Vol. 111: 269–273, 2014
doi: 10.3354/dao02793

DISEASES OF AQUATIC ORGANISMS
Dis Aquat Org

Published October 16

NOTE

Non-structural protein pORF 12 of cyprinid herpesvirus 3 is recognized by the immune system of the common carp *Cyprinus carpio*

Julia Kattlun, Simon Menanteau-Ledouble, Mansour El-Matbouli*

Clinical Division of Fish Medicine, University of Veterinary Medicine, Veterinärplatz 1, 1210 Vienna, Austria

ABSTRACT: Cyprinid herpesvirus 3 is an important pathogen and the causative agent of koi herpesvirus disease, which has been associated with mass mortalities in koi and common carp *Cyprinus carpio*. Currently, the only available commercial vaccine is an attenuated version of the virus. This has led to concerns about its risk to reversion to virulence. Furthermore, the vaccine is currently only available in Israel and the United States. In order to investigate the antigenic profile of the virus, western blot was performed using infected cell culture supernatant and sera from carp that had survived exposure to the virus. Only one antigen could be detected, and mass spectrometry analysis identified the corresponding protein as ORF 12, a putative secreted tumour necrosis factor receptor homologue. In other herpesviruses, such proteins have been associated with the viral infectious process in a number of ways, including the entry into the host cell and the inhibition of apoptosis in infected cells. The reason why only one antigen could be detected during this study is unknown.

KEY WORDS: Koi herpesvirus · Antigenic determinants · Western blot · Fish-acquired immunity

INTRODUCTION

Cyprinid herpesvirus (CyHV-3) is a notifiable pathogen that has been associated with mortalities in the common and koi carp industries. Since its first occurrence in the late 1990s (Hedrick et al. 2000, Ronen et al. 2003), it has spread worldwide and has become one of the major pathogens currently posing a health threat to this aquaculture sector.

It has been reported that fish may develop a natural resistance following viral exposure at a high water temperature (30°C) (Ronen et al. 2003), suggesting that it would be possible to develop an effective vaccine against this pathogen. An attenuated live virus vaccine has been made commercially available in Israel and the USA that proved protective against the virus: vaccination prevented any mortalities while the non-vaccinated control group suffered 95% mortality (Ronen et al. 2003). The vaccine was also able to induce high antibody titers (Perelberg et al. 2008). However, concerns about this new vaccine have surfaced, mostly due to the risk of reversion to a pathogenic form. Additional attempts have been made that aimed at producing an attenuated mutated strain of the virus (Vanderplasschen 2013) as well as DNA vaccines (Zhou et al. 2013, 2014). Both vaccines were reported to be protective and to significantly reduce mortalities following experimental infections. However, they both have yet to be made commercially available. Therefore, the present study was designed to screen for other immunogenic proteins of CyHV-3 and identify alternative antigen and vaccine

*Corresponding author:
mansour.el-matbouli@vetmeduni.ac.at

candidates using sera harvested from carp that had survived an artificial CyHV-3 infection.

MATERIALS AND METHODS

CyHV-3 (also known as koi herpesvirus, KHV-I) was grown in common carp brain (CCB) cell cultures in 25 cm^2 cell culture flasks at 20°C until complete cytopathic effect was visible. Supernatant containing the virus and remaining cell debris was stored in 1 ml aliquots at −80°C until use. Prior to this study, sera were harvested from fish that had survived artificial infection with CyHV-3 as part of a previously conducted experiment. Fish had been infected via intraperitoneal injection with 100 µl of an inoculum containing 10^3 $TCID_{50}$ of CyHV-3 (KHV-I) and kept at a water temperature of 22–23°C for 4 wk. Two fish that had survived infection were euthanized by prolonged immersion in a solution of MS-222 and blood was collected by puncture of the caudal vein. Blood was left to clot overnight at 4°C and subsequently centrifuged at 600 × *g* for 5 min. The sera were collected, pooled and stored at −20°C prior to use. As a control, blood was collected in the same fashion from 2 healthy carp mock-infected with phosphate-buffered saline (PBS; pH 7.4) that was confirmed CyHV-3-free by real-time PCR (Gilad et al. 2004).

Virus-containing cell culture supernatant was thawed at 4°C. The supernatant was then either left unprocessed or centrifuged at 600 × *g* for 10 min in a microcentrifuge. Protease inhibitor cocktail (Sigma-Aldrich) was added, and the preparation was sonicated on ice with a Bandelin Sonopuls 2070 (Bandelin Electronic) ultrasound sonicator using ten 1 s bursts with 65% power.

Two hundred microlitres of the preparation were added to 200 µl of 2× SDS-PAGE sample buffer (Bio-Rad). The same procedure was applied to uninfected cell culture supernatant to act as a negative control. Samples of the supernatant from the virus-infected cell cultures and the uninfected controls were loaded on to 12% acrylamide SDS-PAGE gels overlayed with a 4% stacking gel. These were run at 120 V for 90 min. Afterwards, gels were either immersed in a Coomassie blue staining solution to visualize all existing protein bands or subjected to a western blot: proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane at 40 V overnight at 4°C. Membranes were subsequently washed with 0.1% Tween in PBS (PBST; Sigma-Aldrich) and blocked with 3% blocking solution (Bio-Rad). Following another wash with PBST, one membrane was incubated with the pooled serum from infected fish (diluted 1:500 in 1% bovine serum albumin in PBST), while the other was immersed in uninfected carp serum to act as a control. Membranes were thoroughly washed, and separately incubated with anti-carp IgM monoclonal antibody (Aquatic Diagnostics) diluted 1:66. After another washing step, the third incubation was carried out with goat anti-mouse-horseradish peroxidase conjugate diluted 1:1000 and then developed using an Opti4CN kit (Bio-Rad). All procedures were performed in duplicate. Blots were compared with stained SDS-PAGE gels and the bands of interest, corresponding to regions identified on the western blot, were excised from the gels. Samples were processed by electrospray ionization coupled to mass spectrometry (ESI-MS) analysis, which was performed at the DKFZ, The German Cancer Research Centre of the Helmholtz Association (Heidelberg, Germany).

RESULTS

Results from the western blots were identical between duplicates. Unexpectedly, only one band could be detected in the blot with pooled infected fish serum. This band was roughly 14 kDa in size, and did not appear in lanes containing uninfected cell culture supernatant nor on the membrane incubated with mock-infected carp serum (Fig. 1). ESI-MS identified the viral protein in this particular band as the protein encoded by the open reading frame 12 (pORF 12) of CyHV-3 (GenBank accession no. BAF48825), a putative herpesvirus entry mediator from the family of tumour necrosis factor receptors (TNFRs; Aoki et al. 2007) (see Tables 1 & 2).

DISCUSSION

The fact that only one viral protein could be detected on the western blot was unexpected and is as yet unexplained. It was recently reported that CyHV-3 pORF 12 as well as pORF 134 were amongst the most abundant proteins present in the culture medium of a CCB cell culture infected with CyHV-3 (Ouyang et al. 2013), which might have contributed to its recognition by the immune system. In addition, because our western blots were prepared from cell supernatant, the presence of pORF 12 was expected. The fact that no other antigens could be detected was, however, unanticipated and might suggest a low sensitivity of our screening method, i.e. it only

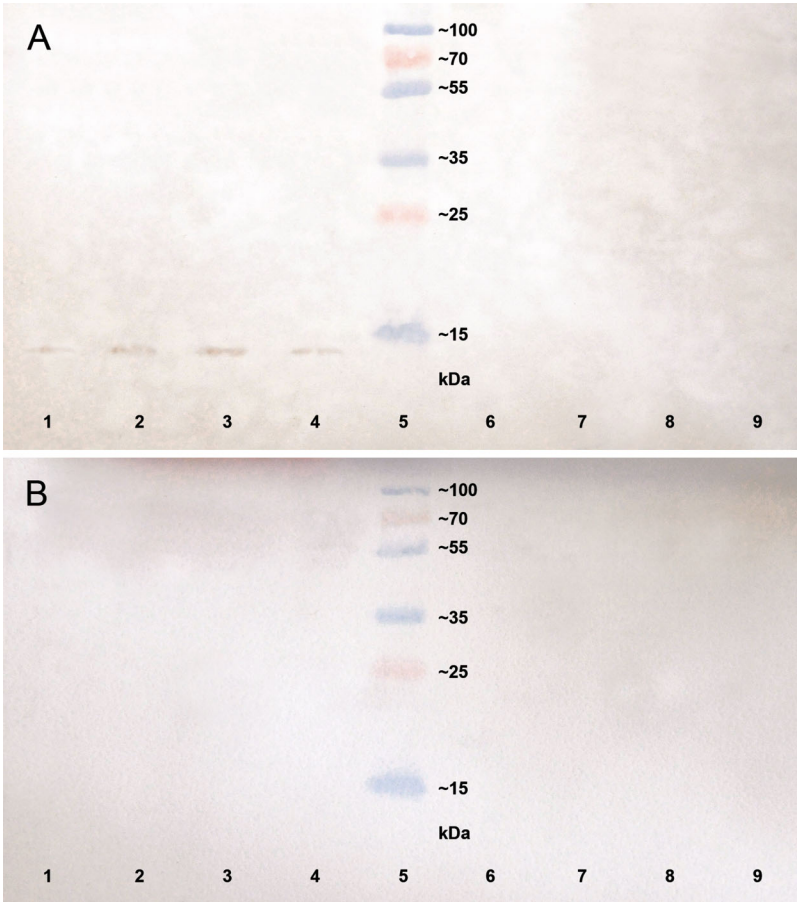

Fig. 1. Western blot with sera from koi herpesvirus (KHV)-infected and uninfected carp *Cyprinus carpio*. Lanes were loaded with either virus-containing sample (lanes 1–4) or uninfected sample (lanes 6–9). Subsequently, blots were incubated with either (A) cyprinid herpesvirus (CyHV-3)-infected or (B) mock-infected carp serum. Lane 5: protein standard

detects the most prevalent antigens. Alternatively, it might reflect that, at least as far as the interactions between this particular isolate of the virus (KHV-I) and the immune system of this particular strain of carp are concerned, this protein dominates the antigenic profile of the virus.

Interestingly, despite being very common in the infected cell culture supernatant, the interleukine (IL-10) homologue pORF 134 was found to be essential neither for viral replication *in vitro* nor for its virulence in common carp (Ouyang et al. 2013).

CyHV-3 genome encodes 2 proteins that have been identified as TNFR homologues: ORF 12, which was identified in this study, as well as ORF 4, which is represented by 2 copies in the virus's genome, and is located on the terminal repeat region (Aoki et al. 2007). Both TNFR homologues as well as the IL-10-homologue encoded by ORF 134 have been hypothesized to play a role in immune evasion mechanisms (van Beurden et al. 2011, Sunarto et al. 2012, Ouyang et al. 2013) in a fashion similar to mechanisms described in pox viruses (Seet et al. 2003).

Interestingly, ORF 12 was recognized as a CyHV-3 gene with late

Table 1. Results from the electrospray ionization-mass spectrometry (EST-MS) analysis. Prot.: protein; sign.: significant; seq.: sequences

Hit no.	Geninfo accession no.	Protein description	Score	Mass (Da)	Prot. matches All	Prot. matches Sign.	Prot. seq. All	Prot. seq. Sign.	Coverage (%)
1	gil74267962	ALB protein (*Bos taurus*)	952	71186	58	17	18	8	29.2
2	gil229552	Albumin	859	68083	56	16	17	7	28.4
3	gil11935049	Keratin 1 (*Homo sapiens*)	656	66198	22	5	12	4	17.9
4	gil62460494	Hemoglobin fetal subunit beta (*Bos taurus*)	606	15963	28	9	10	4	71.7
5	gil209973077	Transferrin (*Bos grunniens*)	475	79775	29	6	10	4	14.1
6	gil27806789	Transthyretin precursor (*Bos taurus*)	474	15831	33	13	9	3	43.5
7	gil114326282	Serotransferrin precursor (*Bos taurus*)	468	79856	24	6	10	4	14.1
8	gil431921648	Keratin, type II cytoskeletal 1 (*Pteropus alecto*)	459	138695	15	6	8	4	6.2
9	gil59857769	Inter-α (globulin) inhibitor H4 (plasma glycoprotein) (*Bos taurus*)	444	101617	26	3	9	2	8.4
10	gil3318722	Chain E, leech-derived tryptase inhibitor TRYPSIN COMPLEX	339	24142	137	13	5	2	26
11	gil27806751	Alpha-2-HS-glycoprotein precursor (*Bos taurus*)	331	39193	21	8	5	2	17
12	gil114051526	Coactosin-like protein (*Bos taurus*)	260	16114	23	0	7	0	39.4
13	**gil129560530**	**Putative herpesvirus entry mediator (cyprinid herpesvirus 3)**	**223**	**19165**	**17**	**13**	**3**	**2**	**22.9**
14	gil442754	Chain A, crystal structure solution	217	15713	15	5	4	2	32.5
15	gil128169	Cobalt-containing nitrile hydratase subunit alpha	200	22991	8	1	4	1	24.1

Table 2. DNA and protein sequences of ORF 12 of cyprinid herpesvirus (CyHV-3) (GenBank accession no. BAF48825)

DNA (501 bp):
TCA ACT TCT CTT TG GTT TTC TGC ACA TAT TCG TCC CCC CCA CGG TTT TCA TTT CGT AGT CGC CCT GAC ATC CAC AGA CTG TGT TCT TAG TTG GAG TAC ACT GTT CCT TTA CGA CTT TGC GGC CAC CGG AAC ATT TGT CAC AGC TAA AAC AGT TCT CAT AAT AAT TGT GTG TGG CAG AGT ATG TCG ATC CAG CAA CAC AAG CTT CAC ATA CTG TTG GTG AAT CTT TGG TAC AAA CCG ATT TAA ATT TTG TGC CGG GCT GGC ACT TCT GAC AGC ATT TCT GAA GCT CAG TCT CGT AGT ATT CCG TCT CAC CGC ATG ATG AGT GTC TAG TTG GTT TGG CTT GAA CAG CCA GGT AGC AGA GTC CAA GAC CCA GAA TCA CAA GCA GCT TCA TGG TTT GAG AAG AAA TCA AGT CGG AGA TGT GAT AGA GTA TAG TAA AAC AAG AGA TTT TAT TGA GCA CAG AAA CAG ACA ATA ATA AAC ATT CAG CTG ATG TTA CAG TGT GAG TCA T

Protein (166 aa):
MTH TVT SAE CLL LSV SVL NKI SCF TIL YHI SDL ISS QTM KLL VIL GLG LCY LAV QAK PTR HSS CGE TEY YET ELQ KCC QKC QPG TKF KSV CTK DSP TVC EAC VAG STY SAT HNY YEN CFS CDK CSG GRK VVK EQC TPT KNT VCG CQG DYE MKT VGG TNM CRK PKR S

expression during the infection process (expression at 8 h post infection), and it is also expressed at 30°C (Ilouze et al. 2012), a temperature at which fish become infected following challenge with CyHV-3 but do not suffer from clinical disease (Ronen et al. 2003). Late-phase expression of viral proteins is generally associated with the synthesis of the viral capsid and envelope, which were extensively analyzed (Michel et al. 2010). However, Michel et al. did not detect ORF 12, suggesting it not to be a structural protein. This is in agreement with the conclusions of Aoki et al. (2007) and Ouyang et al. (2013), who hypothesized ORF 12 to be secreted rather than a structural part of the viral particle.

The fact that ORF 12 is expressed during the late-phase infection despite not being involved in the synthesis of the viral capsid and envelope suggests that it may play a role in facilitating the budding of the virus or in its survival outside of the infected cell.

Tumor necrosis factors (TNFs) are proinflammatory cytokines that are expressed by activated macrophages and lymphocytes in vertebrates. TNFs are able to induce an array of cellular immunological responses varying from apoptosis to expression of genes involved in early and acquired immune response (Rahman & McFadden 2006). Because of their central role in the immune system of their hosts, a number of pathogens have evolved elaborate mechanisms to overcome or evade TNFs and the various responses they mediate. Viruses, in particular, can encode secreted modulatory proteins that are able to block TNFs and TNF-mediated responses at different cellular levels (Rahman & McFadden 2006). For example, they modulate key transduction molecules of the TNF signaling pathway or directly inhibit TNFs interaction with the corresponding receptors.

This latter mechanism is widespread, and a number of viral families have been found to secrete TNFR homologues that bind to extracellular TNFs, sequestering the molecule away from its receptors. For example, such TNFR homologues are particularly common in poxviruses, and have been identified in *Leporipoxvirus*, *Orthopoxvirus* (Cunnion 1999, Xu et al. 2000), cowpox and mousepox viruses (Panus et al. 2002, Saraiva et al. 2002). Interestingly, the pathogenicity of myxoma virus in domestic rabbits could be attenuated when the gene for the soluble secreted TNFR of myxomavirus was deleted from its genome (Upton et al. 1991).

In herpesviruses, TNFRs, like ORF 12 of CyHV-3 in our study, have been associated with different modes of action. Indeed, it has been demonstrated that the glycoprotein D (gD) of herpes simplex virus 1 (HSV-1) interacts with herpes virus entry mediator (HVEM) and that this interaction results in the invasion of resting T cells, monocytes and immature dendritic cells (Montgomery et al. 1996). Interestingly, it has also been shown that addition of anti-HVEM sera, which inhibited the binding of gD on HVEM, had a dose-dependent effect on the invasiveness into HeLa cells.

A TNFR homologue of Epstein-Barr virus (human herpesvirus 4, HHV-4), LMP1, was shown to be required for the conversion of infected B lymphocytes to perpetually proliferating lymphoblasts, as well as having an anti-apoptotic effect in infected cells through upregulation of expression of anti-apoptotic factors (Lam & Sugden 2003, Grimm et al. 2005).

Finally, UL 144 ORF, which is a TNFR homologue of human cytomegalovirus (HCMV, or human herpesvirus 5, HHV-5), inhibits T-cell proliferation by mimicking the inhibitory co-signaling function of the host's HVEM (Cheung et al. 2005).

Taken together, these findings suggest that TNFRs contribute in a variety of ways to the ability of herpesviruses to infect their host and that neutralising antibodies against TNFR homologues might be an efficient means of reducing the pathogenicity of these viruses. However, more research will be required to confirm that this is also the case in CyHV-3. Considering that pORF 12 was found to be immunogenic in the present study, this could make this secreted viral protein an interesting candidate for vaccine development. In addition, targeted deletion mutations could be used to investigate the role of pORF 12 in the invasiveness of CyHV-3, as has been done for IL-10 (Ouyang et al. 2013). It is apparent that more studies on the characterization and immunogenicity of CyHV-3 proteins need to be conducted in order to better understand the virus' interactions with its host's immune system.

Acknowledgements. CCB cells and CyHV-3 virus (KHV-I) were kindly provided by Dr. Sven Bergmann and Dr. Dieter Fichtner; Friedrich Loeffler Institute (FLI), the National Research Center for Animal Health on the Insel Riems, Greifswald, Germany. Funding for this study was provided by the University of Veterinary Medicine Vienna through a Vetmed Success Grant for J.K., and Profile line no. 29111268.

LITERATURE CITED

Aoki T, Hirono I, Kurokawa K, Fukuda H and others (2007) Genome sequences of three koi herpesvirus isolates representing the expanding distribution of an emerging disease threatening koi and common carp worldwide. J Virol 81:5058–5065

Cheung TC, Humphreys IR, Potter KG, Norris PS and others (2005) Evolutionarily divergent herpesviruses modulate T cell activation by targeting the herpesvirus entry mediator cosignaling pathway. Proc Natl Acad Sci USA 102: 13218–13223

Cunnion KM (1999) Tumor necrosis factor receptors encoded by poxviruses. Mol Genet Metab 67:278–282

Gilad O, Yun S, Zagmutt-Vergara FJ, Leutenegger CM, Bercovier H, Hedrick RP (2004) Concentrations of a Koi herpesvirus (KHV) in tissues of experimentally-infected *Cyprinus carpio koi* as assessed by real-time TaqMan PCR. Dis Aquat Org 60:179–187

Grimm T, Schneider S, Naschberger E, Huber J and others (2005) EBV latent membrane protein-1 protects B cells from apoptosis by inhibition of BAX. Blood 105: 3263–3269

Hedrick RP, Gilad O, Yun S, Spangenberg JV and others (2000) A herpesvirus associated with mass mortality of juvenile and adult koi, a strain of common carp. J Aquat Anim Health 12:44–57

Ilouze M, Dishon A, Kotler M (2012) Coordinated and sequential transcription of the cyprinid herpesvirus-3 annotated genes. Virus Res 169:98–106

Lam N, Sugden B (2003) CD40 and its viral mimic, LMP1: similar means to different ends. Cell Signal 15:9–16

Michel B, Leroy B, Stalin Raj V, Lieffrig F and others (2010) The genome of cyprinid herpesvirus 3 encodes 40 proteins incorporated in mature virions. J Gen Virol 91: 452–462

Montgomery RI, Warner MS, Lum BJ, Spear PG (1996) Herpes simplex virus-1 entry into cells mediated by a novel member of the TNF/NGF receptor family. Cell 87: 427–436

Ouyang P, Rakus K, Boutier M, Reschner A and others (2013) The IL-10 homologue encoded by cyprinid herpesvirus 3 is essential neither for viral replication in vitro nor for virulence in vivo. Vet Res 44:53

Panus JF, Smith CA, Ray CA, Smith TD, Patel DD, Pickup DJ (2002) Cowpox virus encodes a fifth member of the tumor necrosis factor receptor family: a soluble, secreted CD30 homologue. Proc Natl Acad Sci USA 99:8348–8353

Perelberg A, Ilouze M, Kotler M, Steinitz M (2008) Antibody response and resistance of *Cyprinus carpio* immunized with cyprinid herpes virus 3 (CyHV-3). Vaccine 26: 3750–3756

Rahman MM, McFadden G (2006) Modulation of tumor necrosis factor by microbial pathogens. PLoS Pathog 2:e4

Ronen A, Perelberg A, Abramowitz J, Hutoran M and others (2003) Efficient vaccine against the virus causing a lethal disease in cultured *Cyprinus carpio*. Vaccine 21: 4677–4684

Saraiva M, Smith P, Fallon PG, Alcami A (2002) Inhibition of type 1 cytokine-mediated inflammation by a soluble CD30 homologue encoded by ectromelia (mousepox) virus. J Exp Med 196:829–839

Seet BT, Johnston JB, Brunetti CR, Barrett JW and others (2003) Poxviruses and immune evasion. Annu Rev Immunol 21:377–423

Sunarto A, Liongue C, McColl KA, Adams MM and others (2012) Koi herpesvirus encodes and expresses a functional interleukin-10. J Virol 86:11512–11520

Upton C, Macen JL, Schreiber M, McFadden G (1991) Myxoma virus expresses a secreted protein with homology to the tumor necrosis factor receptor gene family that contributes to viral virulence. Virology 184:370–382

van Beurden SJ, Forlenza M, Westphal AH, Wiegertjes GF, Haenen OLM, Engelsma MY (2011) The alloherpesviral counterparts of interleukin 10 in European eel and common carp. Fish Shellfish Immunol 31:1211–1217

Vanderplasschen AFC, inventor; Université de Liège, Vanderplasschen AFC, applicant. (2013 Jul 4). A recombinant koi herpesvirus (khv) and vaccine for the prevention of a disease caused by khv. World Intellectual Property Organization WO/2013/098214

Xu X, Nash P, McFadden G (2000) Myxoma virus expresses a TNF receptor homolog with two distinct functions. Virus Genes 21:97–109

Zhou JX, Wang H, Li XW, Zhu X, Lu WL, Zhang DM (2013) Construction of KHV-CJ ORF25 DNA vaccine and immune challenge test. J Fish Dis 37:319–325

Zhou J, Xue J, Wang Q, Zhu X, Li X, Lv W, Zhang D (2014) Vaccination of plasmid DNA encoding ORF81 gene of CJ strains of KHV provides protection to immunized carp. In Vitro Cell Dev Biol Anim 50:489–495

Editorial responsibility: Mark Crane, Geelong, Victoria, Australia

Submitted: April 15, 2014; Accepted: July 23, 2014
Proofs received from author(s): September 9, 2014