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Detection of KHV in Freshwater Mussels and Crustaceans from Ponds with KHV History in Common Carp (*Cyprinus carpio*)

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Abstract

Characterization of asymptomatic KHV carriers may help understand virus transmission and storage. Such information allows farmers to minimize KHV on farms where this virus is present, also in common carp (*Cyprinus carpio*) monocultures. As asymptomatic KHV carriers, freshwater mollusks from the Unionidae family (swan mussels, *Anodonta cygnea*) and crustaceans from the Gammaridae family (scud, *Gammarus pulex*) were studied because of their unique method of feeding by accumulating bacterial and viral particles. The KHV genome was detected by nested PCR and confirmed by PCR recognizing the KHV glycoprotein gene in ORF 56 (KHV-U). Both PCR and nested PCR, which recognize the KHV thymidine kinase gene, always produced negative results in the swan mussels and scud samples.

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Introduction

Mollusks play an important role in water ecosystems because they contribute to utilization of nutritional elements in the water volume (Lewandowski and Stanczykowska, 2000). Swan mussels (*Anodonta cygnea*; Unionidae) have a palaearctic occurrence range, mainly in eutrophic standing waters (Zajac, 2002). However, they are becoming less abundant in Poland because of anthropological factors (Dyduch-Falniowska, 1989).

Freshwater mussels have a special way of receiving nourishment from surrounding water. Inside the shells are not only desired nutritional elements but also dangerous pathogens such as bacteria, parasites, and viruses that are often attached to organic or inorganic compounds (Renault and Novoa, 2004). According to the FAO, annual world production of freshwater mussels has steadily increased from 13,131 tons (US$19,101,500) in 1998 to 139,024 tons (US$94,067,000) in 2007 (FAO, 2009). Swan mussel production in China alone reached 92,000 tons in 2006. Such a great, twelvefold increase brings not only benefits but also potential risks of dangerous diseases, which might be species specific and cause lethal effects.

One of the most dangerous groups of pathogens that cause disease in aquatic organisms is viruses belonging to the Herpesviridae family (Minson et al., 2000). In 2002, about 120 species were identified but many more may exist (Davidson, 2002). Most of the aquatic herpesviruses are not as host-specific as mammalian herpesviruses and cause mortality depending on their life cycles. Host specificity means that viruses and their hosts long ago underwent evolutional processes together and are well adapted to them (Davison, 2002), as shown for Alphaherpesvirinae, Betaherpesviridae, and Gammaherpesviridae (McGeoch et al., 2000). American or eastern oysters (*Crassostrea virginica*) can be infected by a unique herpesvirus (OsHV-1) that infects only mollusks (Farley et al., 1972; Renault et al., 1994). This same herpesvirus was found in carpet shell clams (*Ruditapes decussatus*), Pacific oysters (*Crassostrea gigas*), and great scallops (*Pecten maximus*) by polymerase chain reaction (PCR; Arzul et al., 2001; Renault and Arzul, 2001). PCR is a fast and safe diagnostic, even for specimens that do not show clinical signs of the disease, and helps explain many unexpected disease outbreaks (Barbosa-Solomieu et al., 2004).

First outbreaks of a disease caused by Herpesviridae occurred in common and koi carp (*Cyprinus carpio*) cultures in 1994 (Haenen et al., 2004) and was named koi herpesvirus disease (KHVD). Since 2006, the Fish Genetics Unit of the West Pomeranian University of Technology has been monitoring Herpesviridae viruses among fish and invertebrates in the Odra River drainage. Herpesviridae viruses can enter aquaculture facilities with incoming water. This study examined KHV virus accumulation by filtration or passive or active virus storage inside swan mussels and scuds (*Gammarus pulex*).

Materials and Methods

Sampling and isolation. Swan mussels (10-12 cm; n = 10; Fig. 1) and scuds (n = 15) were caught in a water depth of 1-1.5 m from ponds with KHVD history in common carp in southern Poland. The first analysis was conducted with samples collected in November 2007; the second with samples from August 2008. KHVD outbreaks had occurred in common carp in the farm where samples were collected in 2002-2007 but not in 2008. The mussels were transported to the laboratory and allowed to adapt for five days in an aquarium with a re-circulating system isolated from the environment.
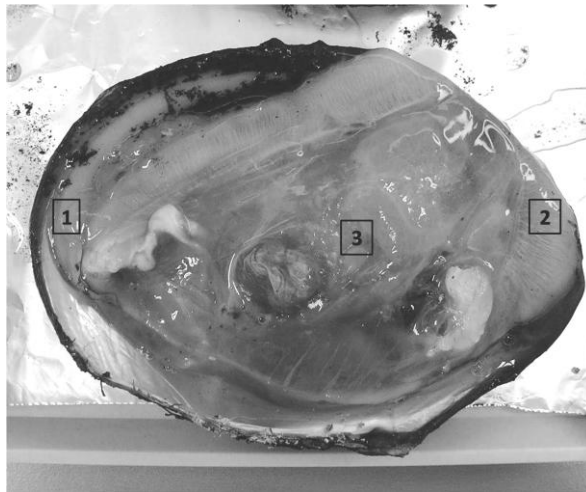

Fig. 1. *Anodonta cygnea*: 1 = mantle, 2 = gills, 3 = gut and digestive glands.

The mussels were opened and water discharged. The first samples were taken with both sides of an earplug from the gills and mantel and placed in an Eppendorf tube containing lysis buffer (QIAmp® DNA Mini Kit, Qiagen). The tissue material from the digestive glands, mantels, and gills of 10 mussels were pooled. For DNA extraction, 25±10 mg of each mussel were used with DNAzol® reagent (Invitrogen) and/or QIAmp® DNA Mini Kit according to the manufacturer's instructions. DNA from earplugs was extracted with the QIAmp® DNA Mini Kit only. Tissues from the same mussels were fixed in Davidson's solution for paraffin embedding for histological analysis and *in situ* hybridization (data not shown).

In addition, two pools of five scuds were ground with sea sand in a mortar and pistil and stored for 18 h at 4°C. After clarification by centrifugation (10 min, 1000 x *g*, 4°C), two aliquots of 0.5 ml of the resultant supernatant from each pool were mixed with 1 ml DNAzol® reagent and prepared according to the manufacturer's instruction. Extracted DNA was pooled from both tubes.

PCR and gel electrophoresis. DNA obtained from mollusk tissues was extracted by two methods (DNAzol and Qiagen kit) and used for PCR according Gilad et al. (2002), Bergmann et al. (2006) using primer pair KHV 1Fn-1Rn, and Bercovier et al. (2005). The primer binding sides were according to accession number DQ657948 in ORF 89 (Gilad et al. 2002), 90 (nested PCR in Bergmann et al., 2006), and 55 thymidine kinase (Bercovier et al., 2005). PCR with primers recognizing the KHV glycoprotein gene (ORF 56, KHV-U) was used for confirmation (Table 1). After PCR assay, all samples were subjected to electrophoresis on a 1.5% agarose gel containing ethidium bromide solution (Roth) for DNA detection.

Table 1. Primer sequences for detection of the KHV glycoprotein gene (accession number DQ177346).

Primer	Position	Primer	Temp	Size
SBM-gp-2F	1059 - 1075	acgtcggcgtgcgccac	60°C	661 bp
SBM-gp-2R	1701 - 1720	ggacgtggtctgccactac	60°C	--

Sequence analysis. PCR products were sequenced directly after elution of the respective DNA fragment with QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Sequencing reaction was carried out using Big Dye Terminator v.1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Thermal cycler conditions (Eppendorf Mastercycler gradient) were as follows: an initial activation step of 96°C for 1 min was followed by 30 cycles of 96°C for 30 s, 65°C for 15 s, and 72°C for 90 s. Subsequently, sequencing products were purified by Sigma Spin Post-Reaction Purification Columns (Sigma Aldrich Chemie GmbH, Munich, Germany) according to instructions. After denaturation with Hi-DiTM Formamide (Applied Biosystems), sequencing reaction was carried out using a 3130 Genetic Analyzer (ABI, Applied Biosystems). Sequence data analysis was performed with Sequence Scanner Software v1.0 (ABI, Applied Biosystems), GCG-X-Win32 (version 11.1.3. UNIX, Accelrys Inc., San Diego, CA, USA). NCBI blast 2 was used for analysis of nucleotide sequence pair percent identity; NCBI blastn was used for comparison with published sequence data.

Results

PCR did not always produce positive signals in the samples despite the fact that KHV DNA was found in all samples by one or two methods, twice replicated. Results from the earplug swabs using column-based extracted DNA according to Gilad et al. (2002), Bergmann et al. (2006), and Bercovier et al. (2005; data not shown) were negative (Fig. 2). Further, desired amplicons were never found in any swan mussel or scud sample. Proper results were

received only when ORF 56 PCR (Fig. 3) confirmed nested PCR with Bergmann et al. (2006) primers (Fig. 4). Moreover, the amount of viral particles varied even within a single animal but at least KHV particles were present in the tissues.
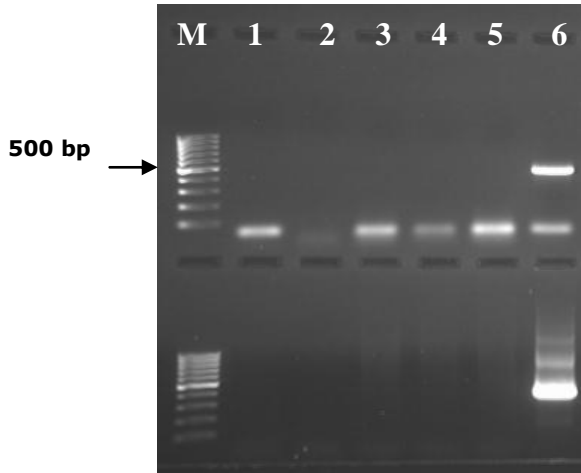

Fig. 2. PCR according to Gilad et al. (2002; upper lane) and nested PCR according to Bergmann et al. (2006; lower lane) with earplug samples from four mollusks: M = 100 bp marker (peqlab), lane 1 = mollusk 1, lane 2 = mollusk 2, lane 3 = mollusk 3, lane 4 = mollusk 4, lane 5 = negative preparation control, lane 6 = positive control (KHV-I) with 484 bp and 414 bp, respectively.
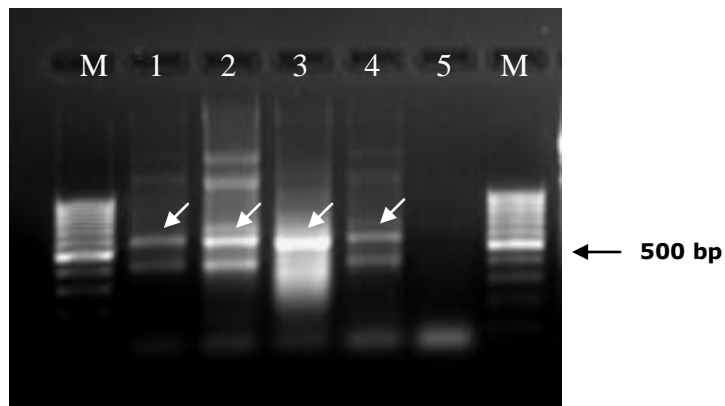

Fig. 3. ORF 56 PCR recognizing 661 bp of the KHV glycoprotein gene fragment in mollusk samples confirmed nested PCR (Bergmann et al., 2006): M = 100 bp marker (peqlab), lane 1 = mantel of mollusk 1, lane 2 = gill of mollusk 2, lane 3 = digestive gland of mollusk 3, lane 4 = digestive gland of mollusk 4, lane 5 = negative preparation control (water). Arrows indicate positive signal at 661 bp.
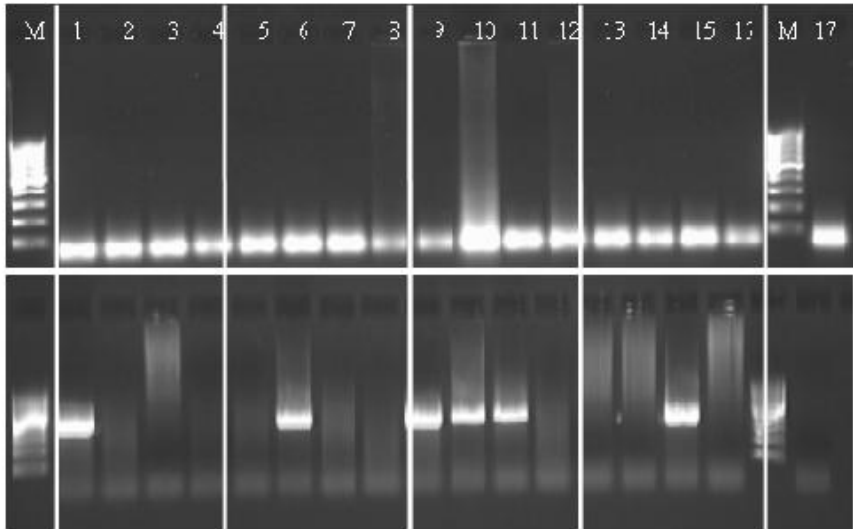
Fig. 4. PCR according to Gilad et al. (2002; upper lane) and nested PCR according to Bergmann et al. (2006; lower lane) with mollusk samples: M = 100 bp marker (peqlab), lanes 1-4 = mantel, gill, and two digestive gland samples of mollusk 1, lanes 5-8 = mantel, gill, and two digestive gland samples of mollusk 2, lanes 9-12 = mantel, gill, and two digestive gland samples of mollusk 3, lanes 13-16 = mantel, gill, and two digestive gland samples of mollusk 4, lane 17 = negative preparation control (water).

The situation was similar in scud samples. After nested PCR (Bergmann et al., 2006), only one sample was KHV DNA positive (Fig. 5). Results of the PCR are summarized in Table 2. For additional confirmation, sequence analysis of the PCR products was done. No mismatches or other changes were found in the consensus nucleotide sequence compared to published data (Aoki et al., 2007).
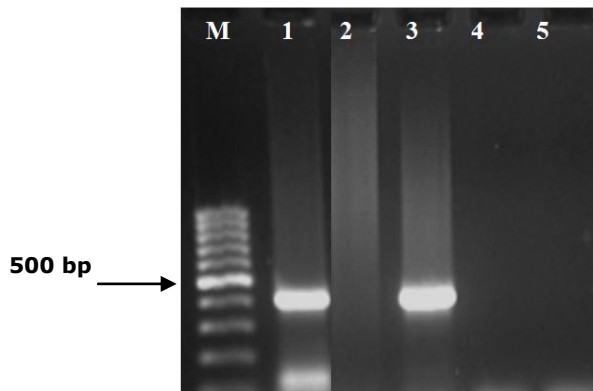

Fig. 5. Detection of KHV in crustaceans and mussels by nested PCR (Bergmann et al., 2006): M = 100 bp marker (peqlab), lane 1 = mollusk 1 (positive control), lane 2 = scud 1, lane 3 = scud 2, lanes 4 and 5 = negative preparation controls.

Table 2. Results of PCR assays with different samples.

Species	486 bp[1]	414 bp[2]	661 bp[3]
Anodonta cygnea 1			n.d.
Mantle	-	+	+
Gills	-	-	n.d.
Digestive gland 1	-	-	n.d.
Digestive gland 2	-	-	n.d.
Anodonta cygnea 2			n.d.
Mantle	-	-	n.d.
Gills	-	+	+
Digestive gland 1	-	-	n.d.
Digestive gland 2	-	-	
Anodonta cygnea 3			n.d.
Mantle	-	+	n.d.
Gills	-	+	n.d.
Digestive gland 1	-	+	+
Digestive gland 2	-	-	n.d.
Anodonta cygnea 4			n.d.
Mantle	-	-	n.d.
Gills	-	-	n.d.
Digestive gland 1		+	+
Digestive gland 2	-	-	n.d.
Gammarus pulex 1	-	+	n.d.
Gammarus pulex 2	-	-	n.d.

n.d. = not done
[1] Gilad et al. (2002)
[2] Bergmann et al. (2006)
[3] KHV glycoprotein gene

Discussion

The aquatic environment is unique because aquatic organisms are constantly surrounded and subjected to potential pathogens. Agents that are not a threat to one species (potential vector) might be a serious existential pathogen for another (susceptible) species. Freshwater mollusk production rises every year, therefore great concern should be focused on this situation. Filtration feeding causes particles to accumulate on mollusks and scuds that might cause mass losses in carp production (Kempter and Bergmann, 2004). Varying amounts of viruses naturally accumulate, depending on physical and chemical water parameters such as turbidity and temperature (Mitchell et al. 1966). Sometimes, viruses are not only stored but also replicated.

In this study, swan mussels and scuds were collected in south Poland from an area where KHV had occurred in carp between 2002 and 2007. Ponds were emptied and surviving fish were harvested for human consumption. No disinfection measures were practiced afterwards in the ponds. No clinical signs of disease were observed during excising of the swan mussel samples. While mussels were sectioned, scuds were ground and supernatants were used for DNA extraction. Thus, it can not be excluded that KHV or DNA particles adhered to the surface of the scuds or mussels. Nevertheless, PCR results from swabs with earplugs from the same mussels were always negative. Only

morphological detection of KHV inside mussel tissue by *in situ* hybridization with KHV specific probes (DNA detection) or immunofluorescence with monoclonal antibodies against KHV (protein or antigen detection) can provide conclusive proof. However, this was not the purpose of the present study and such techniques will be used in a future investigation in which the transfer of KHV to naive carp is shown.

We were able to detect KHV DNA fragments by two PCRs reflecting different genome parts of the virus. Thus, there is a possibility of KHV storage, even perhaps persistence, in swan mussels. The lack of positive results from the very specific and high sensitive PCR according to Bercovier et al. (2005) is explainable. Over the last years we have encountered more and more KHV-infected areas in Poland and Germany where we could not find positive signals with the method of Bercovier et al. (2005) but did occasionally succeed with other PCRs (Yuasa et al., 2005). We assume that this "warm water agent" adapted to European conditions. This TK-negative KHV variant can also be found in the U.K. (Dr. Keith Way, CEFAS UK, OIE reference laboratory for KHVD, KHV Ringtest 2007 and 2008, pers. comm. to Bercovier).

The diagnostic sensitivity of PCR according to Gilad et al. (2002) is much weaker than that of other PCRs. Therefore, it is not unusual that a nested PCR is established or designed when a PCR is not sensitive enough, as in our study. This can also indicate a very weak virus content in or on the mussels or scuds with the possibility that the organism acts as a carrier or vector. However, nested PCRs used for diagnostic purposes always involve the risk of laboratory contamination with template DNA. Therefore we included additional negative controls for each step. All positive controls used the same PCR reaction mix as the samples and the same thermal cycler but were prepared in another laboratory. With these precautions, no contamination was found by nested PCR in the samples during the investigation. The nested PCR products were sequenced, aligned, and compared to published data. The consensus sequences show 100% homology to KHV-I, KHV-U, and KHV-J (Aoki et al., 2007).

Despite the KHV detection in swan mussels and in/on scud by different PCRs reflecting viral genes, there is no clear evidence for virus replication in these animals although there is always the possibility that mussels passively accumulate KHV. Investigations proving replication inside mussel or scud tissue must be undertaken. Invertebrates such as mussels in and on the ground or scuds in the water seem to be able to hold the virus for a long time.

A larger monitoring of the appearance of viral particles in freshwater mollusk species will provide more information about horizontal ways of virus transmission. Fish deaths caused by KHV are still unresolved in many aspects and cases, and require further research and more detailed information on appearance, storage, accumulation, replication, release, and transmission. To conclude, greater efforts should be made to find potential healthy vectors.

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