

Investigation of KHV DNA Methylation during Productive and Latent Infection

by  
Lisa Lin

A THESIS

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## AN ABSTRACT OF THE THESIS OF

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Abstract approved: \_\_\_\_\_

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Koi herpesvirus (KHV), is a highly pathogenic virus causing high mortality in common carp and koi, especially in fry. One of the unique features of herpesvirus infections is their ability to become latent following initial infection. During latent infection, only viral genome persists in the host and remains dormant in the infected cells. It is unknown how KHV latency is established and maintained within the infected cells. DNA methylation has been previously shown to play a role in the latency maintenance of the *gammaherpesviruses*, Epstein-Barr Virus and Kaposi's Sarcoma-Associated Herpesvirus. The expression of viral DNA polymerase gene, ORF79, is required for viral genome replication and CpG islands were found to be present in the ORF79 promoter region. In this study, DNA methylation of the ORF79 promoter was investigated to understand the regulation of KHV genome replication during productive and latent infection. Using bisulfite conversion and PCR amplification with methylation-specific primers, DNA methylation within the ORF79 promoter was found to be present in viral genome isolated from productive infection *in vitro*. Total DNA of white blood cells (WBC) containing a small fraction of KHV DNA was also examined, however, the amount of DNA was too low to assess the status of DNA methylation within ORF79 promoter during latent infection.

Key Words: KHV, ORF79, latency, methylation, bisulfite conversion

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I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes release of my project to any reader upon request.

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Lisa Lin, Author

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## ***Chapter 1: Investigation of KHV DNA Methylation in Productive and Latent Infection***

### **INTRODUCTION**

Cyprinid Herpesvirus 3 (CyHV-3), commonly known as Koi herpesvirus (KHV), is a highly pathogenic virus that affects *Cyprinus Carpio*, including common carp and koi fish. KHV is a member of the *Alloherpesviridae* family within the order of *Herpesvirales*, and affects fish of all ages, but is especially virulent to fry<sup>1</sup>. Clinical symptoms of KHV infection include red or white gill mottling, hemorrhage, pale patches and blisters along the skin, and death can occur within 24-48 hours following onset of symptoms. The virus can be shed through feces, gill, and skin mucus, which can be easily transmitted to naïve fish<sup>2</sup>.

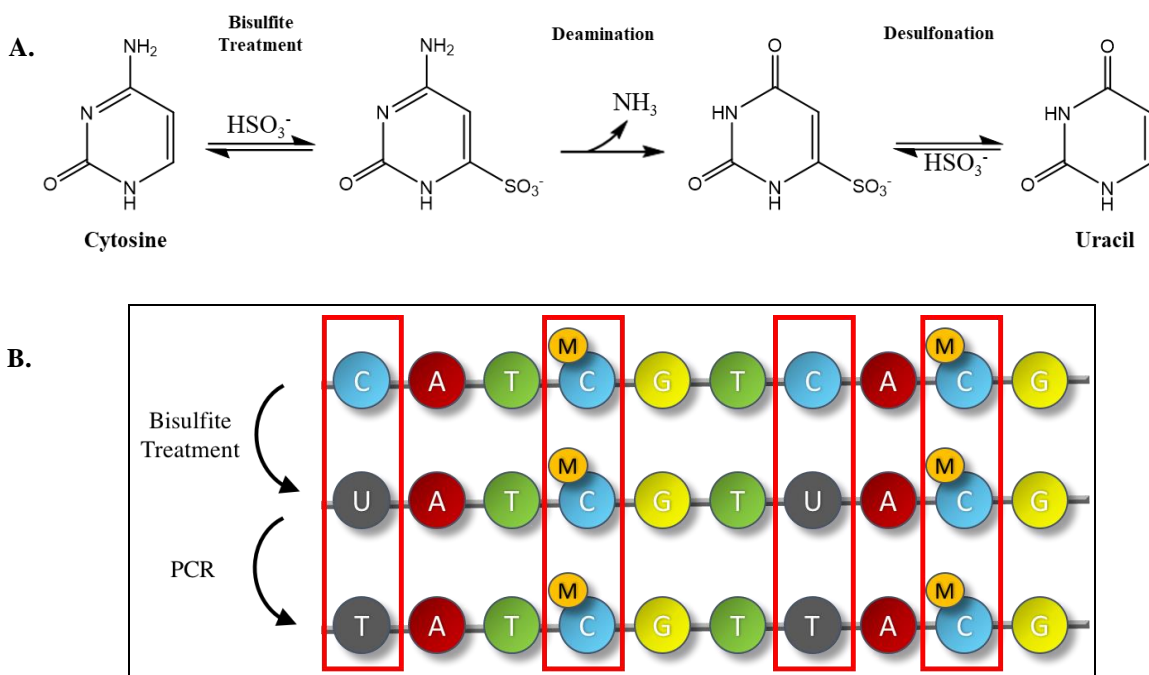
One of the hallmarks of herpesvirus infections is their ability to establish latent infection, when the virus enters a dormant state, shuts down viral gene expression, and avoids immune detection. During the latent period, no viral progeny is produced, and the host cell remains viable. Latency allows herpesviruses to persist within a host for a lifetime. Under stressful conditions, herpesviruses can reactivate from latency and re-enter the lytic cycle where copious amounts of viral progeny (infectious particles) can be produced and spread to other hosts. It has been shown that KHV becomes latent mostly in the B-cells in peripheral blood and during KHV latent infection, only viral DNA can be detected in the white blood cells (WBC) from the latently infected koi<sup>2</sup>. It has also been observed that KHV reactivation often occurs in the spring to autumn months when water temperatures increase from 18°C to 26°C<sup>3</sup>. This temperature increase causes KHV to reactivate from latency, leading to acute infection and the spreading of the KHV to naïve fish. Understanding latency is crucial for developing strategies against herpesvirus infection.



The mechanisms governing herpesvirus latency is still largely unknown. However, it is speculated that viral gene expression is repressed during latency by various mechanisms including epigenetic mechanisms. One of the major epigenetic mechanisms thought to be involved in latency is DNA methylation, a chemical modification of DNA where a methyl group is transferred onto the C5 position of cytosines to form 5-methylcytosine. The majority of DNA methylation occurs on cytosines that precede a guanine nucleotide, or CpG sites. CpG islands are short stretches of DNA containing CpG sites with a GC content of at least 50%, and an observed:expected CpG frequency ratio of at least 0.6. CpG islands are typically located in the promoter region upstream of a gene's transcriptional start site. It is well established that methylation of CpG islands upstream of viral genes can lead to stable epigenetic repression<sup>4</sup>. Methylation of CpG islands can impair transcription factor binding, recruit repressive methyl-binding proteins, and stably silence gene expression. Studies on the Epstein - Barr virus (EBV) latent genome have demonstrated that CpG site methylation occurs upstream of several viral genes during latency stages, which suggests that DNA methylation plays a role in repression of viral gene expression to maintain latency and avoid immune detection by the host<sup>5-8</sup>. DNA methylation at promoters of genes involved in transcription was also demonstrated in the latent infection of Kaposi Sarcoma-Associated herpesvirus (KSHV), and demethylation resulted in viral gene expression and KSHV reactivation, again demonstrating the role of DNA methylation in latency maintenance<sup>9</sup>.

Both EBV and KSHV are members of the subfamily of *Gammaherpesvirinae* within *Herpesviridae*, whereas KHV is a member of the *Alloherpesviridae* subfamily. It is unknown whether epigenetic mechanisms are conserved between these herpesviral families. To date there have been no studies on the role of epigenetic modification in KHV latency establishment and

maintenance. KHV ORF79 encodes a DNA polymerase catalytic subunit and is required for viral DNA replication<sup>1</sup>, and several CpG islands were found within the promoter region of ORF79. We hypothesize that CpG islands within the KHV ORF79 promoter are methylated during latency, which contributes to repression of gene expression during latent infection. Here, we investigate DNA methylation of ORF79 promoter by bisulfite treatment and PCR amplification with primers specific to methylated viral DNA and unmethylated viral DNA. Treatment of DNA with bisulfite ions preferentially deaminates unmethylated cytosines, which, upon alkaline desulfonation, are converted to uracils (Reaction 1)<sup>10</sup>. Bisulfite conversion of 5-methylcytosines occur much more slowly<sup>11,12</sup>.



**Figure 1: The working principle of bisulfite treatment of unmethylated cytosines.** (a) Reaction scheme for bisulfite-driven deamination of unmethylated cytosine to uracil. (b) Conversion of DNA in the presence and absence of 5-methylcytosines.

By selectively converting unmethylated cytosines to uracils via bisulfite treatment, while retaining 5-methylcytosines, the difference between methylated cytosines and unmethylated

cytosines can be detected by PCR using primers specifically designed to the target region and DNA sequencing analysis of the PCR products.

## **MATERIALS AND METHODS**

### KF-1 cell line and viral culture

Koi fin cell line (KF-1) (a gift from Ronald Hedrick, University of California, Davis) was cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA), penicillin (100 U/ml), and streptomycin (100 µg/mL) (Sigma-Aldrich, St. Louis, MO) and incubated at 22°C. The KHV strain from Israel (KHV-I) was a gift from Ronald Hedrick. The virus was cultured in KF-1 cells maintained in DMEM supplemented with 5% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 µg/mL) and incubated at 22°C.

### Purification of KHV DNA

Viral DNA from productive infection was extracted from either KHV-I virions or purified intracellular nucleocapsids as described previously<sup>13</sup>. Briefly, the purified virions or nucleocapsids were digested in 10 mM Tris-HCl (pH 8.0), 100 mM EDTA, 1% *N*-lauroyl sarcosine, and 200 µg/mL proteinase K overnight at 55°C. The viral DNA was extracted twice with an equal volume of phenol-chloroform (1:1 [vol/vol]) and then precipitated with two volumes of ethanol and 1/10 volume of sodium acetate. The precipitate was washed once in 70% ethanol and dissolved in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA).

## CpG Island identification and primer selection: Methylated-Specific and Unmethylation-Specific PCR primers

CpG islands of the ORF79 promoter region were analyzed by MethPrimer 2.0<sup>14</sup>. Primers specific for the KHV ORF79 promoter region amplification were selected based on the annotated KHV genome sequence data available through GenBank (NC\_009127.1). Since bisulfite treatment of unmethylated DNA results in altered DNA sequence, two sets of methylation specific primers were designed to amplify bisulfite treated methylated or unmethylated DNA using MethPrimer 2.0 primer design software.

## Bisulfite Treatment and PCR Amplification

Approximately 4 µg of purified KHV viral DNA was subjected to bisulfite treatment using the protocol provided by the EpiTect Bisulfite Kit (Qiagen, Hilden, Germany). The elution step was performed twice to increase the yield of treated DNA and each conversion reaction resulted in around 60% yield of converted genomic KHV DNA. Hot-start PCR amplification using both methylation and unmethylated specific primer sets were performed for both treated and non-treated viral DNA. PCR reaction was performed in a 50 µL reaction consisting of 1X Mg<sup>2+</sup> PCR Buffer, 0.025 U/µL Taq Polymerase (G-Biosciences, St. Louis, MO), 0.2 mM dNTP, 20 µM of each of the forward and reverse primers, and 400 ng of treated or non-treated viral DNA. Reactions for non-treated viral DNA and methylation-specific primers had 5 µL of Platinum GC Enhancer (Invitrogen, Carlsbad, CA) included because of the high GC content of the non-treated KHV viral DNA. The reaction was subjected to 94°C for 2 minutes, followed by 40 cycles of amplification consisting of denaturation at 94°C for 30 sec, annealing at 45°C for 45 sec, and extension at 72°C for 1 min. The resulting PCR products were run on a 1% agarose gel

and stained with SYBR<sup>TM</sup> Safe DNA Gel Stain (ThermoFisher Scientific, Waltham, MA) to detect for successful amplification by either the methylation specific or non-methylated specific primers.

### Sequencing Analysis

PCR products were purified using ChargeSwitch<sup>TM</sup> PCR Clean-Up Kit (Invitrogen, Carlsbad, CA), and sequenced using Sanger sequencing to determine if the bisulfite treatment of the KHV viral DNA resulted in sequence changes. Sequences were aligned to the ORF79 KHV promoter sequence obtained through GenBank (NC\_009127.1) with Geneious alignment using Geneious 11.1.3 bioinformatics software.

### Source of koi and sampling

Latently infected KHV<sup>+</sup> koi were obtained from a local pet store in Corvallis, Oregon with previous history of KHV outbreaks in the summers of 2015 and 2016. All fish have been previously confirmed to be infected with KHV<sup>3</sup>. Four 4-foot diameter tanks containing six koi each were set up and maintained at 15°C in accordance with the Animal Care and Use Committee guidelines at the John L. Fryer Aquatic Animal Health Laboratory in Corvallis, Oregon. The fish were quarantined for several months to ensure that all KHV infection was in the latency stage. To determine whether the promoter region of ORF79 is methylated during KHV latent infection, koi were anesthetized with MS-222 (100 ppm) and blood samples were collected via caudal vein puncture. Blood samples were stored in 0.35% sodium citrate.

### Total white blood cell isolation from koi

Approximately 2 mL of whole blood containing sodium citrate anti-coagulant was diluted in equal volume of RPMI-1640 medium. WBC were separated through Ficoll-Paque PLUS gradient according to the manufacturer's instructions (GE Healthcare, Little Chalfont, UK). Briefly, diluted total blood was placed on top of the Ficoll-Paque gradient and centrifuged at 400 x g for 40 minutes at 20°C. Total WBC were isolated and washed once with RPMI-1640 solution and twice more with PBS before total DNA extraction.

### Total DNA Extraction from WBC and Methylation Analysis

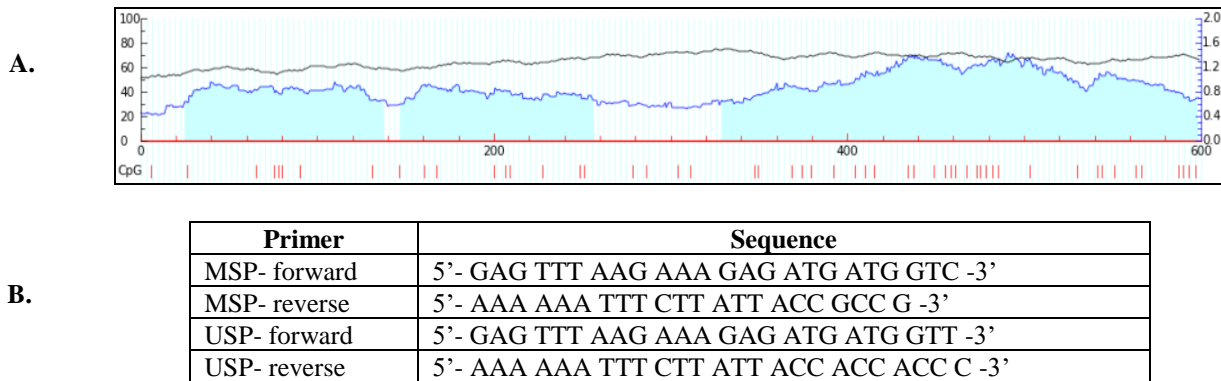
Total WBC at  $10^6$  cells/mL were treated in 400  $\mu$ L of hypotonic buffer (50 mM Tris-HCl pH 8.0, 5 mM EDTA, 2% Triton X-100, 50  $\mu$ g/mL RNase) overnight at 4°C. Total DNA released from WBC following hypotonic buffer was recovered by centrifugation at 10,000 x g for 10 min to remove cellular organelles and other debris. DNA in the supernatant was isolated using DNA binding columns provided by the EZNA Tissue DNA extraction kit (Omega Bio-tek, Norcross, TA). DNA concentration was measured through  $A_{260}$  measurement. Total WBC DNA isolated from 2 mL of whole koi blood ranged from 1 - 10 ng/ $\mu$ L. An alternative method was also used to isolate total DNA from WBC. Briefly, DNA from lysed WBC were extracted via phenol-chloroform extraction, precipitated with ethanol, and re-dissolved in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). Extracted WBC DNA was subjected to bisulfite treatment using the protocol provided by the EpiTect Bisulfite Kit (Qiagen, Hilden, Germany). Each conversion reaction used approximately 2  $\mu$ g of extracted WBC DNA and resulted in around 60% yield of treated WBC DNA. Treated and non-treated WBC DNA were subject to hot-start PCR similar to

as described above, and PCR products were run on a 1% agarose gel for methylation analysis of the ORF79 promoter region.

## RESULTS AND DISCUSSION

### Identification of CpG Islands within ORF79 Promoter

To determine if CpG sites or islands exist in the ORF79 promoter, the DNA sequence upstream of ORF79 translational start site (-769 to -170) including the ORF79 promoter region was analyzed by MethPrimer 2.0 for CpG island prediction. As shown in Fig. 2A, three potential islands within the query sequence were detected with GC contents of 50%, and observed:expected CpG frequency ratios of 0.6. This suggests that ORF79 promoter sequence has potential to be regulated via DNA methylation during KHV infection.



**Figure 2: Location of CpG Islands within KHV ORF79 promoter and MSP Primer design via MethPrimer**

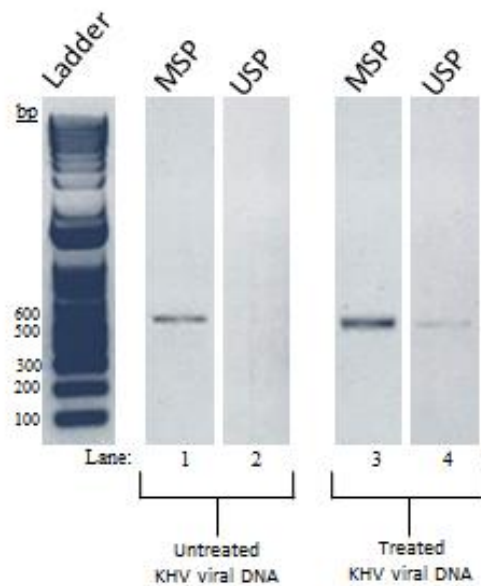
**2.0.** (a) CpG Islands were predicted in the promoter region upstream of the ORF79 translational start site using MethPrimer 2.0. The light-blue shaded regions are the predicted CpG islands. (b) Following CpG Island prediction, two primer pairs, USP and MSP, were designed to amplify either bisulfite treated unmethylated or bisulfite treated methylated DNA respectively.

To analyze the ORF79 promoter methylation status within KHV genome during different infection stages, two sets of primers were selected which flank the CpG islands (Fig. 2B): methylated DNA-detecting (MSP) primers, and unmethylated DNA-detecting (USP) primers. The MSP primers will detect methylated DNA sequences that are resistant to bisulfite treatment due to protection from methylation, while the USP primers will amplify unmethylated cytosines that were converted to uracils from bisulfite treatment.

#### Detection of DNA methylation within viral genome from productive infection

To determine whether DNA methylation occurs within the ORF79 promoter in viral genome isolated from productive infection *in vitro*, the viral KHV DNA was extracted and treated or untreated with bisulfite following the manufacturer's instructions provided by the EpiTect Bisulfite Kit (Qiagen). DNA samples from treated or untreated viral genome were then examined by PCR primers specific to either the methylated or unmethylated ORF79 promoter. Since the untreated DNA has not undergone sequence conversion, the MSP primers are able to amplify untreated viral DNA. USP primers will not be able to amplify the untreated DNA since USP primers are only specific to converted sequences. As shown in Fig. 3, PCR product at the expected size, ~500 bp, was amplified from untreated KHV viral DNA when MSP primers were used (Fig. 3 lane 1), but not USP primers (Fig. 3, lane 2), suggesting that MSP is specific to ORF79 promoter sequences. With treated viral DNA, PCR product at the predicted size was amplified when USP primers were used (Fig. 3, lane 4). However, interestingly, PCR products at the predicted size were also amplified from bisulfite treated viral DNA when MSP primers were used (Fig. 3, lane 3), which suggests that the region targeted by the MSP primers are methylated.

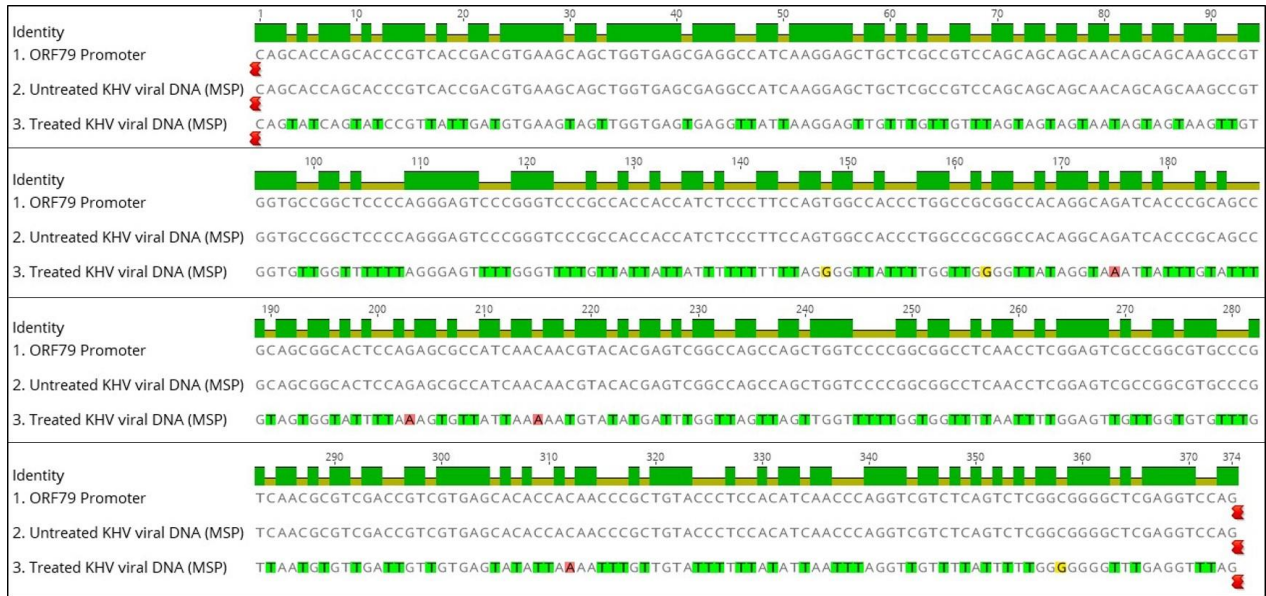




**Figure 3: PCR amplification with treated or untreated KHV viral DNA from productive infection.** PCR amplification was performed with MSP (lanes 1 and 3) and USP (lanes 2 and 4) primers specific to bisulfite treated or untreated KHV DNA, respectively. PCR products were run on a 1% agarose gel. The molecular weight ladder used is 1kb plus from Invitrogen.

To confirm that PCR amplification occurred correctly, PCR products amplified by both MSP and USP primers were sequenced. PCR products amplified by MSP primers were successfully sequenced. Untreated viral DNA sequence amplified by MSP primers is identical to the ORF79 promoter sequence of KHV (Fig. 4). This confirms that PCR reaction with MSP primers worked properly. Interestingly, treated viral DNA sequence amplified by MSP primers showed altered DNA sequence of all cytosines to thymines. Since treated viral DNA was also able to be amplified with USP primers, this indicates that DNA sequence selected for the MSP primers are methylated, but the sequence between the primers are mostly unmethylated and underwent sequence change during bisulfite treatment. The PCR product from USP primers

failed to be sequenced, indicating either that the USP primers are unsuited for the sequencing reaction or that there was not enough PCR product generated to be successfully sequenced.

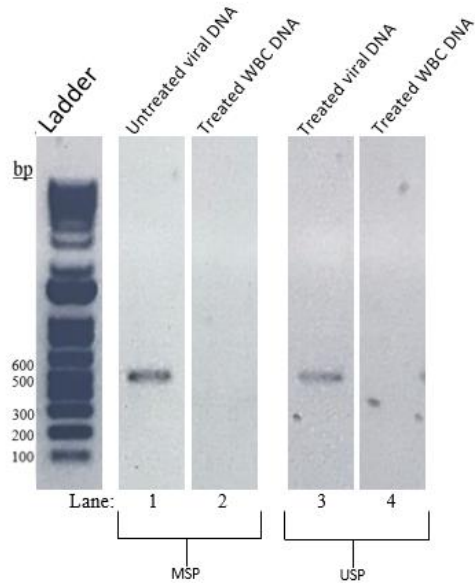


**Figure 4: DNA sequence alignment between reference DNA and MSP PCR products using untreated and treated KHV viral DNA as template.** Sequences were aligned to each other with Geneious 1.1.3. Sequence 1 is the ORF79 promoter sequence obtained from the complete KHV genome available at Genbank (NC\_009127.1). Sequence 2 represents the untreated KHV viral DNA amplified with MSP primers. Sequence 3 represents treated KHV viral DNA amplified with MSP primers.

#### Analysis of DNA methylation within viral genome from WBC of KHV latently infected koi

Previous studies have demonstrated that KHV becomes latent in the peripheral white blood cells (WBC) of koi, especially in B-cells<sup>15</sup>. KHV genome can be detected by PCR in koi latently infected by KHV. To investigate whether DNA methylation occurs in the ORF79 promoter during latency, total DNA was extracted from the WBC of latently infected KHV<sup>+</sup> koi.

WBC total DNA were treated by bisulfite and then examined by PCR using both MSP primers and USP primers.



**Figure 5: DNA Methylation Analysis of Total DNA of WBC from KHV latently infected koi.** Total DNA was isolated from white blood cells from latently infected koi fish, subjected to bisulfite treatment, and amplified with either MSP (lanes 1 and 2) or USP (lanes 3 and 4). Neither MSP nor USP were able to amplify a product for the treated total DNA of WBC (lanes 2 and 4). Untreated KHV viral DNA (lane 1) was used as a positive control for MSP, and treated KHV viral DNA (lane 3) was used as a positive control for USP.

As shown in Fig. 5, PCR products at the anticipated size were produced in treated viral DNA from productive infection by USP primers (Fig. 5, lane 3) and in untreated viral DNA from protective infection by MSP primers (Fig. 5, lane 1). This is in agreement with the results shown in Fig. 4. However, no product was amplified from either treated or untreated WBC total DNA using neither MSP nor USP primers (Fig. 5, lanes 2 and 4). This suggests that the amount of KHV DNA in WBC total DNA is below the detection limit. This is most likely due to the scarcity of KHV genome in WBCs during latency as it has been previously reported that only 1-2

copies of the KHV genome is present in 0.1-0.5% of WBCs<sup>14</sup>. Obtaining sufficient KHV genome may require isolation of the fraction of B-cells that harbor the KHV latency before performing the DNA extraction.

## CONCLUSIONS

The ORF79 gene of KHV encodes a catalytic subunit of DNA polymerase, which is required for viral DNA synthesis during virus infection. The ORF79 gene is turned on early during productive infection during genome replication. Following viral genome replication, ORF79 is turned off by later genes which encode the structural proteins required for virion production<sup>16</sup>. It is not surprising to find CpG islands within the ORF79 promoter. However, it is unknown if ORF79 is regulated by CpG methylation in infected koi cells. This study demonstrated that CpG methylation does not occur within the ORF79 promoter flanked by the MSP primers during productive KHV infection in koi cells *in vitro* as shown by the sequence analysis of the treated KHV viral DNA (Fig. 4).

The bisulfite conversion and PCR amplification with methylation-specific primers can be used successfully in studying the role of DNA methylation in KHV infection during the productive phase, when viral genome is abundant within the host, but not during the latent phase when viral genome is scarce. During latency, KHV DNA has been detected in specifically the IgM<sup>+</sup> B-cells of the peripheral WBC<sup>17</sup>. Sorting total WBCs from whole blood into IgM<sup>+</sup> and IgM<sup>-</sup> cells via magnetic beads and extracting DNA from only IgM<sup>+</sup> cells would increase the yield of KHV<sup>+</sup> cells and allow investigation of DNA methylation status during latent state. To determine if DNA methylation plays a role in latency and reactivation, this method could be used to monitor DNA methylation changes during the reactivation stages for koi fish switching from

latent to lytic infection. The versatility of this method in analyzing KHV DNA methylation status holds much promise for helping to unravel the mechanisms by which KHV persists within host cells. The information gained from this study helps pave the way to developing strategies to combat herpesvirus latency in medicine.

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