



KHV Detection Based on Thermal Lysis - Nested PCR (Polymerase Chain Reaction)

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Author's contribution

The sole author designed, analysed, interpreted and prepared the manuscript.

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ABSTRACT

In the case of common carp (*Cyprinus carpio*) and goldfish (*Carrasius auratus*) that were lightly infected with *Koi Herpes Virus* (KHV), the use of the one-step PCR method was insensitive to detect the presence of KHV DNA. The nested PCR method has been developed for a more sensitive detection system for KHV infection. This study aims to test the sensitivity of one-step and nested PCR methods in detecting the KHV virus in lightly infected common carp to prevent early disease attacks. DNA extraction of the test samples used thermal lysis and PBS (Phosphate Buffered Saline). DNA isolate as a KHV template was used to amplify KHV sequences using KHV-TKf: 5'-GGGTACCTGTACGAG-3' and KHV-TKr: 5'-CACCCAGTAGATTATGC-3' primers in the one-step PCR method. The nested PCR method uses two pairs of primers, namely the KHV-TKf: 5'-GACACCACATCTGCAAGGAG-3' and KHV-TKr: 5'-GACACATGTTACAATGGTGGC-3') and the CEFAS primer pair (5'-CGTCGTGAGGAATACGACG-3' and 5'-ACCGTACAGCTCGTACTGG-3') for amplification of KHV sequences. The one-step PCR method was insensitive for detection of KHV in common carp and goldfish infected with viruses without clinical symptoms, and was sensitive enough to detect viruses with mild symptoms with a DNA fragment size of 409 bp using TK primers. The nested PCR method was more sensitive in detecting KHV DNA in fish without clinical symptoms with a DNA fragment size of 348 bp using CEFAS primers.

Keywords: *Sensitivity; one step; nested PCR; koi herpes virus; comon carp; goldfish.*

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1. INTRODUCTION

Since the KHV disease began to spread in Indonesia in 2002, almost all traditional goldfish cultivation activities and floating net cages have failed to harvest, because this disease causes high mortality (80%). The results of research on common carp, concluded that Koi Herpes Virus or Carp Interstitial Nephritis And Gill Necrosis Virus (CNGV) is a pathogen that triggers mortality [1]. Intensive aquaculture, goldfish exhibitions, and almost unrestricted domestic and international trade and implementation of quarantine programs are the causes of the disease's rapid global spread [2]. The clinical symptoms of KHV are often nonspecific and death occurs rapidly when the temperature ranges from 18-22°C. Discoloration and some gill necrosis are the most common signs of infection [1,3] and there is bleeding in the fins and body. After five days of being infected with the virus, it can achieve 100% mortality in a short time. KHV disease has been diagnosed in goldfish and consumption common carp and is believed to be in the body of infected common carp as potential carriers of the virus.

The level of KHV virus attack on carp and koi determines the sensitivity of the PCR method used, this is related to the abundance of viruses that can still be detected by one step or nested (twice amplification) PCR. This detection sensitivity is related to the minimum number of viruses that can be detected by PCR [4]. In the case of common carp fingerlings that were heavily infected with KHV, the use of one step PCR gave positive results, on the other hand, in cases of very mild infection, the one step PCR method gave negative KHV results. This has led to many complaints from common carp and goldfish farmers, when fish fingerlings were tested negative for KHV by one step PCR, after several months of maintenance, it turned out that many of the fingerlings had the disease.

The development of test methods other than one-step PCR and the use of specific primers is needed to improve the detection sensitivity of KHV in low concentrations. The use of nested PCR with specific primers (TK and CEFAS, Center for Environment Fisheries and Aquaculture Science) that copies the KHV genome thymidine kinase (TK) gene sequence and is needed to improve the diagnosis of KHV infection in common carp and goldfish which are more sensitive than the one-step test. PCR [1,4]. KHV DNA extraction using thermal

lysis (high heating) technique for cell lysis, is a relatively easy DNA extraction method with shorter processing than using a commercial KHV kit. The KHV detection method that combines the DNA extraction process based on thermal lysis and the amplification process of nested PCR with TK primers and CEFAS primers can increase sensitivity to the presence of viruses and shorten the procedure.

2. MATERIALS AND METHODS

2.1 Test Sample

The samples of test fish (fingerlings or consumption fish) were taken from the floating net cages block of Cirata reservoir, West Java, Indonesia (Jatinenggang, Calincing, Cadas Bodas, Ciputri) as shown in Fig. 1. All samples taken are preserved in sample bottles containing preservation solution (glycerol : alcohol = 1: 4) for further processing in the laboratory. The target organs of the test samples (common carp, goldfish) were gills and kidneys for DNA extraction following the IQ2000™ KHV detection kit protocol (Farming IntelliGene Tech. Corp., Taiwan) and the thermal lysis method [1]. Three steps of work in the KHV DNA detection process by PCR include DNA extraction, amplification and electrophoresis.

KHV DNA amplification used the one-step PCR method with the protocol from the KHV IQ 2000 kit and nested PCR using the method from [1] and [4]. The one step PCR method only uses one amplification process, while nested PCR uses two amplification processes. The primers used for one step PCR were only a pair of primers (KHV-TKf: 5'-GGGTTACCTGTACGAG-3' and KHV-TKr: 5'-CACCCAGTAGATTATGC-3') designed in previous study [4]. Nested PCR used 2 pairs of primers, namely primary pair 1 (KHV-TKf and KHV-TKr) and primary pair 2 (KHV-TK-Fn: 5'-CGTCGTGAGGAATACGACG-3' and KHV-TK-Rn: 5'-ACCGTACAGCTCGTACTGG-3') designed by the CEFAS Weymouth Laboratory Fig. 2.

2.2 DNA Extraction

DNA extraction was carried out by two extraction methods, namely based on the IQ2000™ KHV detection kit and thermal lysis protocol. Gill or kidney samples (50 mg) as a source of DNA put in a sterile 1.5 ml microtube. Then 500 µl of DNA extraction buffer was added to the microtube,

and then grinding with sterile plastic chopsticks until smooth on ice. The next step was placing the microtubes into the waterbath for incubation for 10 minutes at 95°C, and centrifuged at 12,000 rpm for 10 min. A total of 200 µl of the supernatant was transferred to a new 1.5 ml microtube and 400 µl of 95% ethanol were

added, then quickly vortexed. Again centrifuged for 5 min at a speed of 12,000 rpm, then the ethanol is discarded on the tissue. The microtube which already contains DNA pellets attached to the bottom of the tube was air dried. Furthermore, 100 µl of TE buffer was added and stored -20°C.

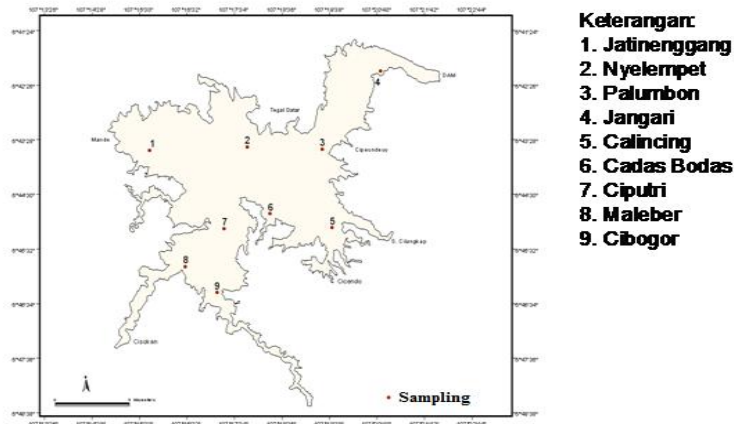


Fig. 1. The location of the test sampling in the floating net cages of the Cirata reservoir

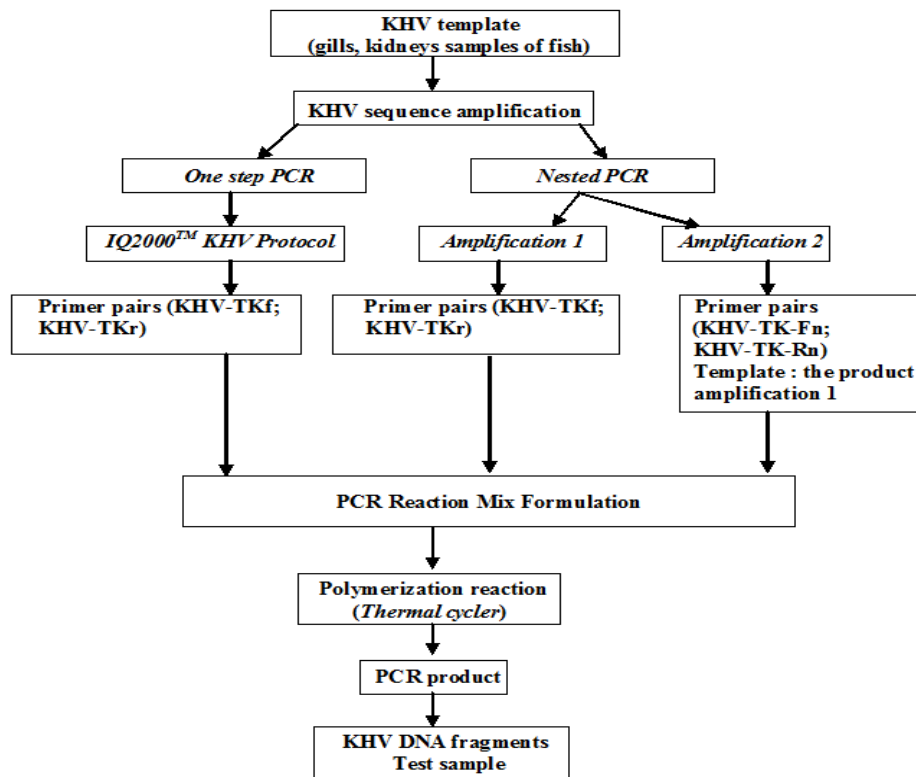


Fig. 2. The process of amplification of KHV DNA fragments using primers and methods different

DNA samples extracted using the thermal lysis method were carried out using high heating techniques after the sample was crushed and homogenized with PBS (Phosphate Buffered Saline) [1]. Tissue samples (gills or fish kidneys) of approximately 50 mg were homogenized with 0.1 M PBS (pH 8.0) of 500 µl and followed by centrifugation of 10,000 rpm for 4 min. The supernatant was then transferred into a new 1.5 ml sterile microtube and about 500 µl of TE (Tris-EDTA) buffer was added, which was then heated at 99 °C for 2 min in a water bath. DNA extraction was completed by centrifugation at 10,000 rpm for 3 min. This supernatant

contains DNA which will be used for PCR testing.

2.3 KHV DNA Amplification

The amplification of KHV fragments was carried out by mixing PCR reagents with the extracted DNA of the test sample and KHV primers according to the protocol used. Each 0.2 ml sterile microtube for the PCR reaction mixture was labeled according to the DNA code of the test sample. The one-step PCR amplification protocol follows the IQ2000™ KHV detection kit and nested PCR method follows are shown in Tables 1 and 2.

Table 1. One step PCR reaction mixture

Material composition	Volume of solution
1. <i>Go Taq Green Master mix 2x</i> (Promega, Madison, WI, USA)	12.5 µl
2. KHV-TKf primer	1 µl
3. KHV-TKr primer	1 µl
4. DNA <i>template</i>	2 µl
5. <i>Nuclease Free Water</i>	8 µl
Total volume	25 µl

Table 2. Nested PCR reaction mixture

Material composition PCR reaction mixture	Nested PCR	
	Amplification 1	Amplification 2
1. <i>Go Taq Green Master mix 2x</i> (Promega, Madison, WI, USA)	12.5 µl	12.5 µl
2. KHV-TKf primer	1 µl	KHV-TK-Fn primer 1 µl
3. KHV-TKr primer	1 µl	KHV-TK-Rn primer 1 µl
4. DNA <i>template</i>	2 µl	DNA <i>template</i> (the product amplification 1) 2 µl
5. <i>Nuclease Free Water</i>	8.5 µl	<i>Nuclease Free Water</i> 8.5 µl
Total volume	25 µl	Total volume 25 µl

Table 3. KHV amplification cycle conditions for the one-step PCR method

Reaction steps	Temperature (°C)	Time	Number of cycles
1. Pra denaturation	94	2 min	1
2. Denaturation	94	1 min	
3. Annealing	55	2 min	30
4. Extension	72	3 min	
5. Final extension	72	7 min	1

Table 4. The condition of KHV thermal amplification cycle using nested PCR method

Reaction steps	Temperature (°C)	Time	Number of cycles
1. Pra denaturation	95	5 min	1
2. Denaturation	95	30 sec	
3. Annealing	52	30 sec	35
4. Extension	72	1 min	
5. Final extension	72	10 min	1

Setting the KHV thermal amplification cycle conditions using the one-step PCR method refer to previous research [5] as presented in Table 3 and nested PCR refers to previous research [1,4] which is shown in Table 4.

The presence of KHV DNA was visualized by UV-transilluminator separated by 1% agarose gel electrophoresis.

3. RESULTS AND DISCUSSION

3.1 DNA Extraction Results

DNA extraction from the test sample was carried out to obtain the filtrate in the form of DNA pellets containing viruses. This extraction process includes the process of tissue lysis, the process of separating and dissolving cell organelles from the cell nucleus, the process of depositing the extracted remains and washing the DNA pellets from debris (extracted residues) so that DNA pellet deposits are obtained [6]. In principle, in order to obtain DNA pellets, the test sample must be perfectly extractable. The results of DNA extraction using the KHV detection kit gave quite decent results Fig. 3, as DNA isolates that would be further processed for amplification of KHV DNA fragments.

Based on Fig. 3, it can be seen that there is a white layer like fine threads which is DNA. These results indicate that the DNA extraction method used for the KHV template is quite feasible given the visible DNA sediment filtrate. KHV DNA will be isolated, if the DNA of the test sample is completely extracted. The results of DNA extraction using thermal lysis are relatively short, efficient in material use and easier to work with compared to the extraction method with the KHV kit [1]. The results of DNA isolation from the test sample produced a clear solution accompanied by a layer of DNA thread (such as clear gel) Fig. 4.

Thermal lysis is a simple method for cell lysis [7]. Cells are heated to 99 °C to induce lysis. One of the advantages of this method is that it can denature protein and RNA components at high temperatures, so that DNA can be released from the cell nucleus. The use of PBS in this method is to collect DNA from high temperature extraction (thermal lysis). This PBS is a salt solution containing sodium chloride, sodium phosphate, and potassium phosphate which has a constant pH which can balance the salt

concentration around cells and prevent osmosis during storage of DNA samples.

3.2 Amplification Products

Furthermore, the DNA template that has been obtained from the above DNA extraction was used for PCR testing with two methods, namely one step PCR and nested PCR. One step PCR amplification was carried out first and then continued with the second amplification (nested PCR). One-step PCR amplification product, showed that KHV DNA was copied by TK primers with a thick band, indicating that the test sample contained KHV virus with an amplicon size of 409 bp (Fig. 5).

PCR products amplified on common carp and goldfish conducted in previous research using TK primers also had a fragment size of 409 bp [8]. The results of research which used a Thymidine Kinase (TK) primer designed from the KHV virus gene encoding the thymidine kinase enzyme in common carp juvenile (weight 60 g) from two culture systems (traditional and floating net cages) was very sensitive to detect the presence of KHV DNA which was amplified in size 409 bp [1]. Thus, the amplicon size in the sample detected in Fig. 5 is the KHV DNA band that infects common carp and goldfish cultivated in Ciputri, Cadas Bodas, Calincing and Jatinegang floating net cages waters of the Cirata reservoir Table 5.

In Table 5 above, it can be shown that both the gill and kidney organs of common carp samples in Ciputri, Cadas Bodas, Calincing and Jatinegang floating net cages are infected with the koi herpes virus which indicates that the use of this TK primer is very sensitive to detect the presence of the virus in the carp's body organs. TK primers can recognize the sequence of the gene encoding for the thymidine kinase (TK) enzyme from a virus which was 409 bp in size (Fig. 5 on the 2nd; the 3rd; 5th; 6th; 10th; 11th, 12th and 13th wells). However, the goldfish's gills and kidneys (Fig. 5, wells 8 and 9) were not detected for the presence of KHV DNA (- KHV) for the one-step PCR product. Specifically for one-step PCR based on TK primers, the detection limit can still be reached by the PCR test, when the virus content is at least 10 phentograms (10 fg) of KHV DNA which is equivalent to the number of 30 virions [4]. Based on this analysis, it can be concluded that the KHV DNA content in goldfish samples (kidney and gill organs) was far below 10 fg, so that the

one-step PCR test cannot detect the virus due to the limited sensitivity of one step PCR [4,9]. The lowest limit that can be detected is around

100 geq (genomic equivalent copies = 100 copies of viral DNA) for one-step PCR [3,10].

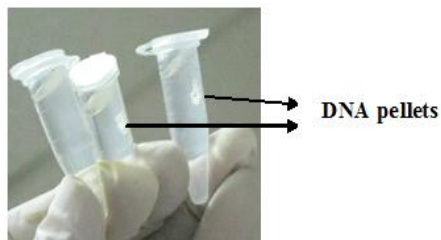


Fig. 3. DNA extraction results with KHV detection kit

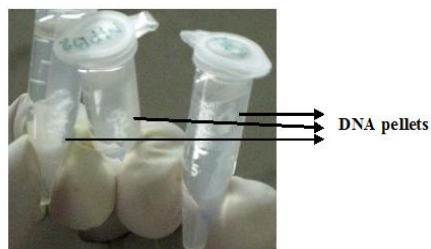


Fig. 4. DNA from the extraction of the thermal lysis method

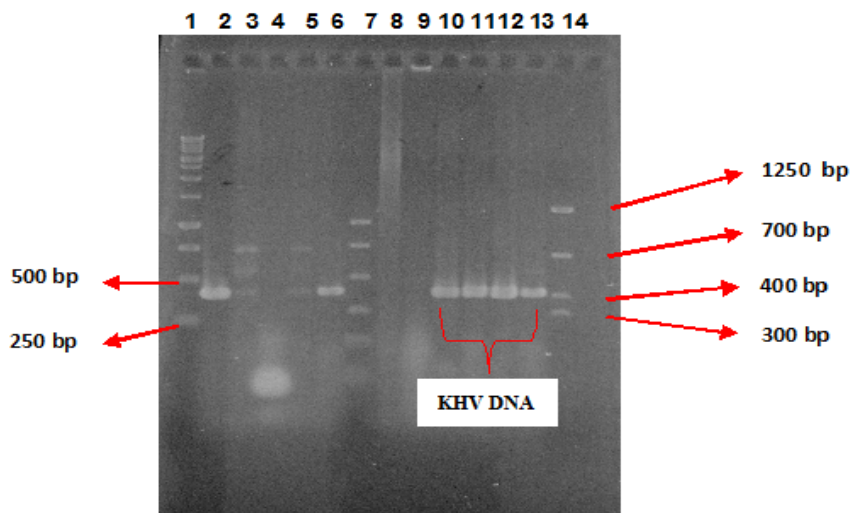


Fig. 5. One step PCR product

1 = DNA ladder 1kb; 2 = common carp gills in Ciputri floating net cages (+ KHV); 3 = common carp kidneys Ciputri floating net cages (+ KHV); 4 = control (-) PCR reaction mixture without DNA template; 5 = common carp gills in Cadas Bodas floating net cages (+ KHV); 6 = common carp kidneys in Cadas Bodas floating net cages (+ KHV); 7 = PCR marker (50 bp, 150 bp, 300 bp, 500 bp 750 bp, 1000 bp); 8 = goldfish gills in Calincing floating net cages (- KHV); 9 = goldfish kidneys in Calincing floating net cages (- KHV); 10 = common carp gills in Calincing floating net cages (+ KHV); 11 = common carp kidneys Calincing floating net cages (+ KHV); 12 = common carp gills in Jatinenggang floating net cages (+ KHV); 13 = common carp kidneys in Jatinenggang floating net cages (+ KHV); 14 = KHV DNA marker (300 bp; 400 bp; 700 bp; 1250 bp).

Sensitivity in detecting viral diseases is very important, especially if the content is far below the detection limit of the PCR test used. There is a need to develop sensitive PCR assays to detect the presence of virus numbers in low concentrations [11]. Increasing the sensitivity of the detection system is required using the nested PCR test [9,12]. The development of detection sensitivity with nested PCR using external and internal specific primers from the KHV thymidine kinase encoding gene sequence can increase its specificity so as to reduce false negative results. Internal primers designed from this sequence are used in the second amplification to act as internal control by multiplying the product of the initial amplification [13,14]. This primer, designed from a one-step PCR product sequence based on TK, was developed by CEFAS (Center for Environment Fisheries and Aquaculture Science) Weymouth Laboratory England in 2007 which amplified the internal sequence of the first amplification product sequence. The results of the electropherogram of nested PCR products tested for common carp and goldfish samples (one-step PCR products) were shown in Fig. 6.

Laboratory primers for test samples of common carp and goldfish showed the presence of KHV DNA bands Fig. 6. The amplified fragment size for gill and kidney samples in common carp and goldfish was 348 bp. This nested PCR product (348 bp), was shorter than the template size (one step PCR product 409 bp), because the primers used in nested PCR are designed on the internal sequence of the one step PCR amplicon [9,13]. Thus, the use of CEFAS primers (KHV-TK-Fn and KHV-TK-Rn) in the second amplification of the one-step PCR products was more sensitive and specific in copying the KHV thymidine kinase encoding gene sequence as shown in Fig. 6 with the nested PCR method. The sensitivity and size specificity of nested PCR products based on CEFAS primers using templates from one-step PCR products were also shown in samples of goldfish (gill and kidneys organs), which detected a KHV DNA fragment size was 348 bp (+ KHV) (Fig. 6 on 7th and 8th wells) where previously the results of the one step PCR test (Fig. 5 on 8th and 9th wells) were negative KHV results. The KHV fragment size in this goldfish sample was also the same as the KHV fragment size using the nested thymidine kinase technique in common carp (*C. carpio*) samples [15]. These results indicate that the sensitive CEFAS primary-based

The results of KHV DNA detection using the nested PCR method with CEFAS Weymouth

Table 5. Detection of the presence of KHV in common carp and goldfish (one step PCR)

Floating net cages		Common carp		Goldfish	
		Gills	Kidneys	Gills	Kidneys
1.	Ciputri	+ KHV	+ KHV		
2.	Cadas Bodas	+ KHV	+ KHV		
3.	Calincing			- KHV	- KHV
4.	Calincing	+ KHV	+ KHV		
5.	Jatinenggang	+ KHV	+ KHV		

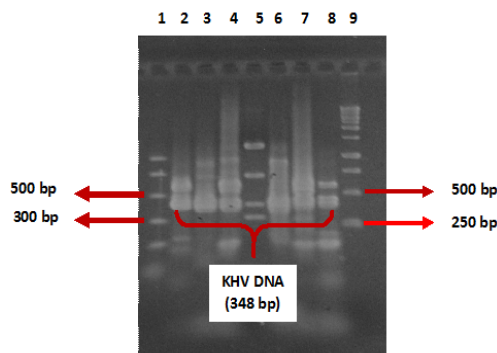


Fig. 6. PCR nested products

1 = PCR marker (50 bp, 150 bp, 300 bp, 500 bp 750 bp, 1000 bp); 2 = common carp gills sample in Jatinenggang floating net cages; 3 = common carp kidneys sample in Calincing floating net cages; 4 = common carp gills sample in Cadas Bodas floating net cages; 5 = KHV DNA marker (300 bp; 400 bp; 700 bp; 1250 bp); 6 = common carp kidneys sample in Ciputri floating net cages (+ control); 7 = goldfish gills sample in Calincing floating net cages; 8 = goldfish kidneys sample in Calincing floating net cages; 9 = DNA ladder 1 kb.

nested PCR test detects KHV viruses below 10 fg (number of virions below 30), as shown by positive KHV results in goldfish gill and kidney samples of the Calincing floating net cages, which previously gave false negative results with the one step PCR test. Detection of virus could change depending on the tissues where the virus is expressed (number of receptor in surfaces) , maybe the number of viral particle is less in tissue and this is the reason in the power of detection of test.

4. CONCLUSION

The one-step PCR method was insensitive to detect KHV DNA in goldfish without clinical symptoms, but it was sensitive enough to detect KHV light attacks mild KHV (DNA fragment size 409 bp) using TK primers. Meanwhile, the Nested PCR method was very sensitive to detect KHV DNA in common carp and goldfish without clinical symptoms using CEFAS primers (DNA fragment size 348 bp). The sensitivity of KHV detection can be increased using nested PCR with specific primers designed from the internal portion of the viral thymidine kinase.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

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