight. The purified KHV was treated with DNase I (Invitrogen/Life Technologies) to remove KHV genome presumptively released from destroyed cells and which is not covered by viral capsid. The virion DNA was extracted using a QIAamp DNA Mini Kit. These DNA samples derived from the infected cells, supernatant, and purified virus were serially diluted 10-fold in distilled water, and DR-PCR and *Sph*-PCR were performed as described above. Cloning plasmids were prepared by ligating the amplicons obtained from the DR-PCR or *Sph*-PCR reactions into pGEM-Easy vector (Promega). The plasmids were propagated in *Escherichia coli* JM109 and purified with a QIAprep Spin Miniprep Kit (Qiagen). The amount of plasmid DNA was measured using a NanoVue spectrophotometer (GE Healthcare) and adjusted to 10^4 copies $µl^{-1}$. As with the DNA of cells, supernatant, and purified virus, the plasmid was diluted and subjected to PCR, and the amplified products were examined by agar electrophoresis as described above.

Detection of KHV in virus-infected CCB cells using various methods

CCB cells, dispersed with 0.25% trypsin (GIBCO/BRL Life Technologies) and suspended in MEM supplemented with 2% FBS, were transferred to three 6-well plates (10 cm^2 $well^{-1}$, Nunc) at 4 × 10^5 cells $well^{-1}$. After incubation overnight at 20°C, the cell culture medium was removed and the KHV stock was used to inoculate CCB cells at a multiplicity of infection (MOI) of 0.1 before incubating for 1 h to facilitate virus adsorption. The virus suspension was then removed and cells were washed 3 times with MEM. After adding new medium, the CCB cells were incubated at 20°C for 48 h. Three wells of the plate were sampled as triplicates at 0, 6, 12, 18, 24, and 48 h post-inoculation (hpi). Cell culture supernatant was collected and total DNA was extracted from 200 µl of the supernatant using a QIAamp DNA Mini Kit. After washing 3 times with MEM, total DNA and RNA were then extracted from infected CCB cells using an All-Prep DNA/RNA Mini Kit (Qiagen). DNA template was used for amplification by DR-PCR, *Sph*-PCR, and real-time PCR (Gilad et al. 2004). KHV mRNA was detected using the RT-PCR method of Yuasa et al. (2012). The viral titer of cell culture supernatant was determined using the 50% tissue culture infectious dose ($TCID_{50}$) method as described by Reed & Muench (1938). Briefly, the supernatants were serially diluted with medium in 10-fold increments, and 50 µl of each dilution were then inoculated onto monolayers of CCB cells in 96-well plates. The cultures were monitored daily for the appearance of cytopathic effect, and $TCID_{50}$ ml^{-1} was determined after 14 d.

RESULTS

PCR targeting the DR junction and sequencing of DR-PCR amplicon

KHV genome was detected from the DNA sample by conventional PCR methods. In this sample, a clear band in the gel was obtained by the newly developed PCR with the DR primer set (Fig. 2). The size of the amplified product (estimated to be approximately 650 bp by electrophoresis) was almost the same as the 666 bp and 654 bp that were estimated based on KHV-U and KHV-I sequences, respectively, when both ends of the DR region at the DR junction were contiguous. Direct sequencing of the PCR product of NRIA0301 with the DR primer set revealed that the size of the amplicon was 655 bp (Fig. 3). Several nucleotide deletions or mutations were observed among terminal sequences of the DR region in the NRIA0301 isolate, KHV-U, and KHV-I (Fig. 3). The DR_R-to-DR_L junction of the NRIA0301 isolate appeared to have a single base insertion (adenine) between the right terminus of DR_R and the left terminus of DR_L (Fig. 3).

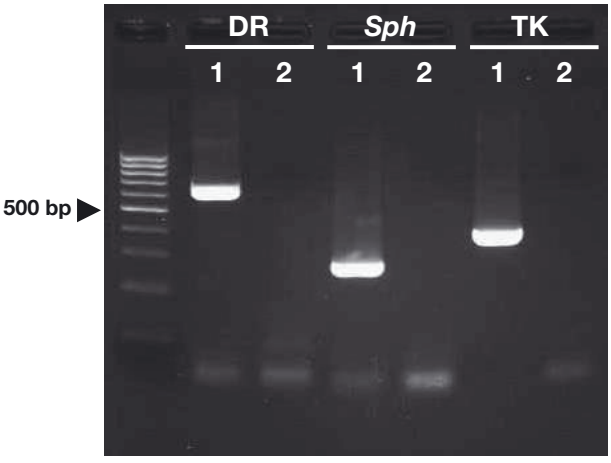

Fig. 2. PCR detection of the koi herpesvirus (KHV) genome from virus-infected cells by different PCR methods. DNA template was prepared from KHV-infected common carp brain (CCB) cells at 4 d post-inoculation. DR: PCR amplification using the direct repeat (DR) primer set; *Sph*: *Sph* method of Yuasa et al. (2005); TK: TK method of Bercovier et al. (2005). Lane 1 is the amplicon from KHV-infected CCB cells at 4 d post-inoculation. Lane 2 contains the negative control (distilled water). The left-most lane contains a 100 bp ladder molecular size marker

Sensitivity and specificity of DR-PCR

When the cloned plasmid was used as template, the detection efficiency of the DR-PCR assay was the same as that of the *Sph*-PCR assay (Fig. 4a). When DNA that had been extracted from infected cells was used, the efficiency of *Sph*-PCR was higher than that of DR-PCR by an order of magnitude (Fig. 4b). On the other hand, no amplicons were obtained when any of the cell culture supernatants were subjected to DR-PCR, but amplification by *Sph*-PCR produced amplicons with 3 of the supernatant dilutions (Fig. 4c). In addition, with the DNA extract from the purified virus, *Sph*-PCR produced a clear band from each template of 10^0 to 10^5 dilutions, but DR-PCR did not produce any band (Fig. 4d). These findings revealed that DR-PCR could be used to detect the circular or concatemeric genome in the virus-infected cells, but not the linear genome of the virion.

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NRIA0301(DR-PCR) AGGGTTAGGGCTCTGGTTGTCTTTGCGTTAGGGTTAGGGCTGTGTGCTGAATCTCCCTGCCAAGTGTTGGGGCTAGGGGTAGGATTAGGGTTAGGGTT
KHV-U(DQ657948)  AGGGTTAGGGCTCTGGTTGTCTTTGCGTTAGGGTTAGGGCTGTGTGCTGAATCTCCCTGCCAAGTGTTGGGGCTAGGGGTAGGATTAGGGTTAGGGTT
KHV-I(DQ177346)  AGGGTTAGGGCTCTGGTTGTCTTTGCGTTAGGGTTAGGGCTGTGTGCTGAATCTCCCTGCCAAGTGTTGGGGCTAGGGGTAGGATTAGGGTTAGGGTT
                 ****************************************************************************************************
                 DR-F1

NRIA0301(DR-PCR) CAGAGTGTTATAGTTGGGACGGTTATGGCTATGGGCTAGGGCTAGGGTTAGGGCTGGAGCTAGAGCGCACTGCTAGGGCTGGTTAGGGTTAGGATTAG
KHV-U(DQ657948)  CAGAGTGTTATAGTTGGGACGGTTATGGCTATGGGCTAGGGCTAGGGTTAGGGCTGGAGCTAGAGCGCACTGCTAGGGCTGGTTAGGGTTAGGATTAG
KHV-I(DQ177346)  CAGAGTGTTATAGTTGGGACGGTTATGGCTATGGGCTAGGGCTAGGGTTAGGGCTGGAGCTAGAGCGCACTGCTAGGGCTGGTTAGGGTTAGGATTAG
                 ****************************************************************************************************

NRIA0301(DR-PCR) GGCTTCGGACTGTGTTAGGGCTAGGGTTAGTGTTAGGGCTAAAATTAGGGCTAGAATTAGGGCTAGAAGTTGGGGCGGTTTTTAAATGGAGCTAACCC
KHV-U(DQ657948)  GGCTTCGGACTGTGTTAGGGCTAGGGTTAGTGTTAGGGCTAAAATTAGGGCTAGAA----------GTTGGGGCGGTTTTTAAATGGAGCTAACCC
KHV-I(DQ177346)  GGCTTCGGACTGTGTTAGGGCTAGGGTTAGTGTTAGGGCTAAAATTAGGGCTAGAA----------GTTGGGGCGGTTTTTAAATGGAGCTAACCC
                 ********************************************************          ******************************

NRIA0301(DR-PCR) TAACACCAGGGGTCTAGGTAAGGCAAGGACCTTGCCACCCTACTTTTCTCTAGAGCTTAACCCTAATTTTTCTAACTGCCCTAACCCTAACC-----C
KHV-U(DQ657948)  TAACACCAGGGGTCTAGGTAAGGCAAGG|CCTTGCCACCCTACTTTTCTCTAGAGCTTAACCCTAATTTTTCTAACTGCCCTAACCCTAACCTAGCCC
KHV-I(DQ177346)  TAACACCAGGGGTCTAGGTAAGGCAAGG|CCTTGCCACCCTACTTTTCTCTAGAGCTTAACCCTAATTTTTCTAACTGCCCTAACCCTAACCTAGCCC
                 ****************************|****************************************************************     *
                 Right terminus of DR_R ◄----|----► Left terminus of DR_L

NRIA0301(DR-PCR) TAACCCTAACCCTAACCCTAACCCTAACC-TAACCCTAGCCCTAGCCCTA------------------ACCCTAACCCTATTCCGTCGTCCAAGACGG
KHV-U(DQ657948)  TAGCCCTAGCCCTAGCCCTAGCCCTAGCCCTAGCCCTAGCCCTAGCCCTAGCCCTAACCCTAACCCTAACCCTATTCCGTCGTCCAAGACGG
KHV-I(DQ177346)  TAGCCCTAGCCCTAGCCCTAGCCCTAGCCCTAGCCCTAGCCCTAGCCCTA------------ACCCTAACCCTAACCCTATTCCGTCGTCCAAGACGG
                 ** ***** ***** ***** ***** ** ** *****************          ******************************

NRIA0301(DR-PCR) CGTGAAACAGCCCCAAGTAGGGTGTCCGCACCACACGACGGTCATTTGCTGGGTGGGAGGAGCCTAAATGGCTTTAAAACTGGCTTCTGCAGCCATGG
KHV-U(DQ657948)  CGTGAAACAGCCCCAAGTAGGGTGTCCGCACCACACGACGGTCATTTGCTGGGTGGGAGGAGCCTAAATGGCCTTAAAACTGGCTTCTGCAGCCATGG
KHV-I(DQ177346)  CGTGAAACAGCCCCAAGTAGGGTGTCCGCACCACACGACGGTCATTTGCTGGGTGGGAGGAGCCTAAATGGCCTTAAAACTGGCTTCTGCAGCCATGG
                 ************************************************************************ ************************

NRIA0301(DR-PCR) TGGACACTTCCAACCCTACCTGGAGCATCAAAACTCATCAAAACCTCTACAAAAACAGCCGCTTCTACTCAAACCTACCGTCTCAAGCCCT 655 bp
KHV-U(DQ657948)  TGGACACTTCCAACCCTACCTGGAGCATCAAAACTCATCAAAACCTCTACAAAAACAGCCGCTTCTACTCAAACCTACCGTCTCAAGCCCT 666 bp
KHV-I(DQ177346)  TGGACACTTCCAACCCTACCTGGAGCATCAAAACTCATCAAAACCTCTACAAAAACAGCCGCTTCTACTCAAACCTACCGTCTCAAGCCCT 654 bp
                 ************************************************************************************
                                                                                                  DR-R1
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Fig. 3. Sequence of direct repeat (DR)-PCR amplicon of the koi herpesvirus (KHV) NRIA0301 isolate and terminal sequences of the DR region in KHV-U and KHV-I. The sequence of NRIA0301 was determined by sequencing of the DR-PCR amplicon (accession number AB968522). The sequences of the right terminus of DR_R and the left terminus of DR_L were derived from registered sequences of KHV-U (DQ657948) and KHV-I (DQ177346). Underlining indicates primer sequences (DR-F1 and DR-R1). Asterisks indicate the consensus sequence among NRIA0301, KHV-U, and KHV-I

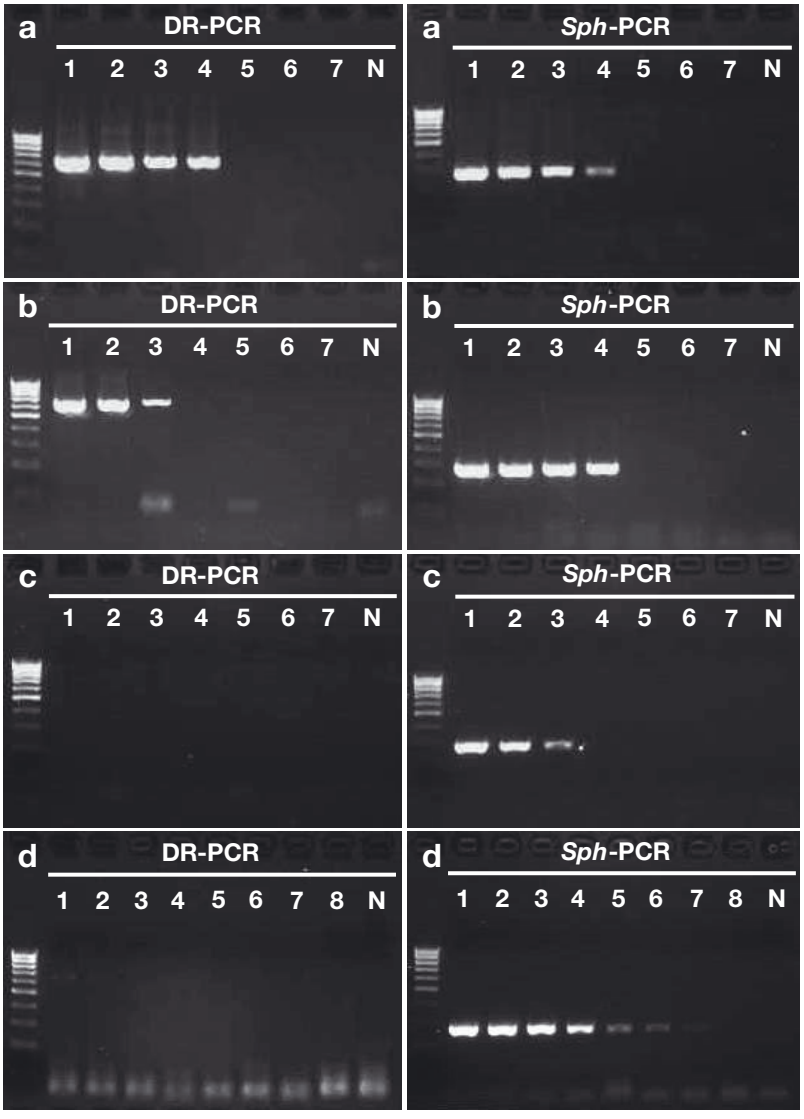

Fig. 4. PCR amplification of 4 DNA templates extracted from (a) cloned plasmid (10^4 copies μl^{-1}), (b) koi herpesvirus (KHV)-infected CCB cells at 48 h post-inoculation, (c) culture supernatants of (b), and (d) purified KHV with 2 primer sets (DR-PCR and *Sph*-PCR). Each template was serially diluted 10-fold with distilled water (Lanes 1 to 8: serial dilutions from 10^0 to 10^{-7}, respectively). N: negative control (distilled water)

Kinetics of KHV genome expression in CCB cells and comparison of viral detection methods

DR-PCR produced a faint band when using template collected at 12 hpi, and clear bands were produced when using templates collected at 18 hpi (Fig. 5a, top). The mRNA of the terminase gene was expressed at 6 hpi, which was before the formation of the circular or concatemeric genome (Fig. 5a, center). Conventional PCR detected viral genome at all sample times, even before the viral replication cycle had started (Fig 5a, bottom). Real-time PCR revealed that the copy numbers of the KHV genome decreased temporarily in CCB cells at 6 hpi, followed by a continuous increase from 12 to 48 hpi. Conversely, although copy numbers and infectious viral titers in the cell culture supernatants were slightly increased at 6 hpi, both had started to show a clear increase at 24 hpi (Fig. 5b).

DISCUSSION

In the present study, we designed a new primer set to detect the presumptive DR_R-to-DR_L junction of the circular or concatemeric genome of KHV and obtained a unique amplicon by PCR amplification of KHV-infected CCB cells using the DR primer set (Fig. 2). The DR-PCR primer sequences (KHV DR-F1 and KHV DR-R1) were conserved among KHV-U, KHV-I, and KHV-J, which suggests that the primer set can be used to detect circular or concatemeric genome of all KHV strains. Fig. 4 shows that DR-PCR with the cloned plasmid had the same sensitivity as *Sph*-PCR. On the other hand, the sensitivity of DR-PCR with KHV-infected CCB cells was lower than that of *Sph*-PCR, and no amplification product was obtained from the cell culture supernatant samples subjected to DR-PCR. This difference indicates that *Sph*-PCR can detect both linear and 'endless' configurations, but DR-PCR can only detect 'endless' genome. PCR analysis of the purified KHV also supports the specificity of DR-PCR (Fig. 4d). Therefore, we consider that the DR-PCR assay employed in this study can specifically amplify the circular or concatemeric DNA of KHV, while *Sph*-PCR cannot.

When we estimated the size of the DR-PCR amplicon based on registered sequences of KHV-U, KHV-I, and KHV-J, the presumptive PCR product of KHV-J (427 bp) was much smaller than that of KHV-U (666 bp) and KHV-I (654 bp). The size of the DR-PCR amplicon was approximately 650 bp (Fig. 2). This phenomenon seemed to be due to the lack of a

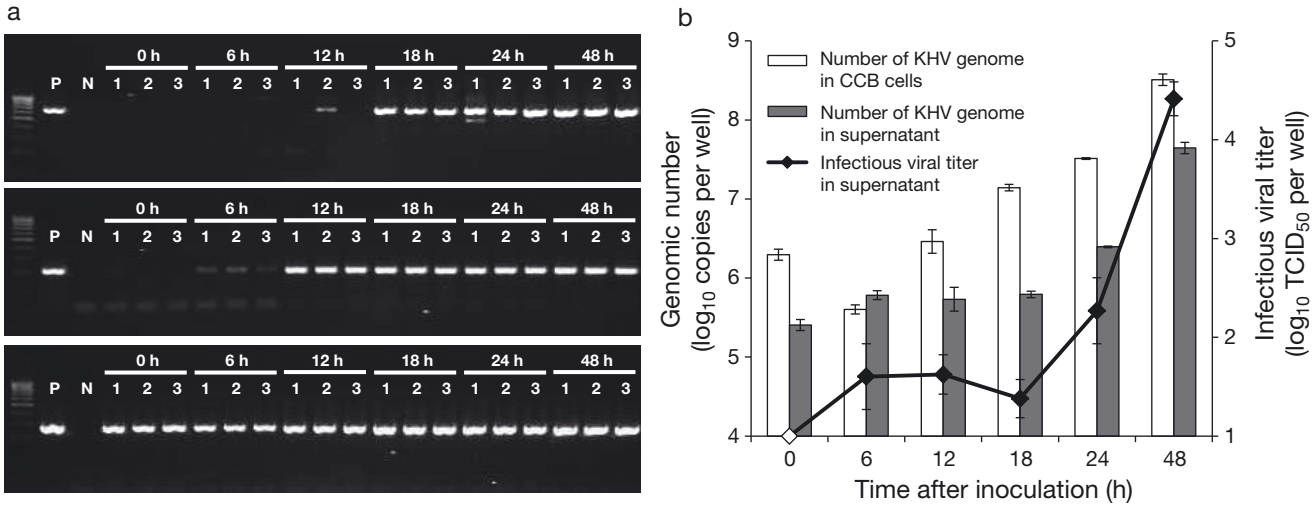

Fig. 5. Kinetics of the koi herpesvirus (KHV) genome in common carp brain (CCB) cells. CCB cells were inoculated with KHV at a multiplicity of infection of 0.1 and incubated at 20°C. Total DNA and RNA of CCB cells and DNA of cell culture supernatant were collected at 0, 6, 12, 18, 24, and 48 h post-inoculation. (a) Detection of KHV genome by DR-PCR (top) and *Sph*-PCR (bottom), and detection of viral mRNA by RT-PCR according to Yuasa et al. (2012) (center). Lane numbers 1 to 3 are triplicate samples at each sample time. P: positive control; N: negative control (distilled water). (b) Quantitation of viral genomic number in virus-infected CCB cells and cell culture supernatant by real-time PCR (Gilad et al. 2004), and infectious viral titer of the cell culture supernatant. Data are means ± SD

terminal sequence of the DR region in the registered KHV-J sequence. Therefore, we did not use the KHV-J sequence to compare the terminal region of DR (Fig. 3).

Our sequencing analysis of the DR-PCR amplicon of the NRIA0301 isolate revealed that the DR junction of KHV had a single nucleotide insertion between DR_R and DR_L (Fig. 3). In the case of HHV-6 which has a 10 kbp DR_L and DR_R region at the end of each genome terminus (Gompels et al. 1995), the DR junction was sequenced using a PCR product, and aberrant junction sequences due to several nucleotide insertions or deletions were observed (Thomson et al. 1994, Gompels & Macaulay 1995). On the other hand, the DR junction of CCV contained 11 to 17 bp of the right terminus of UL sequences following the DR_R region (Gray et al. 1999). While previous studies attributed the existence of nucleotides between DR regions to PCR artifacts (Thomson et al. 1994, Gompels & Macaulay 1995, Gray et al. 1999), our sequence analysis of the DR-PCR amplicon in the time-course experiment showed that the same nucleotide sequences were present in all of the sequenced samples (data not shown). Further investigation is therefore necessary to examine the configuration of the DR junction of KHV.

In the present study, we attempted to clarify the life cycle of KHV in CCB cells by examining mRNA expression, the formation of a circular or concatemeric genome, copy number of the viral genome, and infectious viral titer. Since the genomic copy number and infectious viral titer in the cell culture supernatant increased significantly at 24 hpi, we considered that a single virus propagation cycle requires 24 h to complete (Fig. 5b). Slight increases in the $TCID_{50}$ and genome copies in the supernatant at 6 to 18 hpi can be explained by contamination with inoculum of infectious virus non-specifically attached to CCB cells. These results suggest that the initial replication cycle of KHV in CCB cells is completed at 24 hpi. The target gene for RT-PCR analysis is the putative ATPase subunit of the terminase gene (ORF 33; Aoki et al. 2007), which is responsible for packaging the viral genome and is conserved in herpesviruses; for example, the UL15 gene in HSV-1 (Dolan et al. 1991), U60 and U66 in HHV-6 (Gompels et al. 1995), and ORF62, ORF69, and ORF72 in CCV (Davison 1992). mRNA expression in herpesviruses is classified into immediate early (IE), early (E), and late (L) genes (Honess & Roizman 1974). Ilouze et al. (2012) reported that expression of the terminase gene was detected from 4 hpi in KHV-infected CCB cells, and demonstrated that the terminase gene of KHV was the E gene. In this study, RT-PCR revealed that the KHV terminase gene in CCB cells was initially detected at 6 hpi and was abundant by 12 hpi (Fig. 5a, center). This expression pattern in CCB cells was similar to the result of Ilouze et al. (2012). On the

other hand, the circular or concatemeric genome was detected by DR-PCR at 18 hpi, immediately before packaging of DNA into the viral capsid. Generally, viral DNA synthesis in herpesviruses begins shortly after the appearance of E genes, and the viral genome is mainly synthesized during the latter part of the infection cycle (Lehman & Boehmer 1999). Thus, unlike assays based on mRNA expression, DR-PCR can be used to specifically identify the latter stages of KHV replication, making it better suited for use as an indicator of viral infection in the host.

Many herpesviruses form head-to-tail concatemers as an intermediate stage during viral replication (Jacob et al. 1979, Cebrian et al. 1983, Martin et al. 1991). Indeed, the replicating intermediate of KHV was observed as 'well' DNA, which does not migrate from the sample wells in Southern blot analysis of KHV-infected CCB cells because of its circular or concatemeric configuration (Dishon et al. 2007). In the present study, the copy number of KHV genome in CCB cells increased from 12 hpi, but DR-PCR amplicons were stably detected from 18 hpi onward (Fig. 5). In HHV-6, whose genome structure is the same as that of KHV, equal amounts of both circular and concatemeric DNA were produced as replicating intermediates in host cells (Severini et al. 2003). These findings imply that, like HHV-6, KHV also has 2 replication intermediates and that DR-PCR can detect latter-stage replication intermediates.

This is the first report to show that PCR detection of circular or concatemeric DNA can be used as an indicator of KHV replication at the infection stage. Recently, the host range of KHV has received considerable attention due to the international and domestic live-fish trade, and virus susceptibility has been examined in a variety of potential host species (Yuasa et al. 2013). However, the conventional PCR methods (Gilad et al. 2002, Gray et al. 2002, Bercovier et al. 2005, Yuasa et al. 2005) widely used for KHV disease diagnosis are ineffective for evaluating viral replication in the host. For example, in the time-course experiment of this study, conventional PCR detected the viral genome immediately after virus inoculation and could not distinguish between the inoculum and replicated viruses (Fig. 5a, bottom). Detection of expressed viral mRNA by mRNA-specific RT-PCR is therefore a useful method for confirming virus replication in the host (Yuasa et al. 2012). On the other hand, the DR-PCR method developed in this study is useful for determining whether the host is infected by the virus. In the case of CCV, 'endless' DNA was detected from latently infected fish by PCR assay (Gray et al. 1999). We also confirmed that the circular or concatemeric DNA was detectable in fish surviving KHV infection by DR-PCR, although the mRNA was not detected by the RT-PCR (unpublished data). Consequently, the RT-PCR method is useful for detecting active viral replication in KHV-infected fish, and the DR-PCR assay can be an indicator of intracellular viral infection and can be valuable for evaluating the host range of KHV.

Acknowledgements. This study was supported by grants from the Food Safety and Consumer Affairs Bureau, Ministry of Agriculture, Forestry, and Fisheries of Japan.

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Editorial responsibility: Mark Crane, Geelong, Victoria, Australia

Submitted: March 6, 2014; Accepted: June 20, 2014
Proofs received from author(s): September 26, 2014