

CYPRINID HERPESVIRUS 3: AN INTERESTING VIRUS FOR APPLIED AND FUNDAMENTAL RESEARCH

L'HERPÈSVIRUS CYPRIN 3: UN VIRUS INTÉRESSANT TANT POUR LA RECHERCHE APPLIQUÉE QUE LA RECHERCHE FONDAMENTALE

Par Guillaume FOURNIER¹ and Alain VANDERPLASSCHEN^{*}
(Communication présentée le 3 novembre 2011)

SUMMARY

The koi herpesvirus recently designated cyprinid herpesvirus 3 (CyHV-3) is an emerging agent that causes fatal disease in common and koi carp. Since its emergence in the late 1990s, this highly contagious pathogen has caused severe financial losses in koi and common carp culture industries worldwide. In addition to its economical importance, recent studies demonstrate that CyHV-3 is an interesting topic for fundamental research: for example CyHV-3 encodes the largest genome amongst the order Herpesvirales, and serves as an extreme model for mutagenesis of large DNA viruses. Studies of the CyHV-3 portal of entry in carp suggest that the skin of teleost fish represents an efficient portal of entry for viruses. They also highlight the role of fish epidermal mucus as an innate immune barrier against pathogen entry. In this manuscript, we summarize these advances in CyHV-3 fundamental research.

Keywords: Cyprinid herpesvirus 3, CyHV-3, Koi herpesvirus, KHV, Herpesvirales, Alloherpesviridae.

RÉSUMÉ

L'herpèsvirus de la carpe koi, récemment renommé herpèsvirus cyprin 3 (CyHV-3), est l'agent causal d'une maladie émergente et fatale chez la carpe commune et la carpe koi. Depuis son émergence fin des années 1990, ce pathogène très contagieux a induit des pertes économiques très importantes dans l'élevage industriel des carpes koi et commune. En plus de son intérêt économique, des études récentes démontrent l'intérêt du CyHV-3 comme sujet de recherche fondamentale : par exemple, de par la taille de son génome (le plus grand au sein de l'ordre des Herpesvirales), le CyHV-3 procure un modèle extrême pour l'étude de la mutagenèse des grands virus à ADN. Par ailleurs, l'étude de la porte d'entrée de ce virus chez la carpe suggère que la peau des poissons téléostéens pourrait représenter une porte d'entrée efficace pour certains virus. Le modèle « CyHV-3 - carpe » a également permis de démontrer de manière directe le rôle du mucus épidermique comme composant de l'immunité innée. Dans ce manuscrit, nous résumons les études fondamentales relatives à ces aspects de la recherche sur le CyHV-3.

Mots-clés: herpèsvirus cyprin 3, CyHV-3, herpèsvirus de la carpe koi, KHV, Herpesvirales, Alloherpesviridae.

(1) University of Liège, Belgium

(*) Corresponding author. Mailing address: Immunology-Vaccinology (B43b), Department of Infectious and Parasitic Diseases, Faculty of Veterinary Medicine, University of Liège, B-4000 Liège, Belgium. Phone: 32-4-366 42 64. Fax: 32-4-366 39 08.
E-mail: A.vdplasschen@ulg.ac.be

INTRODUCTION

Common carp (*Cyprinus carpio carpio*) is a freshwater fish and one of the most important species in aquaculture, with a world production of 3.2 million metric tons per year (2009 www.fao.org). In addition to common carp, which is cultivated for human consumption, koi (*Cyprinus carpio koi*), an often-colourful subspecies, is grown for personal pleasure and competitive exhibitions. In the late 1990s, a highly contagious and virulent disease began to cause severe economic losses in these two carp industries worldwide (Michel *et al.* 2010). The observed rapid worldwide spread of the disease has been attributed to the international fish trade and koi shows that occur all around the world (Hedrick *et al.* 2000). The causative agent of the disease was initially called koi herpesvirus (KHV) according to its morphological resemblance to viruses belonging to the order *Herpesvirales* (Hedrick *et al.* 2000). Recently, the virus was renamed cyprinid herpesvirus 3 (CyHV-3; species *Cyprinid herpesvirus 3*, genus *Cyprinivirus*, family *Alloherpesviridae*, order *Herpesvirales*) based on the homology of its genome with previously described cyprinid herpesviruses (Davison *et al.* 2009). The newly designated family *Alloherpesviridae* comprises viruses that infect fish and amphibians. The common ancestor of this family is thought to have diverged from the common ancestor of the *Herpesviridae* family (herpesviruses infecting reptiles, birds and mammals) (*figure 1*) (Waltzek *et al.* 2009).

Because of its economical importance, once isolated, CyHV-3 rapidly became an important subject for applied research. However, recent studies have demonstrated that CyHV-3 is also an interesting fundamental research topic. In the present manuscript, we summarize recent advances made in CyHV-3 fundamental researches on the mutagenesis of its large genome and on the identification of its portal of entry in carp.

MUTAGENESIS OF CYHV-3 USING BAC CLONING AND RECOMBINATION TECHNOLOGIES

The genome of CyHV-3 is a 295 kb linear dsDNA molecule consisting of a large central portion flanked by two 22 kb repeat regions called the left repeat (LR) and the right repeat (RR) (Aoki *et al.* 2007). The genome size is similar to that of CyHV-1 infecting catfish, but is much larger than that of other members of the order *Herpesvirales*, which generally range from 125 to 240 kb. The sequence of CyHV-3 genome revealed a significant number of original DNA sequences with no homology to any other known viral sequences. Moreover, it contains highly divergent DNA sequences encoding polypeptides, which resemble those of several dsDNA viruses, like herpesvirus, poxvirus, iridovirus and other large DNA viruses (Aoki *et al.* 2007).

Since its first isolation, an increasing number of studies have been devoted to CyHV 3. They reported data related to viral gene content, to viral pathogenesis, to the epidemiology of the

infection, to the diagnostic of the infection and to control methods (Michel *et al.* 2010). However, until very recently, no information has been published on the role of individual CyHV-3 genes in the biology of the infection or in the pathogenesis. Two reasons can explain this lacuna. Firstly, the CyHV-3 genome sequence has been published only very recently (Aoki *et al.* 2007). Secondly, prolonged CyHV-3 cultivation *in vitro* leads to spontaneous attenuation of the virus making the production of CyHV-3 recombinants difficult using classical homologous recombination in eukaryotic cells (Ronen *et al.* 2003).

Manipulation of large herpesvirus genomes has been facilitated by the use of bacterial artificial chromosome (BAC) vectors (Wagner *et al.* 2002). These vectors allow the stable maintenance and efficient mutagenesis of the viral genome in *Escherichia coli* (*E. coli*) followed by the reconstitution of progeny virions by transfection of the BAC plasmid into permissive eukaryotic cells. Several herpesviruses have been successfully propagated as infectious BAC clones. The 235 kb genome of Human Cytomegalovirus (HCMV) was before the BAC cloning of CyHV-3 the largest herpesvirus genome which has been BAC cloned (Borst *et al.* 1999).

BAC cloning represents an obvious approach to avoid the problems described above in production of CyHV-3 recombinants. However, the large size of its genome and its abundant repetitive sequence content (Aoki *et al.* 2007) represent two intrinsic features of CyHV-3 that could render its BAC cloning difficult or even impossible. Recently, we described for the first time the cloning of the CyHV-3 genome as a stable and infectious BAC clone (Costes *et al.* 2008). Several recombinant strains were derived from the BAC clone using homologous recombination in eukaryotic cells and prokaryotic recombination technology. The availability of a CyHV-3 BAC is an important advance that will allow the study of viral genes involved in CyHV-3 pathogenesis, as well as the production of attenuated recombinant candidate vaccines.

THE MAJOR PORTAL OF ENTRY OF CYHV-3 IN CARP IS THE SKIN

Several authors postulated that the gills might be the portal of entry of CyHV-3 in carp (Gilad *et al.* 2004; Miyazaki *et al.* 2008). Recently, we challenged this hypothesis using *in vivo* bioluminescent imaging. Taking profit of the BAC cloning of CyHV-3, a recombinant strain expressing luciferase (LUC) as a reporter protein was produced (Costes *et al.* 2009). Using this strain, bioluminescent imaging and an original system to perform percutaneous infection restricted to the posterior part of the fish, the skin covering the fin and body was shown to mediate the entry of CyHV-3 into carp (Costes *et al.* 2009) (*figure 2*). This study, together with an earlier report addressing the portal of entry of a rhabdovirus (infectious hematopoietic necrosis virus) in salmonids (Harmache *et al.* 2006), suggests that the skin of teleost fish represents an efficient portal

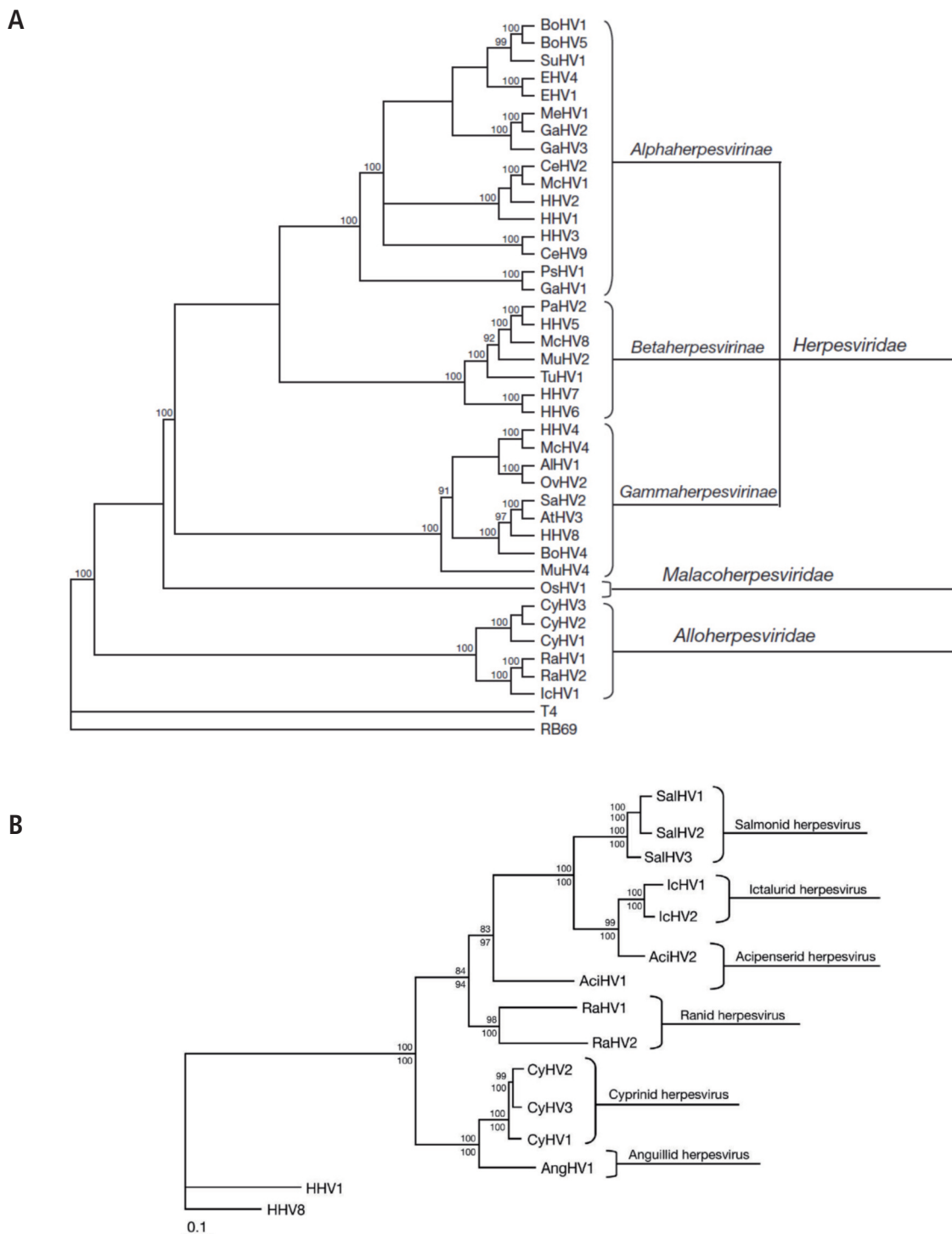


Figure 1: (A) Cladogram depicting relationships among viruses in the order Herpesvirales, based on the conserved regions of the terminase gene. The Bayesian maximum likelihood tree was rooted using bacteriophages T4 and RB69. Numbers at each node represent the posterior probabilities (values > 90 shown) of the Bayesian analysis. (B) Phylogenetic tree depicting the evolution of fish and amphibian herpesviruses, based on sequences of the DNA polymerase and terminase genes. The maximum likelihood tree was rooted with two mammalian herpesviruses (HHV-1 and HHV-8). Maximum likelihood values (>80 are shown) and Bayesian values (>90 are shown) are indicated above and below each node, respectively. Branch lengths are based on the number of inferred substitutions, as indicated by the scale bar. AIHV-1: alcelaphine herpesvirus 1; AtHV-3: ateline herpesvirus 3; BoHV-1, -4, -5: bovine herpesvirus 1, 4, 5; CeHV-2, -9: cercopithecine herpesvirus 2, 9; CyHV-1, -2: cyprinid herpesvirus 1, 2; EHV-1, -4: equid herpesvirus 1, 4; GaHV-1, -2, -3: gallid herpesvirus 1, 2, 3; HHV-1, -2, -3, -4, -5, -6, -7, -8: human herpesvirus 1, 2, 3, 4, 5, 6, 7, 8; IcHV-1: ictalurid herpesvirus 1; McHV-1, -4, -8: macacine herpesvirus 1, 4, 8; MeHV-1: meleagrid herpesvirus 1; MuHV-2, -4: murid herpesvirus 2, 4; OsHV-1: ostreid herpesvirus 1; OvHV-2: ovine herpesvirus 2; PaHV-1: panine herpesvirus 1; PsHV-1: psittacid herpesvirus 1; RaHV-1, -2: ranid herpesvirus 1, 2; SaHV-2: saimirine herpesvirus 2; SuHV-1: suid herpesvirus 1; TuHV-1: tupaiid herpesvirus 1. Reproduced with permission from Waltzek et al. (2009) (Waltzek et al. 2009).

COMMUNICATION

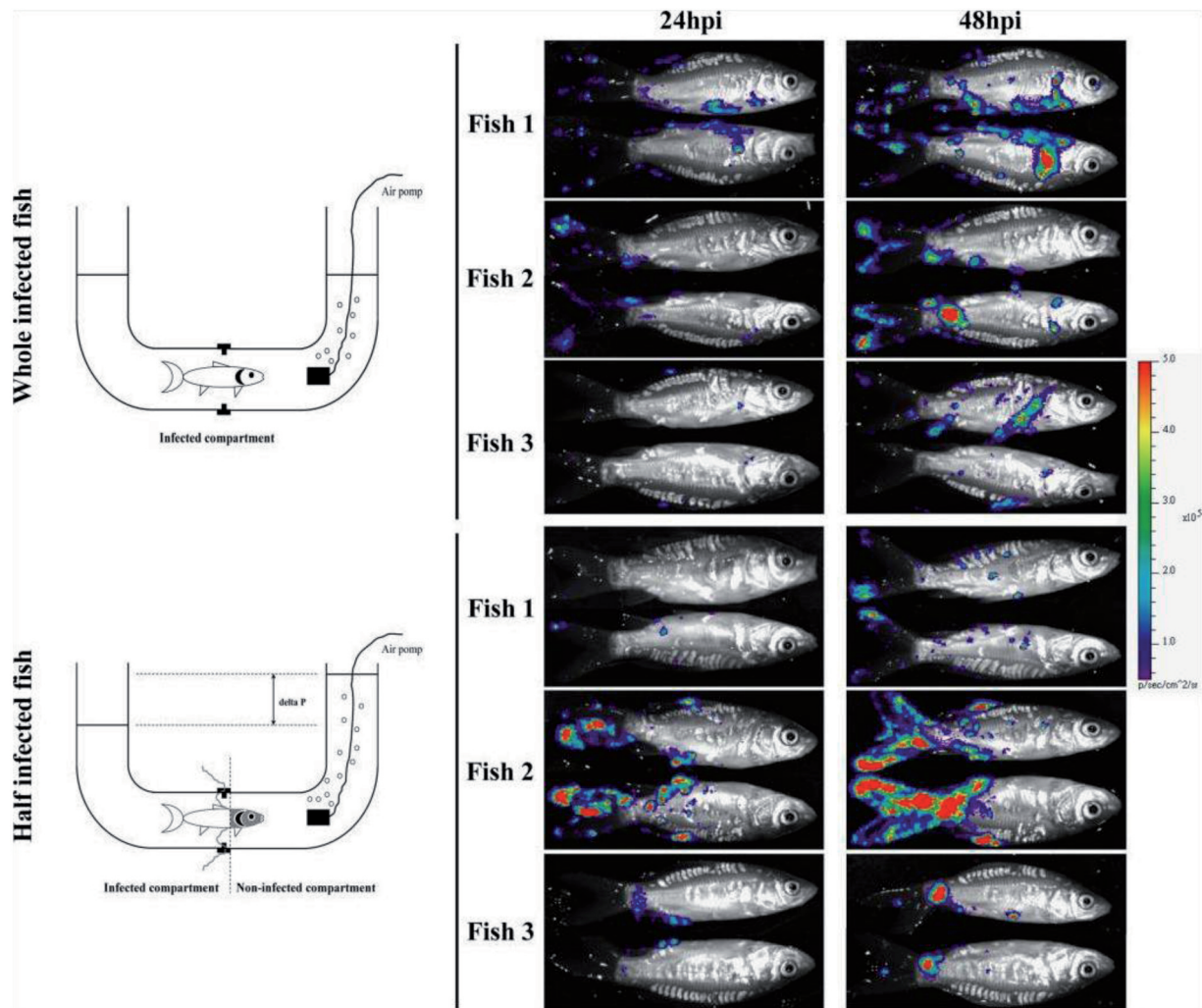


Figure 2: The skin of carp is acting as a portal of entry for CyHV-3. A schematic representation of the system used to restrict viral inoculation to the fish skin is shown on the left. The lower drawing presents the conditions under which fish ($n = 6$) were inoculated by restricted contact of the virus with the skin located posterior to the anterior part of the dorsal fin. The upper drawing presents control conditions under which fish ($n = 6$) were inoculated in the system but without the latex diaphragm dividing the fish body into two isolated parts, allowing the virus to reach the entire fish body. The fish were infected by bathing them for 24 hours in water containing a recombinant CyHV-3 strain able to emit bioluminescence. All fish were analyzed 24 hours post-infection (hpi) by bioluminescence imaging. After an additional incubation period of 24 hours in individual tanks containing fresh water, they were reanalyzed by bioluminescence imaging (48 hpi). Three representative fish are shown. The images are presented with standardized minimum and maximum threshold values for photon flux. Reproduced with permission from Costes et al. (2009)(Costes et al. 2009).

of entry for viruses. It is important to note that the epidermis of the skin of teleost fish is a stratified squamous epithelium. Unlike its mammalian counterpart, it is living and capable of mitotic division at all levels, even at the outermost squamous layer. The scales are dermis structures covered by the epidermis.

EPIDERMAL MUCUS INHIBITS CYHV-3 BINDING TO EPIDERMAL CELLS

Fish skin is a complex limiting structure providing mechanical, chemical and immune protection against injury and pathogenic microorganisms (Fontenot & Neiffer, 2004). Its mucus layer

confers an innate immune protection against pathogen entry. Two types of mechanisms explain the protection conferred by mucus. Firstly, the mucus forms an efficient mechanical barrier that is constantly moving downstream along the fish and off of trailing edges. Like the muco-ciliary escalator of the respiratory tract of pulmonate animals, fish mucus reduces pathogen access to epithelial cells. Secondly, the mucus contains numerous proteins such as for example immunoglobulins, enzymes and lytic agents able to neutralise microorganisms (Palaksha et al. 2008; Subramanian et al. 2008). It is generally accepted that chemical and physical (for example, ectoparasite infestations, rude handling or injuries) stresses that affect skin mucus increase fish susceptibility to infection by pathogens (Roberts & Ellis, 2001). However, despite the abundance of studies on

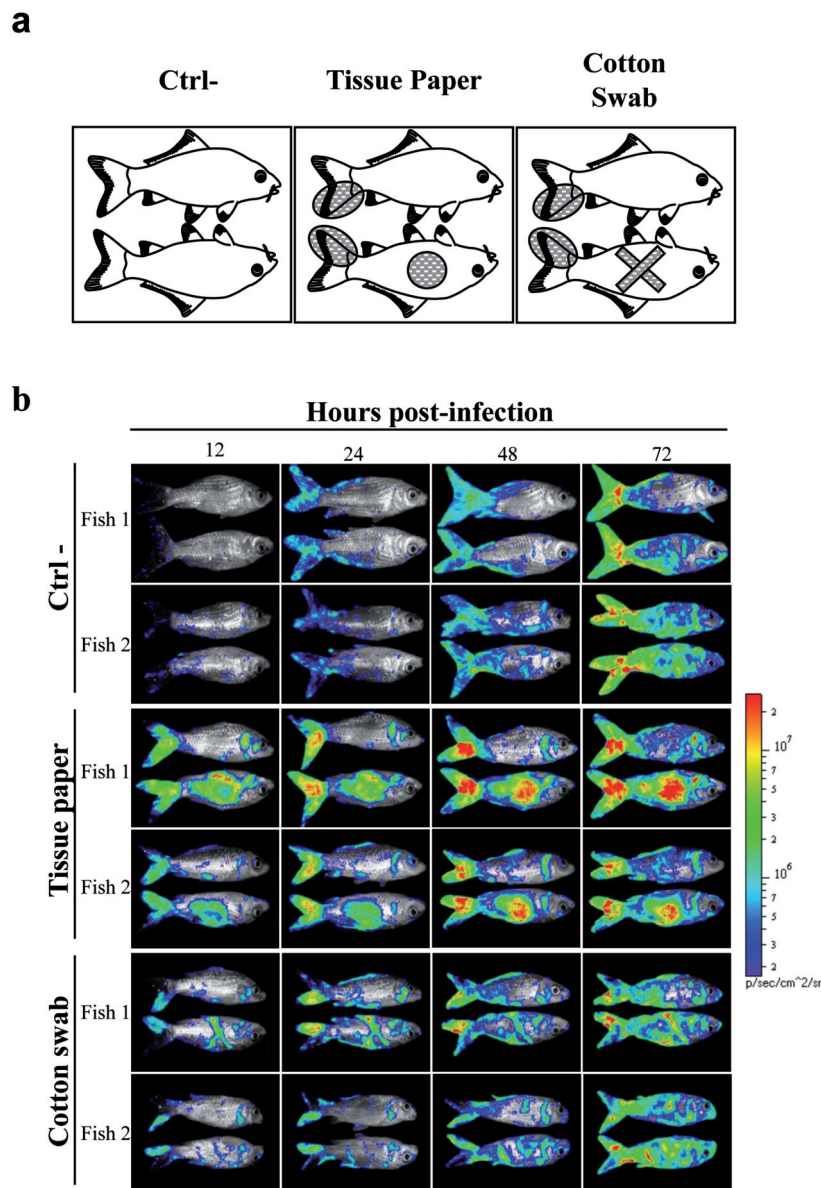


Figure 3: Epidermal mucus removal and epidermis erosion enhance CyHV-3 entry in carp as revealed by bioluminescence imaging. (a) Schematic diagram representing the area (in grey) of the fish skin to which the indicated physical treatments (rubbing with a soft tissue paper or with a cotton swab) were applied just before viral inoculation. Each panel represents the same fish lying on its left and right side. (b) Each physical treatment was applied to a group of 7 fish. Immediately after skin treatment, fish were inoculated by immersion in water containing a recombinant CyHV-3 strain expressing luciferase. The fish were analyzed by bioluminescence imaging at the indicated time post-inoculation. Each fish was analyzed lying on its right and its left side. Two representative fish are shown per group. The images collected over the course of the experiment are presented with standardized minimum and maximum threshold values for photon flux. Reproduced with permission from Raj et al. (2011) (Stalin Raj et al. 2011).

fish skin immunity and skin bacterial infection, there are few direct *in vivo* evidence on the role of skin mucus as a first line of innate immune protection against bacterial infection, and none against viral infection (Fouz *et al.* 1990; Kanno *et al.* 1989; Takahashi *et al.* 1986). Recently, we took advantage of the “CyHV-3 carp” model of infection to investigate by using bioluminescence imaging the effect of mucus removal and progressive epidermal lesions on CyHV-3 entry in carp. Physical treatments inducing removal of the mucus up to complete erosion of the epidermis were applied on a defined area of carp skin just before inoculation by immersion in infectious water (*figure 3*). CyHV-3 entry in carp was drastically enhanced on the area of the skin where the mucus was removed with or without associated epidermal lesion. To investigate whether skin mucus inhibits CyHV-3 binding to epidermal cells, tail fins with an intact mucus layer or without mucus were inoculated *ex vivo*.

While electron microscopy examination revealed numerous viral particles bound on the fins inoculated after mucus removal, no particle could be detected after infection of mucus-covered fins. Finally, anti-CyHV-3 neutralising activity of mucus extract was tested *in vitro*. Incubation of CyHV-3 with mucus extract reduced its infectivity in a dose dependent manner. All together, these data highlights the role of fish skin mucus as an innate immune protection against viral epidermal entry.

CONCLUSION

Because CyHV-3 causes several financial and economical losses in both koi and common carp culture industries worldwide, it is an important topic for applied science. However, several aspects of CyHV-3 make it an interesting and original fundamental science subject.

ACKNOWLEDGMENTS

Guillaume Fournier is a Research Fellow of the Belgian "Fonds pour la formation à la Recherche dans l'Industrie et dans l'Agriculture". The CyHV-3 program of the laboratory of Immunology-Vaccinology is supported by grants of the University of Liège (Crédit d'Impulsion) and an FRFC grant of the FNRS (2.4622.10).

REFERENCES

- Aoki, T., Hirono, I., Kurokawa, K., Fukuda, H., Nahary, R., Eldar, A., Davison, A. J., Waltzek, T. B., Bercovier, H., Hedrick, R. P. 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.
- Borst, E. M., Hahn, G., Koszinowski, U. H., Messerle, M. 1999. Cloning of the human cytomegalovirus (HCMV) genome as an infectious bacterial artificial chromosome in *Escherichia coli*: a new approach for construction of HCMV mutants. *J Virol.* 73: 8320-8329.
- Costes, B., Fournier, G., Michel, B., Delforge, C., Raj, V. S., Dewals, B., Gillet, L., Drion, P., Body, A., Schynts, F., Lieffrig, F., Vanderplasschen, A. 2008. Cloning of the koi herpesvirus genome as an infectious bacterial artificial chromosome demonstrates that disruption of the thymidine kinase locus induces partial attenuation in *Cyprinus carpio* koi. *J Virol.* 82: 4955-4964.
- Costes, B., Raj, V. S., Michel, B., Fournier, G., Thirion, M., Gillet, L., Mast, J., Lieffrig, F., Bremont, M., Vanderplasschen, A. 2009. The major portal of entry of koi herpesvirus in *Cyprinus carpio* is the skin. *J Virol.* 83: 2819-2830.
- Davison, A. J., Eberle, R., Ehlers, B., Hayward, G. S., McGeoch, D. J., Minson, A. C., Pellett, P. E., Roizman, B., Studdert, M. J., Thiry, E. 2009. The order Herpesvirales. *Arch Virol.* 154: 171-177.
- Fontenot, D. K. & Neiffer, D. L. 2004. Wound management in teleost fish: biology of the healing process, evaluation, and treatment. *Vet Clin North Am Exot Anim Pract.* 7: 57-86.
- Fouz, B., Devesa, S., Gravningen, K., Barja, J. L., Toranzo, A. E. 1990. Antibacterial action of the mucus of turbot. In *Bulletin of European Association of Fish Pathologists*, pp. 56-59.
- Gilad, O., Yun, S., Zagmutt-Vergara, F. J., Leutenegger, C. M., Bercovier, H., Hedrick, R. P. 2004. Concentrations of a Koi herpesvirus (KHV) in tissues of experimentally infected *Cyprinus carpio* koi as assessed by real-time TaqMan PCR. *Dis Aquat Organ.* 60: 179-187.
- Harmache, A., LeBerre, M., Droineau, S., Giovannini, M., Bremont, M. 2006. Bioluminescence imaging of live infected salmonids reveals that the fin bases are the major portal of entry for Novirhabdovirus. *J Virol.* 80: 3655-3659.
- Hedrick, R. P., Gilad, O., Yun, S., Spangenberg, J., Marty, R., Nordhausen, M., Kebus, M., Bercovier, H., Eldar, A. 2000. A herpesvirus associated with mass mortality of juvenile and adult koi, a strain of common carp. *J Aquat Anim Health* 12: 44-55.
- Kanno, T., Nakai, T., Muroga, K. 1989. Mode of transmission of vibriosis among ayu *Plecoglossus altivelis*. *Journal of Aquatic Animal Health* 1: 2-6.
- Michel, B., Leroy, B., Stalin Raj, V., Lieffrig, F., Mast, J., Wattiez, R., Vanderplasschen, A. F., Costes, B. 2010. The genome of cyprinid herpesvirus 3 encodes 40 proteins incorporated in mature virions. *J Gen Virol.* 91: 452-462.
- Miyazaki, T., Kuzuya, Y., Yasumoto, S., Yasuda, M., Kobayashi, T. 2008. Histopathological and ultrastructural features of Koi herpesvirus (KHV)-infected carp *Cyprinus carpio*, and the morphology and morphogenesis of KHV. *Dis Aquat Organ* 80:1-11.
- Palaksha, K. J., Shin, G. W., Kim, Y. R., Jung, T. S. 2008. Evaluation of non-specific immune components from the skin mucus of olive flounder (*Paralichthys olivaceus*). *Fish Shellfish Immunol.* 24: 479-488.
- Roberts, R. J. & Ellis, A. E. 2001. The anatomy and physiology of teleosts. In *Fish Pathology*, Third Edition, pp. 12-54. London, United Kingdom: W. B. Saunders.
- Efficient vaccine against the virus causing a lethal disease in cultured *Cyprinus Ronen*, A., Perelberg, A., Abramowitz, J., Hutoran, M., Tinman, S., Bejerano, I., Steinitz, M., Kotler, M. 2003. *carpio*. *Vaccine* 21: 4677-4684.
- Stalin Raj, V., Fournier, G., Rakus, K., Ronsmans, M., Ouyang, P., Michel, B., Delforges, C., Costes, B., Farnir, F., Leroy, B., Wattiez, R., Melard, C., Mast, J., Lieffrig, F., Vanderplasschen, A. 2011. Skin mucus of *Cyprinus carpio* inhibits cyprinid herpesvirus 3 binding to epidermal cells. *Vet Res.* 42: 92.
- Subramanian, S., Ross, N. W., MacKinnon, S. L. 2008. Comparison of antimicrobial activity in the epidermal mucus extracts of fish. *Comp Biochem Physiol B Biochem Mol Biol.* 150: 85-92.
- Takahashi, Y., Itami, T., Konegawa, K. 1986. Enzymatic-properties of bacteriolytic substances in skin mucus and intestine of carp. *Fish Pathology* 21: 187-191.
- Wagner, M., Ruzsics, Z., Koszinowski, U. H. 2002. Herpesvirus genetics has come of age. *Trends Microbiol.* 10: 318-324.
- Waltzek, T. B., Kelley, G. O., Alfaro, M. E., Kurobe, T., Davison, A. J., Hedrick, R. P. 2009. Phylogenetic relationships in the family Alloherpesviridae. *Dis Aquat Organ.* 84: 179-194.