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Antibody screening identifies 78 putative host proteins involved in Cyprinid herpesvirus 3 infection or propagation in common carp, *Cyprinus carpio L.*

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Abstract

Cyprinid herpesvirus 3 (CyHV-3) is the aetiological agent of a serious and notifiable disease afflicting common and koi carp, *Cyprinus carpio* L., termed koi herpesvirus disease (KHVD). Significant progress has been achieved in the last 15 years, since the initial reports surfaced from Germany, USA and Israel of the CyHV-3 virus, in terms of pathology and detection. However, relatively few studies have been carried out in understanding viral replication and propagation. Antibody-based affinity has been used for detection of CyHV-3 in enzyme-linked immunosorbent assay and PCR-based techniques, and immunohistological assays have been used to describe a CyHV-3 membrane protein, termed ORF81. In this study, monoclonal antibodies linked to N-hydroxysuccinimide (NHS)-activated spin columns were used to purify CyHV-3 and host proteins from tissue samples originating in either CyHV-3 symptomatic or asymptomatic fish. The samples were next analysed either by polyacrylamide gel electrophoresis (PAGE) and subsequently by electrospray ionization coupled to mass spectrometry (ESI-MS) or by ESI-MS analysis directly after purification. A total of 78 host proteins and five CyHV-3 proteins were identified in the two analyses. These data can be used to develop novel control methods for CyHV-3, based on pathways or proteins identified in this study.

Keywords

electrospray ionization mass spectrometry; immunochemistry; koi herpesvirus; polyacrylamide gel electrophoresis; protein purification

Introduction

Cyprinid herpesvirus 3 (CyHV-3), formerly known as koi herpesvirus (KHV), is the aetiological agent of a serious and notifiable disease afflicting common and koi carp, *Cyprinus carpio* L., termed koi herpesvirus disease (KHVD), and it has spread via carp trade and koi shows (Pikarsky *et al.* 2004; Pokorova *et al.* 2005; Ilouze *et al.* 2008). CyHV-3 has been isolated from kidney, gill, spleen, intestine, liver and brain of dead fish and causes a high mortality rate, between 80 and 100% (Hedrick *et al.* 2000). The most common clinical signs of the disease are white patches, sunken eyes, enlargement of the spleen and kidney, and necrosis in the gills in infected fish (Hedrick *et al.* 2005). CyHV-3 is known to infect carp at temperatures between 15 and 25 °C (Gilad *et al.* 2003). The genome of CyHV-3

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consists of approximately 295 thousand base pairs (kB), coding for 156 novel putative proteins (Aoki *et al.* 2007).

Several studies have used immunochemistry, which takes advantage of affinity based on antibody avidity to an antigen, in investigations into CyHV-3 (Rosenkranz *et al.* 2008; Soliman & El-Matbouli 2009). In a recent study, polyclonal antibodies against CyHV-3 were used for viral detection purposes (Soliman & El-Matbouli 2009), and the technique may be more sensitive than other traditional PCR-based methods (Gilad *et al.* 2002; Bercovier *et al.* 2005; El-Matbouli, Rucker & Soliman 2007; Bergmann *et al.* 2010; Soliman & El-Matbouli 2010). A sensitive enzyme-linked immunosorbent assay (ELISA) was developed for the detection of CyHV-3 by Adkinson, Oren & Hendrick (2005), and a different monoclonal antibody against ORF68 has also been developed by Aoki *et al.* (2011). Another monoclonal antibody, derived against ORF81, localized to cytoplasmic regions of cells, including endoplasmic reticulum or Golgi apparatus probably during protein synthesis, and into the viral envelope in mature virons (Rosenkranz *et al.* 2008).

Knowledge about protein interactions can be used to understand how viruses enter host cells and propagate during infection (Guo *et al.* 2011; Blondot *et al.* 2012). Although some genes in CyHV-3 maintenance and replication have been identified (Fuchs *et al.* 2011), little is known about the protein interactions in viral propagation and even less is known whether this virus interacts with any endogenous common carp proteins. A recent report (Michel *et al.* 2010) used polyacrylamide gel electrophoresis (PAGE) and chemical-based protein purification techniques to identify 40 CyHV-3 proteins incorporated into mature virons. Eighteen fish proteins were also identified in the study including one common carp protein (Michel *et al.* 2010). Although a number of methods for protein purification have been described (de Souza *et al.* 2008; Moen *et al.* 2011), purification by antibody-based avidity is a powerful technique to identify *in vivo* protein binding partners (Tuxworth *et al.* 2005; Gotesman, Hosein & Gavin 2010; Gotesman, Hosein & Gavin 2011).

During mass spectrometry, samples are ionized and filtered based on mass, charge and shape, and subsequently, a detector measures the sample's mass-to-charge ratio (Cameron 2012). Analysis of samples containing polymers, peptides and proteins of molecular weights beyond 20 kDa using an electrospray ionization source coupled with a quadruple mass spectrometer (ESI-MS) was first proposed by Fenn *et al.* (1989). Since then, ESI-MS has developed to be a sensitive tool for detecting samples at femtomolar concentrations in nanomole quantities and a routine method for characterizing non-volatile and thermally labile bio-molecules that are not amenable to analysis by other conventional techniques (Ho *et al.* 2003). Over the last decade, ESI-MS has emerged as an important technique in proteomics for confirmation of amino acid sequence and for characterization of post-translational modifications (Griffiths *et al.* 2001). We used antibody purification in conjunction with ESI-MS analysis to identify novel proteins that may be used as potential targets for the inhibition of CyHV-3 replication.

Materials and methods

Fish specimens

Two naturally infected fish, which showed signs of severe necrosis of the gills and skin, and loss of mucus on the skin, were used as CyHV-3 asymptomatic samples. One fish that lacked any signs of CyHV-3 was used as a control.

Tissue preparation

Tissue extracts (pooled from gill, liver, kidney, spleen and brain) were separately sampled aseptically from two different CyHV-3 symptomatic and one asymptomatic koi carp, and the samples were separately homogenized in minimum essential medium. Part of each homogenate was used for DNA extraction using a DNeasy Blood Tissue Kit (Qiagen) in accordance with the manufacturer's instructions. Another aliquot of the homogenate was used for propagation of CyHV-3 on common carp brain cell line (CCB). Extracted DNA was subjected to conventional PCR according to Bercovier *et al.* (2005) and real-time PCR according to Gilad *et al.* (2004). CyHV-3 from symptomatic fish was successfully propagated on CCB cells and subsequently confirmed by PCR amplification of target DNA segments by conventional (Fig. 1) and real-time PCR as previously described. However, neither conventional PCR nor real-time PCR detected CyHV-3 in the asymptomatic fish sample. The remaining aliquots were separately used to prepare tissue lysate for electrospray ionization mass spectrometry (ESI-MS) analysis.

Tissue lysate preparation

Each homogenate from the previous step was separately lysed in a 1:1 ratio with a non-denaturing lysis buffer: 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 20 mM ethylene diamine tetraacetic acid (EDTA), 1% Na-deoxycholate, 1% Triton X-100 (Williams 2000) and protease inhibitor cocktail (50 μL mL^{-1} of lysis buffer). Subsequently, each lysate was vigorously vortexed and centrifuged at 16 000 ***g*** for 15 min. The supernatant was transferred to a fresh 1.5-mL Eppendorf tube and recentrifuged at 16 000 ***g*** for an additional 15 min. Supernatant from the second centrifugation for each fraction was separately used for affinity purification as described in the following sections.

Preparation of monoclonal antibody-linked spin columns

Monoclonal antibodies against CyHV-3 glycoprotein (KHV/10A9 #171103, Insel Riems, Germany) were conjugated to N-hydroxysuccinimide (NHS)-activated 33-mg-capacity agarose spin column (Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, 50 μL of CyHV-3 monoclonal antibody was resuspended into 350 μL of phosphate-buffered saline (PBS): 13.7 mM NaCl, 0.27 mM KCl, 10 mM Na_2HPO_4, 0.2 mM KH_2PO_4, pH 7.4 to make a total solution of 400 μL, and incubated overnight at 4 °C with mild shaking of 300 rpm in an Eppendorf Thermomixer Comfort. The spin columns were emptied and washed twice with PBS after overnight incubation at 4 °C with mild shaking. Subsequently, the spin columns were quenched with 400 μL of 1 M ethanolamine (pH 7.4) by incubation for 1 h at 4 °C with mild shaking. Finally, the spin columns were emptied by centrifugation at 16 000 ***g*** for 30 s and washed six times with PBS, and optical density at 280 (OD_{280}) of the final flow-through was zero.

Protein purification

Non-denatured whole-cell extracts from CyHV-3 tissue samples originating in either symptomatic or asymptomatic fish were separately incubated overnight at 4 °C with mild shaking at 300 rpm in monoclonal antibody-linked spin columns (previously described). Next, the spin columns were cleared, and the flow-through was saved for gel electrophoresis. The spin columns were washed eight times with PBS, and the OD_{280} of the final flow-through was zero. The spin columns were subsequently eluted with 500 μL of 0.1 M glycine (pH 3.0) by incubation with mild shaking for 1 h at 4 °C, and the pH was immediately neutralized by addition of 50 μL of 1 M Tris base (pH 8.0). The columns were immediately washed with PBS, and both the eluate and the first wash fraction were concentrated, using a dry vacuum concentrator (Eppendorf) to a final volume of 55 μL.

Next, the samples were either analysed by ESI-MS or further concentrated to 10 μL and analysed by sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE

The 10 μL concentrated sample was mixed with 10 μL of 2× Laemmli sample buffer (100 mM Tris–HCl, pH 6.8, 200 mM 2-mercaptoethanol, 4% SDS, 0.2% bromophenol blue, 20% glycerol) and heated at 99 °C with mild shaking (300 rpm) for 10 min. Proteins were separated in 12% polyacrylamide resolving gel and 5% stacking gel prepared according to Sambrook & Russell 2001). Gels were run using the Mini-Protein Tetra Cell (Bio-Rad laboratories GmbH) at 120 V for 70 min in 1× running buffer (25 mM Tris, 250 mM glycine, pH 8.3, 0.1% SDS). Gel loading was equal for all lysates. Proteins were visualized by staining with Coomassie Brilliant Blue R-250 (Sigma-Aldrich). The broad range (10–200 kDa) ProSieve Unstained Protein Marker II (Lonza), which contain 11 bands, 10, 15, 20,30, 40, 50, 70, 100, 120, 150 and 200 kDa, was used to estimate the molecular weight of the separated proteins.

Electrospray ionization mass spectrometry (ESI-MS) analysis

After staining with Coomassie Blue, the PAGE-gel was cut at sites corresponding to regions where bands of interest were visualized and sent for electrospray ionization coupled to mass spectrometry (ESI-MS) analysis. The other samples, which consisted of the entire antibody-purified products originating in either symptomatic or asymptomatic fish, were also separately analysed by ESI-MS. Electrospray ionization mass spectrometry analysis was performed by the DKFZ, The German Cancer Research Centre in the Helmholtz Association (Heidelberg, Germany).

Results

Protein purification and gel electrophoresis

Protein purification by affinity was used to analyse host and viral proteins involved in CyHV-3 infection. Tissue samples originating in CyHV-3 symptomatic and asymptomatic fish were processed by anti-CyHV-3 monoclonal antibody conjugated to spin columns (as described in the Materials and methods). The two symptomatic samples were eluted with respective optical densities of 0.264 and 0.552 at 280 (OD_{280}). After concentration, the OD_{280} of the samples was above 3.0. The third sample, originating from asymptomatic and PCR-negative fish, was also purified, and the eluate had an OD_{280} of 0.012. The sample with an original OD of 0.264 was analysed on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). One band corresponding to ~10 kDa and another band corresponding to ~60 kDa were visualized (Fig. 2).

ESI-MS analysis

Two separate samples of anti-CyHV-3 monoclonal antibody–purified CyHV-3 tissue samples originating in symptomatic fish and a tissue sample originating in asymptomatic, and PCR-negative fish were analysed by electrospray ionization coupled to mass spectrometry (ESI-MS) analysis.

The first sample, which had an original OD_{280} of 0.264 after purification, consisted of two slices that showed bands in Coomassie-stained gel as previously described. The other sample, which had an original OD_{280} of 0.552 after purification, was analysed without any modifications except for concentration. The two analyses identified a total of five CyHV-3 proteins, two of which were overlapping (40%), and a total of 78 carp proteins, 28 of which were overlapping (36%). In the sample originating from asymptomatic fish, only one

CyHV-3 was identified in conjunction with the 12 carp proteins identified, of which eight (66%) were identical to ones identified in the gel separation.

Gel separation—In the gel separation sample, 56 proteins were identified by ESI analysis of anti-CyHV-3 monoclonal antibody–purified tissue samples originating in CyHV-3 symptomatic fish. Two CyHV-3 proteins were identified: the major capsid protein and glycoprotein (Table 1a). The remaining 54 proteins, corresponding to proteins originating from carp were classified into seven different groups (Table 2a). Five of the carp proteins or their homologs were previously identified in Michel *et al.*'s (2010) report, in conjunction with beta-actin (Kuznetsov, Langford & Weiss 1992), which was grouped with three other cytoskeletal proteins. Also, seven proteins involved in host defence regulation in addition to 12 proteins that are involved in protein modification were identified. Fifteen haem-harbouring proteins in conjunction with three transferrins were also identified, and the remaining eight proteins were unclassified.

In solution—The second sample, which had an original OD of 0.552 at 280, was analysed directly by ESI-MS, and 57 proteins were identified. In addition to the two CyHV-3 proteins identified in the previous section, three additional CyHV-3 proteins were identified: ORF22, ORF23 and ORF150 (Table 1b). Additionally 52 carp proteins were identified, which were classified into seven different groups as in the previous section (Table 2b). Similarly, five of the carp proteins or their homologs were previously identified in Michel *et al.* (2010) in conjunction with beta-actin, which was grouped with vimentin, another cytoskeletal protein. Also, six proteins involved in host defence regulation in addition to 12 proteins that are involved in protein modification were identified. Ten haem-harbouring proteins in conjunction with two transferrins were also identified, and the remaining 15 proteins were unclassified.

Asymptomatic fish group—The third sample, which consisted of anti-CyHV-3 monoclonal antibody–purified tissue samples originating in CyHV-3 asymptomatic fish, identified only one CyHV-3 protein, glycoprotein, ORF56 (Table 1c), and 12 carp proteins that include one homolog to the Michel *et al.* (2010) study, three cytoskeletal proteins, two host defence–related proteins, one protein involved in protein modification, two haem-harbouring proteins and three unclassified proteins (2c).

Discussion

The importance of understanding proteomics such as protein–protein interactions is fundamental in understanding biological systems (Watson James 2003). In conjunction with the traditional tools used to describe protein interactions, such as immunochemistry and yeast two-hybrid system, analysis by mass spectrometry has become a very useful tool to study protein interactions (Cameron 2012). We used antibody-based purification coupled with mass spectrometry to identify protein targets during Cyprinid herpesvirus 3 infection that could ultimately be used to develop novel methods for CyHV-3 control in koi and in common carp. During this study, 78 host proteins involved in Cyprinid herpesvirus 3 infection or propagation and five potential immunogenic CyHV-3 protein targets were identified in CyHV-3 diseased fish. Additionally, four host proteins were identified in CyHV-3 asymptomatic fish. The major capsid protein identified in this study corresponds to ORF92 previously identified in Michel *et al.* (2010); however, the remaining four proteins, ORF22, ORF23, glycoprotein (ORF56) and ORF150, were previously undetected. To enhance our understanding of the CyHV-3 proteins, Prosite (Sigrist *et al.* 2010) and Simple Modular Architect Research Tool (SMART) by Letunic, Doerks & Bork (2012) were used to elucidate important domain structures from the five CyHV-3-identified proteins (Table 3).

We compared proteins identified in this purification to the ones identified by Michel *et al.* (2010) to assess the validity of our assay. The comparison is complicated because the data set used in the previous study was composed before the entire genome for *C. carpio* was available, and therefore, all but one of the proteins (elongation factor) listed in the previous report come from either zebra fish, *Danio rerio*, or Atlantic salmon, *Salmo salar*. However, two proteins (elongation factor, and lactate dehydrogenase) were identical matches for proteins identified in the aforementioned studies. Rab8a was also included in as a match because of the high sequence homology amongst the Rab family of proteins (Bright *et al.* 2010). Similar logic was used to include heat shock proteins amongst the proteins also identified in the Michel *et al.*'s (2010) study. Although glutathione S-transferase rho was identified in both isolations and is a ras-related protein, it was not included in the Michel *et al.* (2010) list because ClustalW alignment did not show significant homology between the two proteins. Interestingly, our study identified several cytoskeletal proteins including actin (Hosein *et al.* 2003; Williams *et al.* 2006) and vimentin, and the Michel *et al.* (2010) study also identified cytoskeleton proteins that include two of the ubiquitous dynamic cytoskeletal proteins tubulin and actin (Gavin 1997), and a cofilin-like protein that may act as a regulator of actin dynamics (De La Cruz 2009; Shiozaki *et al.* 2009).

Electrospray analysis of samples originating in symptomatic fish identified seven proteins from the in-gel analysis and six proteins from the in-solution analysis that are involved in the host defence pathway. Two of the proteins, natural killer cell enhancing factor (Fujiki *et al.* 1999) and complement C3-H1 (Nakao *et al.* 2000), were observed in each of the two samples originating in symptomatic fish; therefore, a total of 11 proteins were identified. In addition to the C3-H1 protein, three other proteins (complements C3-S, C4-1 and C4-2) were identified in the complement host defence pathway (Kato *et al.* 2004). Of the remaining five host defence proteins, granzyme A/K is a member of the serine protease family (Huang *et al.* 2010) along with natural killer cell enhancing factor, which is a component of natural kill cells, and macrophage migration inhibitory factor (MIF) is a member of the cytokine signalling pathway (Bernhagen *et al.* 1993). Belcourt *et al.* (1995) demonstrated that granulin-1 localizes to macrophages in common carp and gold fish, *Carassius auratus auratus*, and granulin-3 which was identified in the gel analysis also is likely part of the host defence pathway. Lysozyme C and lysozyme G were also identified in this analysis; ClustalW alignment did not show significant homology between the two proteins and are phylogenetically unlinked (Savan, Aman & Sakai 2003). Overexpression of endogenous lysozyme C has antimicrobial and antiviral activities in grouper, *Epinephelus coioides*, spleen cells (Wei *et al.* 2012). Although lysozyme C and lysozyme G share very low sequence homology in the grass carp, *Ctenopharyngodon idellus*, they share similar expression profiles and were both shown to have antimicrobial activities, which may indicate that lysozyme G also has antiviral activities consistent with the previous study (Ye *et al.* 2010).

Protein modification is the final method in protein regulation, such as in the modifying gene regulation via the histone code (Jenuwein & Allis 2001); the ubiquitin code and other proteins modifiers are used to activate, repress, target, stabilize or degrade proteins (Sims & Reinberg 2008; Harper & Schulman 2006; Williams & Schwarzbauer 2009). A total of 17 proteins involved in protein modification were identified in this study. Seven of the proteins overlapped in both the gel and unmodified solution analysis. Two members of the protein degradation pathway, an ubiquitin fusion protein along with a proteasome activator subunit, were identified in the gel solution (Table 2a), and accordingly, one of the CyHV-3 proteins identified (Table 3a) has a RING-finger domain, which is known to mediate E3 ubiquitin-ligase activity (Joazeiro & Weissman 2000). Both analyses identified glutathione S-transferase rho; in addition, four other glutathione-related proteins were also identified in the unmodified solution (Table 2b), and these proteins are known to regulate inflammatory

response and modulate cytoskeletal proteins (Zhang *et al.* 1995). Interestingly, alpha-1-antitrypsin homolog was identified in both analyses, and trypsin 1 was identified in the untreated sample; one may wonder whether interaction of these proteins is modulated during CyHV-3 disease.

Cyprinid herpesvirus 3 is also known as KHV, which was once called carp interstitial nephritis and gill necrosis virus (CNGV) because the disease was initially described to affect the kidneys and gills (Hutoran *et al.* 2005; Pokorova *et al.* 2005; Ilouze *et al.* 2008). Recently, there has been debate as to whether the skin, especially at points of abrasion, is the site for viral entry (Costes *et al.* 2009; Raj *et al.* 2011; Fournier *et al.* 2012). However, we are unaware of a study that describes how the virus spreads to other major organs such as liver, spleen and brain (Gilad *et al.* 2004; Dishon *et al.* 2007). Abundance of blood-related proteins such as the globins and transferrins may lead one to speculate whether CyHV-3 propagates via the blood transport system.

Amongst the unclassified list, a total of 21 proteins were grouped. Three proteins were identified in both analyses, the gel solution contained an additional six proteins, and the unmodified solution contained an additional 12 proteins (Table 3). CyHV-3 is known to affect kidneys, spleen and brain of carp (Ronen *et al.* 2003; Pikarsky *et al.* 2004; Miyazaki *et al.* 2008); several proteins that localize to CyHV-3-affected areas were identified including nephrosis precursor, fetuin short form and three other glial-associated proteins (Kálmán 1998; Tsai *et al.* 2004).

Initial attempts to control CyHV-3 outbreaks were carried out by a short exposure to CyHV-3 at permissible temperature (i.e. 15–25 °C) and subsequent treatment at non-permissible temperature (30 °C) to stimulate an immunogenic response against CyHV-3 (Ronen *et al.* 2003). However, several studies indicate that CyHV-3 stays dormant in survivors of CyHV-3 disease (Bretzinger *et al.* 1999; Bergmann *et al.* 2009; Eidea *et al.* 2011) and that CyHV-3 can even resurface to propagate and infect naïve fish (St-Hilaire *et al.* 2005; St-Hilaire *et al.* 2009). The third sample that was analysed came from a fish with no signs of CyHV-3 disease and was free of CyHV-3 according to analysis by conventional PCR (Bercovier *et al.* 2005) and real-time PCR (Gilad *et al.*, 2004); however, ESI-MS detected glycoprotein (ORF56), indicating that the sample may have been a latent carrier for CyHV-3 disease (Table 1c). Interestingly, in the asymptomatic carrier, four novel proteins were identified in conjunction with the 8 proteins previously identified. The four novel proteins include an additional homolog to heat shock proteins, termed Hsc70-1 (Wu *et al.* 2011), and a cell cycle regulator cyclin B in addition to the two host defence proteins. Granulins share very high sequence homology (Belcourt *et al.* 1995), and therefore, granulin-2 may not be a novel finding in the asymptomatic study. However, Toll-like receptor 22 (TLR-22) was not detected in the other two analyses and has been demonstrated to have antiviral function (Lv *et al.* 2012).

A few regions of the world are relatively free of the Cyprinid herpesvirus such as India and Australia (McColl *et al.* 2007; Rathore *et al.* 2009); however, CyHV-3 has become an epidemic threat to the world carp industry, especially in Europe, Israel, Indonesia and the largest aquaculture carp producer, China (Bergmann *et al.* 2006; Bondad-Reantaso, Sunarto & Subasinghe 2007; Dishon *et al.* 2007; Sunarto *et al.* 2007; Dong *et al.* 2011). Several studies have explored the method of infection, latency and detection of CyHV-3 (Bergmann *et al.* 2009, 2010; Costes *et al.* 2009). However, relatively few studies have explored CyHV-3 on a molecular level (Michel *et al.* 2010; Fuchs *et al.* 2011). We used anti-body-based purification followed by electrospray ionization analysis to investigate molecular interactions during CyHV-3 infections. Seventy-eight endogenous host proteins along with five putative immunogenic CyHV-3 proteins involved in CyHV-3 replication and

propagation were identified in this investigation. Additionally, four endogenous proteins were identified in samples originating from CyHV-3 asymptomatic fish. CyHV-3 infection has been recently shown to modulate gene expression in the proteasome degradation pathway, granulin, and other protein modifying enzymes identified in this report (Rakus *et al.* 2012). The antibody against glycoprotein (ORF56) was able to isolate CyHV-3 proteins in a sample that was KHV negative according to PCR methods detecting the TK gene. The glycoprotein may also be highly immunogenic and a good target for detection of CyHV-3 in asymptomatic fish by ELISA or even by PCR analysis. The diversity of proteins identified in this study opens the door for further analysis of host proteins involved in CyHV-3 replication, propagation and repression. For example, Toll-like receptor 22 appears to be involved in suppressing CyHV-3 disease in infected fish. Therefore, stimulating the overexpression of TLR-22 may be an alternative method of controlling CyHV-3 disease.

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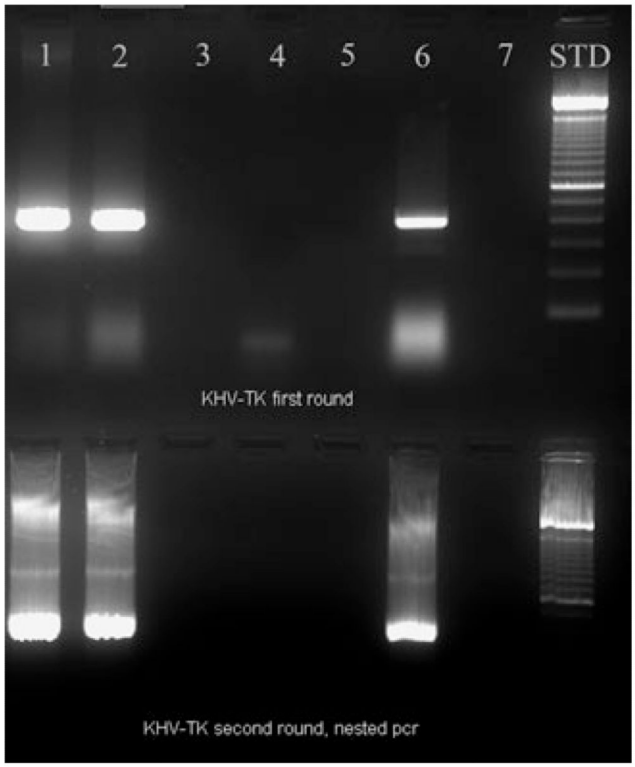

Figure 1.
PCR analysis of infected fish samples. Lane 1: DNA extract from the symptomatic fish that was later used for PAGE and subsequent ESI-MS analysis. Lane 2: DNA extract from the symptomatic fish that was later used directly for ESI-MS analysis. Lane 3: empty. Lane 4: DNA extract from the asymptomatic fish that was later used directly for ESI-MS analysis. Lane 5: empty. Lane 6: DNA extract from CyHV-3 positive control. Lane 7: empty. Fifteen microlitres of sample was added to lanes 1–7. Lane 8: 1 μL of Bio-Rad 1 kb DNA Ladder.

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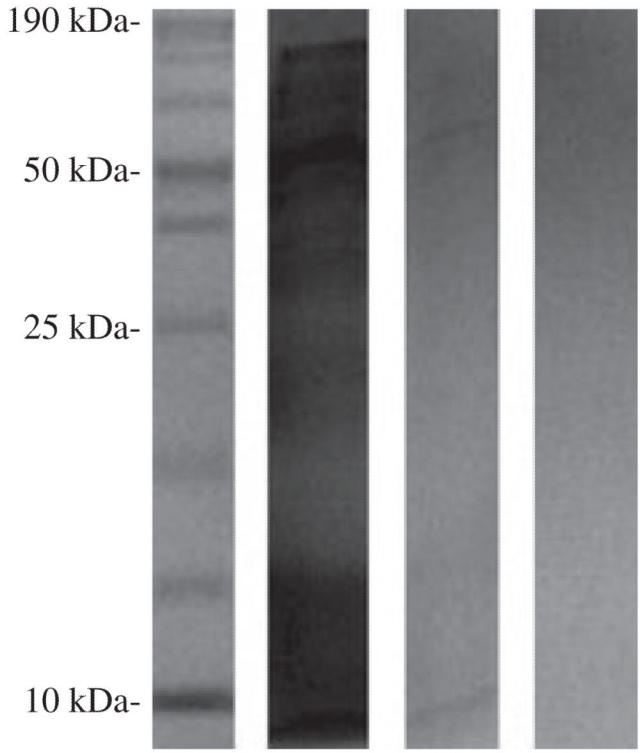

Figure 2.
PAGE analysis of immunoprecipitation. Lane 1: 5 μL of the ProSieve Unstained Protein Marker II (By Lonza), 10–200 kDa was used, which consists of 11 bands: 10, 15, 20, 30, 40, 50, 70, 100, 120, 150 and 200 kDa. The 120, 150 and 200 kDa did not separate well and appear as 1 band. Lane 2: Flow-through of non-denatured lysate (see methods). Lane 3: Monoclonal antibody–purified peptides. Two bands are observed: one at ~10 kDa and another band corresponding to ~60 kDa. Lane 4: First phosphate-buffered saline wash after elution with glycine (pH 3.0). Forty microlitres of the respective samples was run in lanes 2–4.

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Table 1

CyHV-3 proteins identified by ESI-MS analysis

Accession no.	Protein description	Score	Mass [Da]	Matches	Coverage [%]
(a) Two CyHV-3 proteins identified by eiectrospray analysis from gei slices originating in symptomatic fish samples					
gi|65306693	Major capsid protein [Cyprinid herpesvirus 3]	278	140437	7	4.8
gi|129560573	Glycoprotein [Cyprinid herpesvirus 3]	43	100710	2	2
(b) Five CyHV-3 proteins identified by electrospray analysis from unmodified lysate solution originating in symptomatic fish samples					
gi|61696095	Major capsid protein [Cyprinid herpesvirus 3]	25	140436	1	0.9
gi|l29560573	Glycoprotein [Cyprinid herpesvirus 3]	24	100710	1	0.9
gi|l29560669	0RF150 [Cyprinid herpesvirus 3]	23	70515	1	1.1
gi|l31840052	0RF22 [Cyprinid herpesvirus 3]	22	67563	1	1
gi|l31840053	ORF23 [Cyprinid herpesvirus 3]	26	38808	1	2.1
(c) One CyHV-3 proteins identified by electrospray analysis from unmodified lysate solution originating in asymptomatic fish					
gi|129560573	Glycoprotein [Cyprinid herpesvirus 3]	24	100710	1	1.1

CyHV-3, Cyprinid herpesvirus 3.

Table 2

Cyprinus carpio proteins identified by ESI-MS analysis

Accession no.	Protein description	Score	Mass [Da]	Matches	Coverage [%]
(a) Fifty-four *C. carpio* proteins identified by electrospray analysis from gel slices originating in symptomatic fish samples grouped into 7 aforementioned categories					
Michel *et al.* 2010 or homolog proteins					
gi|28628941	Elongation factor 1-alpha	**128**	**50 325**	**3**	**6.1**
gi|l7369826	Lactate dehydrogenase A chain	**115**	**36 539**	**2**	**5.4**
gi|l5628189	Heat shock protein 90 alpha	28	25 336	1	5.3
gi|l4388583	Warm-temperature acclimation-related 65-kDa protein	78	50 640	3	6.6
gi|218511593	RAB8A	**23**	**23 823**	**1**	**5.3**
Cytoskeletal proteins					
gi|42560193	Beta-actin	**496**	**42 068**	**24**	**25.1**
gi|1703140	Alpha-actin-1	326	42 274	14	18.8
gi|13365501	Integrin beta-2 chain	173	87 597	5	7.3
gi|6226787	Vimentin	**56**	**52 516**	**4**	**3.5**
Host defence proteins					
gi|4126587	Complement C3-H1	**223**	**184 985**	**6**	**4**
gi|9453863	Complement C4-1	67	193 004	1	0.8
gi|9453865	Complement C4-2	98	194 643	3	1.9
gi|3399699	Natural killer cell enhancing factor	**44**	**22 410**	**1**	**5.5**
gi|297718575	Granzyme A/K	26	29 149	2	4.3
gi|7939556	Lysozyme C	49	17 241	2	11.7
gi|2829695	Granulin-3	47	6995	2	24.6
Protein modification enzymes or inhibitors					
gi|439153	Serine protease inhibitor	**288**	**45 960**	**12**	**13.4**
gi|416561	Alpha-1-antitrypsin homolog	**372**	**42 044**	**22**	**23.7**
gi|56709493	Putative glyceraldehyde-3-phosphate dehydrogenase	**21**	**33 540**	**2**	**2.2**
gi|4027925	Creatine kinase M1-CK	**37**	**42 980**	**1**	**3.4**
gi|73762632	Carbonic anhydrase	**80**	**28 716**	**3**	**11.9**
gi|373503076	Glutamate dehydrogenase	**910**	**54 750**	**46**	**47.1**
gi|112901127	Glutathione S-transferase rho	**35**	**26 700**	**1**	**6.6**
gi|152925970	Ubiquitin fusion protein	279	14 987	19	41.4
gi|91798526	Proteasome activator PA28 subunit	37	28 579	1	2.8
gi|217272706	Monoamine oxidase	442	59 621	13	16.3
gi|6690508	Janus kinase 3	22	131 078	1	0.7
gi|300676317	Catalase	21	49 187	1	3.1
Globins					
gi|2208883	Alpha-globin	**529**	**15 421**	**35**	**52.4**
gi|2208891	Alpha-globin	**488**	**15 566**	**35**	**54.5**
gi|2208889	Alpha-globin	**390**	**15 574**	**28**	**61.5**
gi|122392	Alpha-globin	**595**	**15 437**	**45**	**62.2**
gi|6009727	Alpha-2-macroglobulin-1	631	162 274	22	11.8

Accession no.	Protein description	Score	Mass [Da]	Matches	Coverage [%]
gi|6009729	Alpha-2-macroglobulin-2	541	157 876	18	10
gi|6009731	Alpha-2-macroglobulin-3	434	88 297	14	15.1
gi|140368180	Alpha-2-macroglobulin 4	368	101 340	12	11.4
gi|22135548	Beta-globin	**584**	**16 520**	**41**	**63.3**
gi|2208895	Beta-globin	**732**	**16 617**	**67**	**87.2**
gi|1644253	Beta-globin	**718**	**16 645**	**63**	**87.2**
gi|1644251	Beta-globin	706	16 542	68	87.2
gi|3953518	Immunoglobulin heavy chain	**1053**	**50 331**	**54**	**47.6**
gi|55700034	Immunoglobulin heavy chain	78	40 300	4	6.4
gi|85067845	Myoglobin isoform 2	**29**	**16 278**	**1**	**5.4**
Transferrins					
gi|18034630	Transferrin variant A	**1882**	**75 595**	**100**	**47.1**
gi|189473163	Transferrin variant F	**986**	**75 036**	**45**	**26**
gi|189473165	Transferrin variant G	1003	75 083	47	26.9
Unclassified					
gi|29501366	Fetuin short form	**43**	**34 332**	**2**	**7**
gi|13445027	Apolipoprotein A-I	**83**	**20 797**	**4**	**16.1**
gi|435737	Glial fibrillary acidic protein	**119**	**24 944**	**69**	**9.3**
gi|151558991	Vitellogenin B1	2221	148 683	100	39.6
gi|52782167	Vitellogenin B2	964	179 640	48	13.4
gi|14009437	Mitochondrial ATP synthase	32	59 699	1	2.2
gi|47605558	Mitochondrial synthase subunit beta	71	55 327	2	4.1
gi|10566900	Myeloid protein-1	35	17 849	1	7.5
(b) Fifty-two *C. carpio* proteins identified by electrospray analysis from unmodified lysate solution originating in symptomatic fish samples grouped into 7 aforementioned categories					
Michel *et al.* 2010 or homologs					
gi|28628941	Elongation factor 1-alpha	**97**	**50 325**	**5**	**6.7**
gi|l7369826	Lactate dehydrogenase A chain	**110**	**36 539**	**2**	**6.3**
gi|55669145	Lactate dehydrogenase B-type subunit	150	36 669	3	9
gi|33598990	Constitutive heat shock protein HSC70-2	329	70 779	12	12.3
gi|218511593	RAB8A	**26**	**23 823**	**1**	**5.3**
Cytoskeletal proteins					
gi|42560193	Beta-actin	**457**	**42 068**	**15**	**24.8**
gi|6226787	Vimentin	**127**	**52 516**	**6**	**3.5**
Host defence proteins					
gi|4126587	Complement C3-H1	**362**	**184 985**	**9**	**5.2**
gi|4126593	Complement C3-S	179	185 561	5	2.9
gi|3399699	Natural killer cell enhancing factor	**93**	**22 410**	**3**	**9.5**
gi|84569882	Natural killer cell enhancing factor	251	22 014	10	47.7
gi|165880803	MIF	26	12 525	1	14.8
gi|47117021	Lysozyme g	88	20 425	3	10.8
Protein modification enzymes or inhibitors					

Accession no.	Protein description	Score	Mass [Da]	Matches	Coverage [%]
gi|439153	Serine protease inhibitor	**83**	**45 960**	**2**	**9**
gi|416561	Alpha-1-antitrypsin homolog	**261**	**42 044**	**7**	**15.6**
gi|56709493	Putative glyceraldehyde-3-phosphate dehydrogenase	**146**	**33 540**	**2**	**9.2**
gi|4027925	Creatine kinase M1-CK	**34**	**42 980**	**1**	**1.6**
gi|73762632	Carbonic anhydrase	**285**	**28 716**	**7**	**40.4**
gi|373503074	Glutamate dehydrogenase	**63**	**54 737**	**2**	**3.1**
gi|112901127	Glutathione S-transferase rho	**51**	**26 700**	**1**	**4.4**
gi|95832156	Pi-class glutathione S-transferase	69	23 794	2	10.1
gi|300676299	Glutathione peroxidase 1	37	19 182	1	10
gi|343481094	Glutathione synthetase	22	41 342	1	2.5
gi|353351682	Trypsin 1	114	26 940	1	6.6
gi|117518748	Enolase	21	16 206	1	4.1
Globins					
gi|2208891	Alpha-globin	**527**	**15 566**	**44**	**73.4**
gi|2208883	Alpha-globin	**494**	**15 421**	**35**	**69.2**
gi|2208887	Alpha-globin	492	15 449	36	69.2
gi|2208889	Alpha-globin	**372**	**15 574**	**24**	**69.2**
gi|122392	Alpha-globin	**659**	**15 437**	**56**	**81.1**
gi|1644251	Beta-globin	**652**	**16 542**	**47**	**78.4**
gi|2208895	Beta-globin	**568**	**16 617**	**46**	**70.9**
gi|22135548	Beta-globin	**485**	**16 520**	**26**	**54.4**
gi|3953518	Immunoglobulin heavy chain	**177**	**50 331**	**5**	**12.8**
gi|85067845	Myoglobin isoform 2	**86**	**16 278**	**2**	**12.9**
Transferrins					
gi|18034630	Transferrin variant A	**420**	**75 595**	**17**	**12.3**
gi|189473165	Transferrin variant G	**321**	**75 083**	**12**	**10.2**
Unclassified					
gi|29501366	Fetuin short form	**64**	**34 332**	**3**	**7.3**
gi|13445027	Apolipoprotein A-I	**226**	**20 797**	**7**	**32.2**
gi|435737	Glial fibrillary acidic protein	**417**	**24 944**	**41**	**28**
gi|435739	Glial fibrillary acidic protein	504	24 531	44	33.2
gi|49356890	Glia-derived neurotrophic factor	25	11 004	1	8.6
gi|2252655	Nephrosin precursor	340	31 629	13	27.5
gi|281333450	Heart-type fatty-acid-binding protein	156	14 704	5	21.8
gi|15778562	Vitellogenin	294	148 794	12	7.8
gi|84569880	Translationally controlled tumour protein	84	19 152	2	8.2
gi|281429776	Adipocyte fatty-acid-binding protein	124	15 278	2	16.4
gi|1173446	Somatoliberin	28	4976	1	17.8
gi|585105	Ependymin	385	24 433	67	41.9
gi|40549335	Cytochrome P4501C1	24	59 583	1	1.3
gi|219935425	Mineralocorticoid receptor	24	107 380	1	0.5
gi|169674670	Liver-basic fatty-acid-binding protein b	24	14 255	1	15.9

Accession no.	Protein description	Score	Mass [Da]	Matches	Coverage [%]
(c) Twelve *C. carpio* proteins identified by electrospray analysis from unmodified lysate solution originating in asymptomatic fish samples grouped into 7 aforementioned categories					
Michel *et al.* 2010 or homolog proteins					
gi|32394421	Muscle-specific heat shock protein Hsc70-1	51	70 660	2	6.1
Cytoskeletal proteins					
gi|42560193	Beta-actin	**78**	**42 068**	**4**	**8.8**
gi|1703140	Alpha-actin-1	**77**	**42 274**	**3**	**9**
gi|6226787	Vimentin	**32**	**52 516**	**1**	**1.5**
Host defence proteins					
gi|7939556	Lysozyme C	**264**	**17 241**	**20**	**43.4**
gi|313509543	Toll-like receptor 22	30	109 136	3	0.7
gi|2829694	Granulin-2	43	7215	1	17.5
Protein modification enzymes or inhibitors					
gi|152925970	Ubiquitin fusion protein	**99**	**14 987**	**5**	**24.2**
Globins					
gi|122392	Alpha-globin	**88**	**15 437**	**4**	**16.1**
gi|85067845	Myoglobin isoform 2	**25**	**16 278**	**1**	**5.4**
Unclassified					
gi|435737	Glial fibrillary acidic protein	**80**	**24 944**	**30**	**5.1**
gi|162423638	Cyclin B	27	45 068	1	2

MIF, migration inhibitory factor.

The 54 and 52 identified proteins from either gel slices or unmodified samples, respectively, were classified into seven groups that include: Michel *et al.* 2010, cytoskeletal, host defence, protein modification or inhibitors, globins, transferrins, unclassified. Proteins that showed up in both analyses are in bold.

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Table 3

Description of CyHV-3 proteins identified. Prosite (Sigrist *et al.* 2010) and Simple Modular Architect Research Tool (SMART) by Letunic *et al.* (2012) were used to identify important domains in the 5 CyHV-3 proteins identified in this study. Amino acid number is referred to by the abbreviation aa

Accession no.	Protein description	Protein length	Domain of interest	Spanning region
(a) Glycoprotein and 0RF150 modelled using Prosite				
gi|129560573	Glycoprotein	897 aa	Putative AMP binding	173-184 aa
gi|129560669	0RF150	628 aa	Zinc finger Ring-type	24-66 aa
(b) The remaining three proteins, 0RF22, 0RF23 and 0RF92 (major capsid protein), modelled by SMART				
gi|65306693	Major capsid protein	1268 aa	MHC II beta	461-513 aa
gi|131840052	0RF22	588 aa	Transmembrane domain	22-217 aa
gi|131840053	0RF23	335 aa	Interleukin-10 (IL-10)	24-163 aa