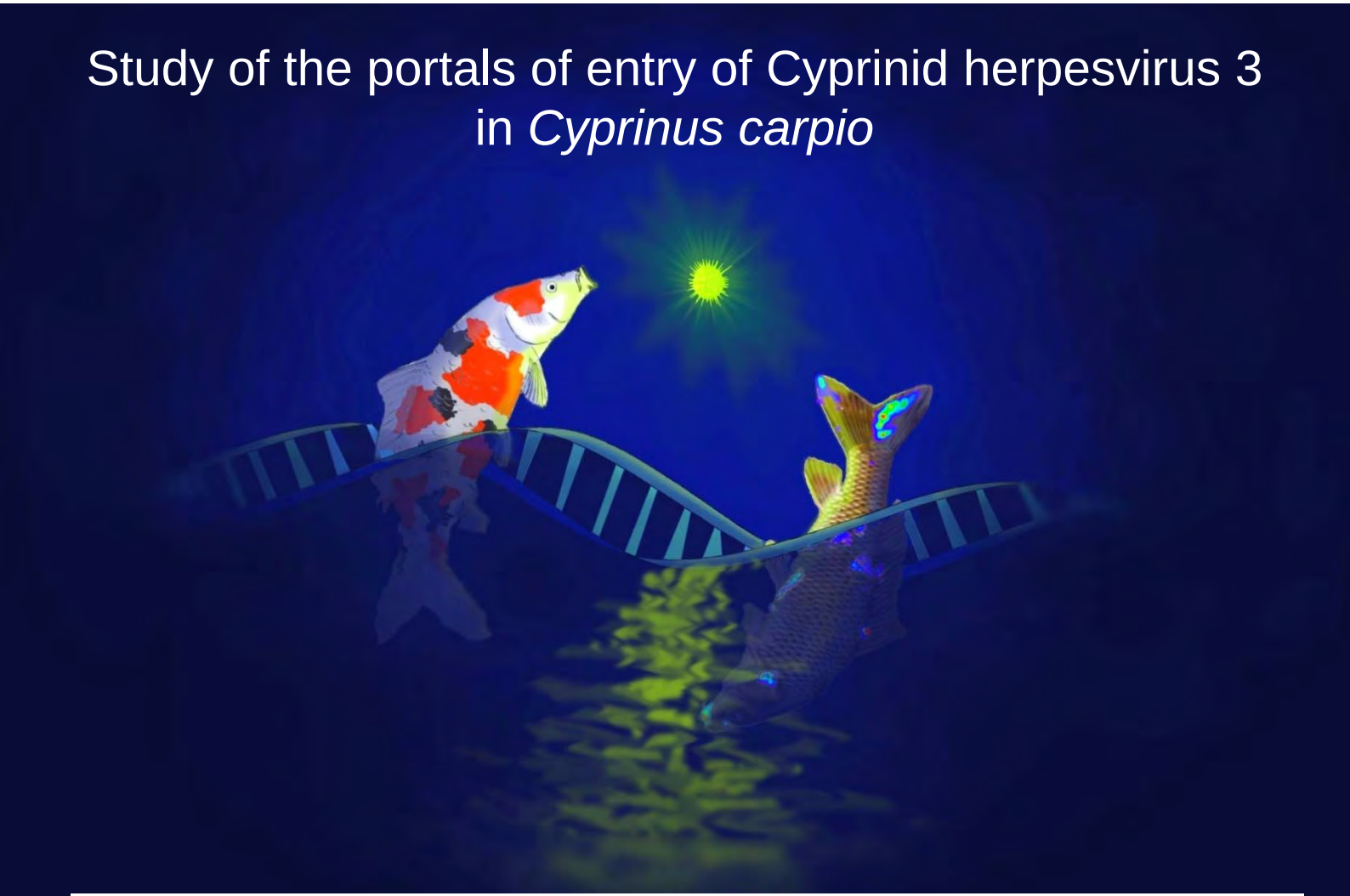
Académie Universitaire Wallonie - Europe
Université de Liège
Faculté de Médecine Vétérinaire
Département des Maladies Infectieuses et Parasitaires
Service d'Immunologie et de Vaccinologie

Etude des portes d'entrée de l'Herpèsvirus cyprin 3 chez *Cyprinus carpio*

Study of the portals of entry of Cyprinid herpesvirus 3 in *Cyprinus carpio*



Guillaume FOURNIER

Thèse présentée en vue de l'obtention du grade
de Docteur en Sciences Vétérinaires

Année académique 2011-2012

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Promoteur : Prof. Alain Vanderplasschen

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« La science progresse en indiquant l'immensité de l'ignoré. »

Louis Pauwels

Remerciements

Liège, le 15 février 2012

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List of abbreviations

α gene :	immediate early gene
Aa :	amino acid
AbHV-1	abalone herpesvirus 1
AcHV	atlantic cod herpesvirus
AciHV-1 :	acipenserid herpesvirus 1
AciHV-2 :	acipenserid herpesvirus 2
ADN :	acide désoxyribonucléique
AlHV-1 :	alcelaphine herpesvirus 1
Amp :	ampicillin
AngHV-1 :	anguillid herpesvirus 1
Ap :	anterior pharynx
AtHV-3 :	ateline herpesvirus 3
ATPase	adenosine triphosphatase
Au :	goldfish fin cell
Aw :	abdominal wall
β gene :	early gene
Ba :	branchial arch
BAC :	bacterial artificial chromosome
Bc :	body cavity (2nd study)
Bc :	buccal cavity (4th study)
BoHV-1 :	bovine herpesvirus 1
BoHV-2 :	bovine herpesvirus 2
BoHV-4 :	bovine herpesvirus 4
BoHV-5 :	bovine herpesvirus 5
Bp	base pair
Ca	caudal fin
CaF-2 :	carp fin cell (=CFC)
CCB :	*Cyprinus carpio* brain cell
CCG :	*Cyprinus carpio* gill cell
CCO :	channel catfish ovary cell
CCV :	channel catfish virus (=IcHV-1)
cDNA :	complementary DNA
CEFRA :	centre de formation et de recherche en aquaculture
CeHV-2 :	cercopithecine herpesvirus 2
CeHV-9 :	cercopithecine herpesvirus 9
CER :	centre d'économie rurale
CFC :	carp fin cell
CHSE-214 :	chinook salmon embryo cell
CHV :	carp herpesvirus (=CyHV-1)
CMC :	carboxymethylcellulose
CME :	clarified mucus extract
CNGV :	carp interstitial nephritis and gill necrosis virus
Cp :	chewing pad
CPE :	cytopathic effect
CS :	clinical signs
Cy :	cytosol
CyHV-1 :	cyprinid herpesvirus 1 (CHV)
CyHV-2 :	cyprinid herpesvirus 2
CyHV-3 :	cyprinid herpesvirus 3
Δ :	deleted
Da :	dalton
DIVA :	differentiation of infected and vaccinated animal

DMEM :	dulbecco’s modified essential medium
dpi :	days post-infection
dpt :	days post-transfection
dsDNA :	double-stranded DNA
Do :	dorsal fin
E :	esophagus
E gene :	early gene
EBV :	epstein-barr virus (=HHV-4)
EEDV :	epizootic epitheliotrope disease virus (=SalHV-3)
EGFP :	enhanced green fluorescent protein
EHV-1 :	equid herpesvirus 1
EHV-4 :	equid herpesvirus 4
ELISA :	enzyme-linked immunosorbent assay
EM :	electron microscopy
emPAI :	exponentially modified protein abundance index
EPC :	epitheliuma papulosum cyprinid cell
ER :	external repeat
ER :	endoplasmic reticulum
FAO :	food and agriculture organisation
FCS :	fetal calf serum
FHM :	fathead minnow cell
FL strain :	strain isolated by François Lieffrig
FNRS :	fonds national de la recherche scientifique
FRFC :	fonds de la recherche fondamentale collective
FRIA :	fonds pour la formation à la recherche dans l’industrie et l’agriculture
FV-4 :	frog virus 4 (=RaHV-2)
Fw :	forward
*gal*K :	galactokinase
GaHV-1 :	gallid herpesvirus 1
GaHV-2 :	gallid herpesvirus 2
GaHV-3 :	gallid herpesvirus 3
GAM :	goat anti-mouse
γ gene :	late gene
gB, gC, gD, gG	glycoproteins B, C, D, G
GC :	guanidine chloride
GFHNV :	goldfish hematopoietic necrosis virus
Gi :	gills
GPCR :	G-protein coupled receptor
Gr :	gill raker
HCMV :	human cytomegalovirus (=HHV-5)
HHV-1 :	human herpesvirus 1
HHV-2 :	human herpesvirus 2
HHV-3 :	human herpesvirus 3
HHV-4 :	human herpesvirus 4
HHV-5 :	human herpesvirus 5
HHV-6 :	human herpesvirus 6
HHV-7 :	human herpesvirus 7
HHV-8 :	human herpesvirus 8
hpi :	hours post-infection
HVA :	herpesvirus angillae (=AngHV-1)
I :	intestine
ICCD :	intensified charge coupled device camera
IcHV-1 :	ictalurid herpesvirus 1
IcHV-2 :	ictalurid herpesvirus 2

IcmHV :	Ictalurus melas herpesvirus (=IcHV-2)
ICTV :	international committee on taxonomy of viruses
IE gene :	immediate early gene
IFN :	interferon
Ig :	immunoglobulin
IL-10 :	interleukin 10
INRA :	Institut national de la recherche agronomique
IP :	intraperitoneal
IR (L, S) :	internal repeat (large, small)
IVIS :	in vivo imaging system
Kana :	kanamycin
Kb (p) :	kilobase (pairs)
KCF-1 :	koi caudal fin-1 cell
kDa :	kilo Dalton
KFC :	koi fin cell
KF-1 :	koi fin cell
KHV (I, J, U)	koi herpesvirus (Israel, Japan, USA)
KSHV :	kaposi's sarcoma-associated herpesvirus
L gene :	late gene
LAMP :	loop-mediated isothermal amplification
LAT :	latency transcript
Lba :	left branchial arch
Lo :	left operculum
Lp :	lingual process
LR :	left region
LTHV :	lucké tumor herpesvirus (=RaHV-1)
LTR :	left terminal repeat
LUC :	luciferase gene
MC :	mortality in carp during cohabitation
MCMV :	murine cytomegalovirus
MEM :	minimum essential medium
MOI :	multiplicity of infection
MS :	mass spectrometry
MuHV-1 :	murine herpesvirus 1
MuHV-2 :	murine herpesvirus 2
MuHV-4 :	murine herpesvirus 4
MYA :	million years ago
OGP :	N-octyl β-D-glucopyranoside
ORF :	open reading frame
OsHV-1 :	ostreid herpesvirus 1
PBS :	phosphate-buffered saline
p.f.u. :	plaque forming unit
pi :	post-infection
pORF :	protein coded by the open reading frame
RaHV-1 :	ranid herpesvirus 1
RaHV-2 :	ranid herpesvirus 2
rpm :	rotations per minute
RR :	right region
RT :	reverse transcription
RTG-2 :	rainbow trout gonad cell
RTR :	right terminal repeat
RTp :	room temperature
SaHV-1 :	saimiriine herpesvirus 1
SalHV-1 :	salmonid herpesvirus 1

SalHV-2 :	salmonid herpesvirus 2
SalHV-3 :	salmonid herpesvirus 3
SDS :	sodium dodecyl sulfate
TK :	thymidine kinase
TmpK :	thymidylate kinase
TNFR :	tumor necrosis factor receptor
Tol/FL :	silver carp fin cell
TX :	triton X-100
U_L	long unique sequence
U_S	short unique sequence
v/v :	volume/volume
w/v :	weight /volume

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Preamble

The common carp, cultivated for human consumption, is one of the most important freshwater species in aquaculture with a world production of 3.2 million metric tons per year (estimation from the FAO for 2009). While the common carp is a cheap source of animal proteins, its coloured subspecies koi is grown for personal pleasure and competitive exhibitions and can be sold for thousands of Euros per animal. In the 1990s, a highly contagious and fatal disease started to cause severe economic losses in these two carp industries worldwide. The causative agent of the disease was initially called koi herpesvirus (KHV). It has been recently renamed cyprinid herpesvirus 3 (CyHV-3) and classified in the *Alloherpesviridae* family of the *Herpesvirales* order.

The structure of this manuscript is as follows. It starts with an introduction devoted to the *Herpesvirales* order and to CyHV-3. The objectives of the thesis are then briefly exposed followed by the result section organised into four chapters corresponding to four published or in press publications. In the last section of this manuscript “Discussion et perspectives”, the main results are discussed and potential perspectives are presented. According to the rules applied to PhD theses in Veterinary sciences, this last section is in French.

Introduction

Introduction

1st chapter:

The order *Herpesvirales*

Introduction

At the border of living and non-living, viruses are submicroscopic biological agents consisting of nucleic acid and protein shell which may be multilayered. They can't replicate in the extracellular medium and reproduce as obligate intracellular parasites in the host organism. Since the description of the tobacco mosaic virus at the end of the 19th century, thousands of viruses were described in every ecosystem. They infect bacteria, plants and animals (Dimmock *et al.*, 2007). The International Committee on Taxonomy of viruses (ICTV) developed universal systems for classifying viruses. In the current ICTV taxonomy, six orders have been established, the *Caudovirales*, the *Herpesvirales*, the *Mononegavirales*, the *Nidovirales*, the *Picornavirales* and the *Tymovirales* (King *et al.*, 2012).

Members of the order *Herpesvirales* are enveloped viruses with a linear double-stranded DNA (dsDNA) genome. They share an identical structure consisting in a densely packed DNA core in an icosahedral capsid. The capsid is embedded in a complex proteinaceous layer called the tegument. A lipid envelope containing numerous viral glycoproteins forms the outermost structure of the viral particle (McGeoch *et al.*, 2008). Most of the members of the order *Herpesvirales* have been shown to realize two distinct phases in their life cycle: lytic replication characterized by a transcription program where immediate-early (IE), early (E), and late (L) genes are expressed successively; and latency, consisting of the maintenance of the viral genome as a non-integrated episome and the expression of a limited number of viral genes and microRNAs (Roizman & Pellet, 2007). Upon reactivation, latency reverses to a lytic replication.

The origin of the order *Herpesvirales* has been estimated at several hundred million years ago (Davison, 2002). So far, approximately 135 members have been isolated from oyster, fish, amphibian, reptile, bird and mammal species, including human(Davison *et al.*, 2009). Herpesviruses have mainly co-evolved with their host and in most cases are well adapted to them. This adaption is demonstrate that the ability of most herpesviruses to persist in the host species without inducing lethal infection.

The order *Herpesvirales* contains three families, the *Herpesviridae* (infecting reptiles, birds and mammals), the *Alloherpesviridae* (infecting fish and amphibians) and the *Malacoherpesviridae* families. Below, we will first provide a general and brief description of the structure, the genome, the common biological properties and the replication cycle of the members of the order *Herpesvirales*. Next, we will discuss briefly the biological specificities of the three families.

Virus structure

Every virus classified in the order *Herpesvirales* possesses an identical structure (Ackermann, 2004). Their genome is protected by an icosahedral capsid with diameter of approximately 100 nm. The capsid is composed of 162 capsomers (150 hexons and 12 pentons) (Figure 1). This nucleocapsid is surrounded by an amorphous layer of proteins termed tegument, which contains proteins mainly

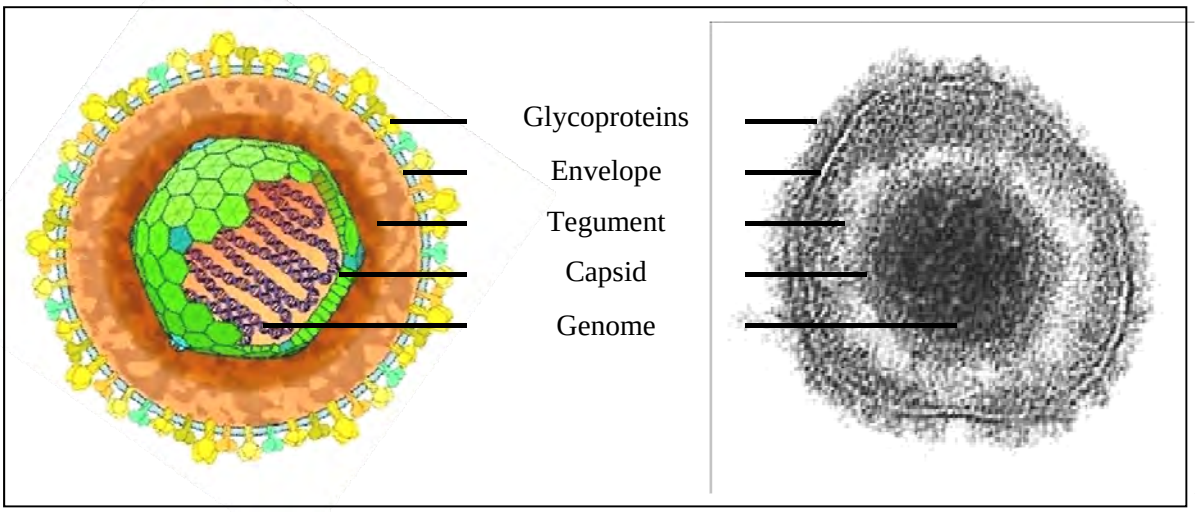

Figure 1. Herpesvirus structure. Schematic representation and electron microscopy picture of a viral particle.

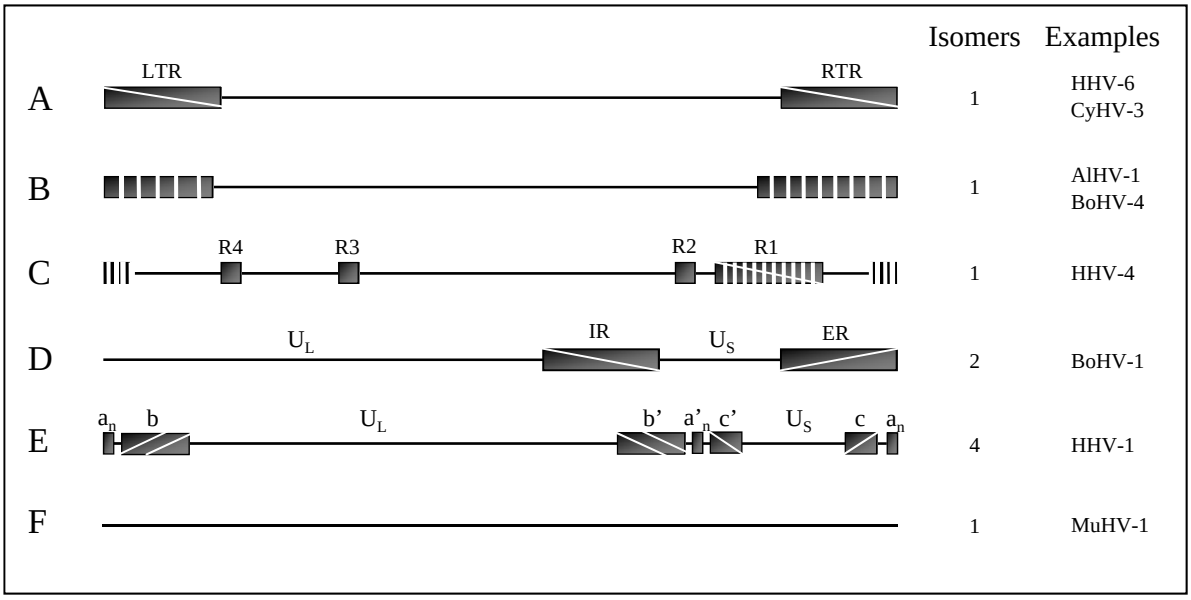

Figure 2. The order *Herpesvirales* regroups 6 classes of genome. Horizontal lines represent unique regions. Rectangles represent left and right terminal repeats (LTR and RTR, respectively) for A group; internal repeats R1 to R4 of the C group and internal and external repeats (IR and ER) for the D group. Terminal repeats of the E group are constituted by two parts. One is composed by *n* copies of the *a* sequence near the larger *b* sequence. The other one is composed by the repeated *a* sequences followed by a *c* sequence. Terminal sequences a_nb and ca_n are inversed and are separated by long (U_L) and short (U_S) unique sequences. In the B group, terminal sequences are repeated a variable number of times at each extremity. In the D group, U_S can be inverted compared to the U_L giving two different isomers. In the E group, U_L and U_S regions can also be inverted generating four different isomers. Terminal repeats were not described in the F group. *Human herpesvirus 1* (HHV-1), *4* (HHV-4) and *6* (HHV-6), *Alcelaphin herpesvirus 1* (AlHV-1), *Bovin herpesvirus 1* (BoHV-1) and *4* (BoHV-4), *Murin herpesvirus 1* (MuHV-1) and *Cyprinid herpesvirus 3* (CyHV-3) were chosen as examples (adapted from Roizman *et al.*, 2007).

involved in gene expression regulation. Finally, a lipid envelope bearing viral glycoproteins is covering the elements listed above to form a spherical particle of approximately 150 to 300 nm in diameter (Figure 1).

Genomic features

Herpesvirus genome is a long dsDNA molecule, linear in the capsid, but circular once it penetrates the nucleus of the host cell (Roizman & Pellet, 2007). Depending of the virus species, the guanine plus cytosine (G+C) percentage varies from 31 to 75% while the genome length varies from 120 to 295 kilobase pairs (kb) (Aoki *et al.*, 2007; Roizman & Pellet, 2007). The genome contains variable internal and terminal repeated sequences. Based on the arrangement of these sequences, herpesvirus genomes have been classified in 6 different groups (Figure 2) (Roizman & Pellet, 2007). All herpesvirus genomes contain at their termini conserved signals for packaging of the DNA into capsids (Roizman & Pellet, 2007).

Common biological properties

Herpesviruses seem to share 4 important biological properties (Ackermann, 2004). Firstly, they encode their own enzymes for nucleic acid synthesis. Secondly, both viral DNA replication and assembly of the nucleocapsid take place in the nucleus of the infected cell. Thirdly, production of progeny viral particles leads to the lysis of the infected cell. Finally, even if this is not firmly demonstrated for the *Alloherpesviridae* and *Malacoherpesviridae* families, all studied herpesviruses are able to establish a latent infection in their natural host.

Biological cycle

Herpesviruses have two distinct phases in their life cycle: lytic and latent infection. The characterization of these two phases is based on the study of the members of the *Herpesviridae* family.

Lytic infection

The herpesvirus multiplication cycle is illustrated in Figure 3. It starts with the virion attachment on the host cell surface mediated by the interaction of viral glycoproteins with their cellular receptors. For example, human herpesvirus 1 (HHV-1) first binds to the cells through interaction of glycoproteins gC and gB with some cellular proteoglycans such as heparan sulfate (Spear, 2004). A stronger attachment is then mediated by the interaction of gD to its specific cellular receptor (Spear, 2004).

After fusion of the viral envelope with the plasma membrane (or eventually endocytic vesicles), the nucleocapsid and tegument proteins are delivered in the cytoplasm where microtubules bring the nucleocapsid surrounded by the tegument close to the nucleus (Figure 3)(Sodeik *et al.*,

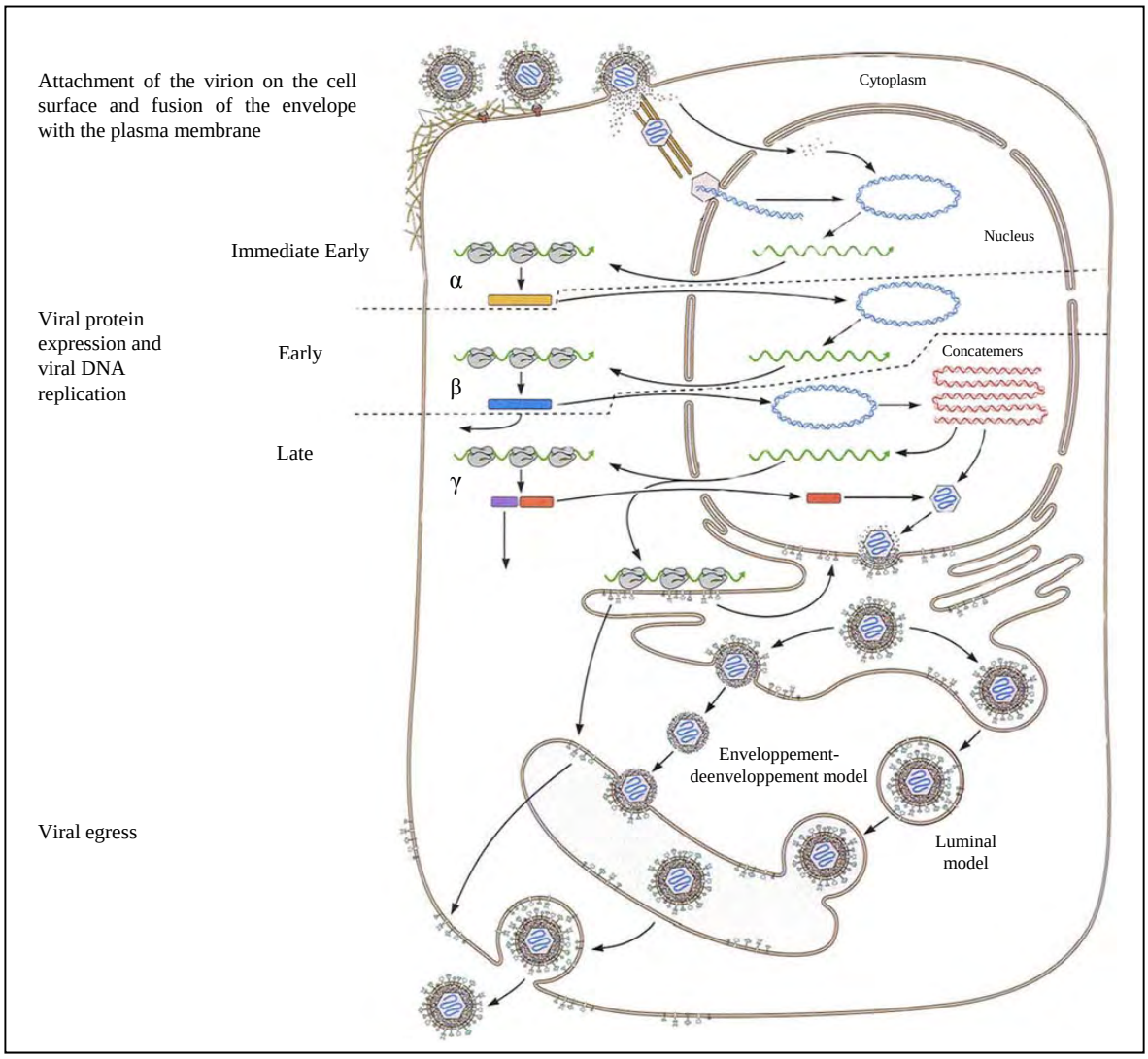

Figure 3. Schematic representation of the lytic infection of herpesviruses (adapted from Flint *et al*., 2000).

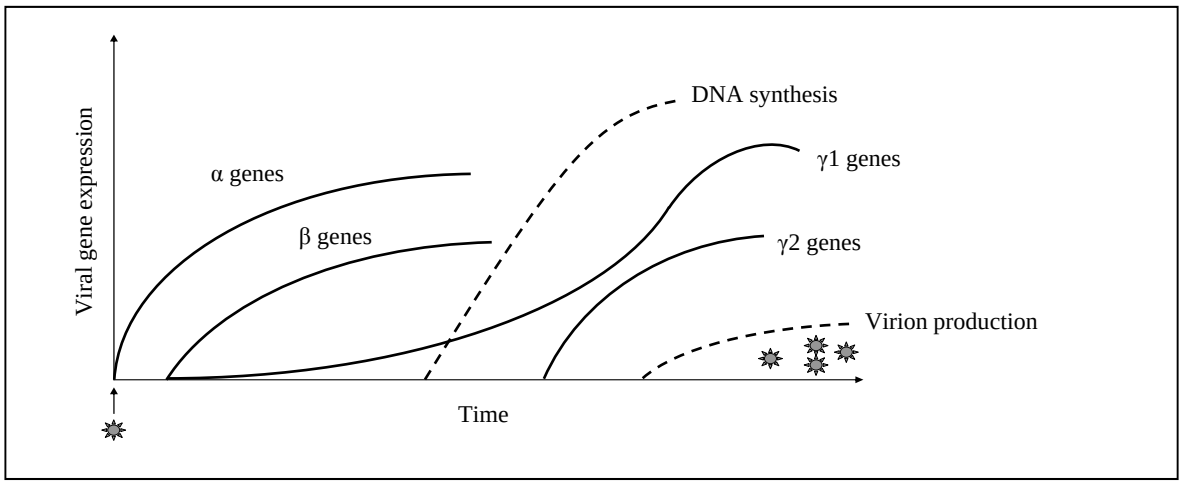

Figure 4. Kinetic of herpesvirus gene expression illustrated in a relative manner.

1997). The genome is then released and enters the nucleus through a pore of the nuclear membrane. As soon as the genome enters in the nucleus, the viral DNA circularizes prior to viral protein synthesis (Garber *et al.*, 1993). This circularization is realized by direct ligation of single unpaired 3' end nucleotides present at both ends of the genome (Davison, 1984). Tegument proteins migrate with genome into the nucleus where they regulate virus and cellular gene expression.

Herpesvirus gene expression is characterized by a transcription program where immediate-early (IE or α), early (E or β), and late (L or γ) genes are expressed successively (Figures 3 and 4) (Honess & Roizman, 1974; 1975; Jones & Roizman, 1979). IE gene expression is initiated by tegument proteins which interact with cellular transcriptional proteins, such as RNA polymerase II, to activate the transcription. IE genes encode mainly for transcription factors which inhibit IE gene expression and promote E gene expression. The maximum of E gene expression is usually observed between 4 and 8 hours post-infection (Figure 4). They are mainly coding for enzymes involved in nucleotide metabolism and viral DNA replication (Figure 3). Similarly as IE genes, E genes down regulate their own expression while stimulating the expression of L genes. Maximum L gene expression occurs after virus DNA replication (Figures 3 and 4). L genes are further divided in L1 (or γ1) and L2 (or γ2) subclasses. L1 gene expression is increased by viral DNA synthesis genes while L2 gene expression starts only after the synthesis of the viral genome (Figure 4) (Wagner *et al.*, 1998). Most of the L genes code for the proteins incorporated in mature virions; these proteins are called structural proteins. The structural proteome of a virus is defined as all the proteins which enter in the virion composition. Produced capsid proteins encoded by L genes are assembled in the nucleus to form the nucleocapsid containing newly synthesized viral DNA (Figure 3).

The replication of the viral genome is initiated from one or several origins of replication. Specific viral proteins are involved in viral DNA synthesis through a rolling-circle mechanism (Ackermann, 2004; Jacob *et al.*, 1979). This process generates concatemers consisting of complexe structure of high molecular weight made of several genomic units linked head-to-tail (Figure 3). A viral protein complex brings concatemers close to the portal complex of a capsid through which a single genomic unit is internalized and cleaved from the concatemer(Mettenleiter *et al.*, 2009).

Different models were proposed for the egress of the nucleocapsid from the nucleus to the extracellular space (Granzow *et al.*, 2001; Johnson & Spear, 1982; Wild *et al.*, 2005). In the envelopment-deenvelopment model (Figure 3), the temporary enveloped virus in the peri-nuclear space fuses with the external nuclear membrane to deliver the naked capsid in the cytoplasm. Tegument proteins are associated with the capsid before it buds into *trans*-golgi vesicles to form the envelope (Browne *et al.*, 1996; Granzow *et al.*, 2001; Masse *et al.*, 1999; Smith, 1980). The virion is finally released from the cell by exocytosis or cell lysis (Figure 3) (Flint *et al.*, 2000; Mettenleiter, 2004; Mettenleiter *et al.*, 2009). In the luminal model, the capsids bud in the internal nuclear membrane then migrate in the endoplasmic reticulum (ER). The enveloped virions are then (i) incorporated in a transport vesicle and delivered in the golgi apparatus (vesicular model) or

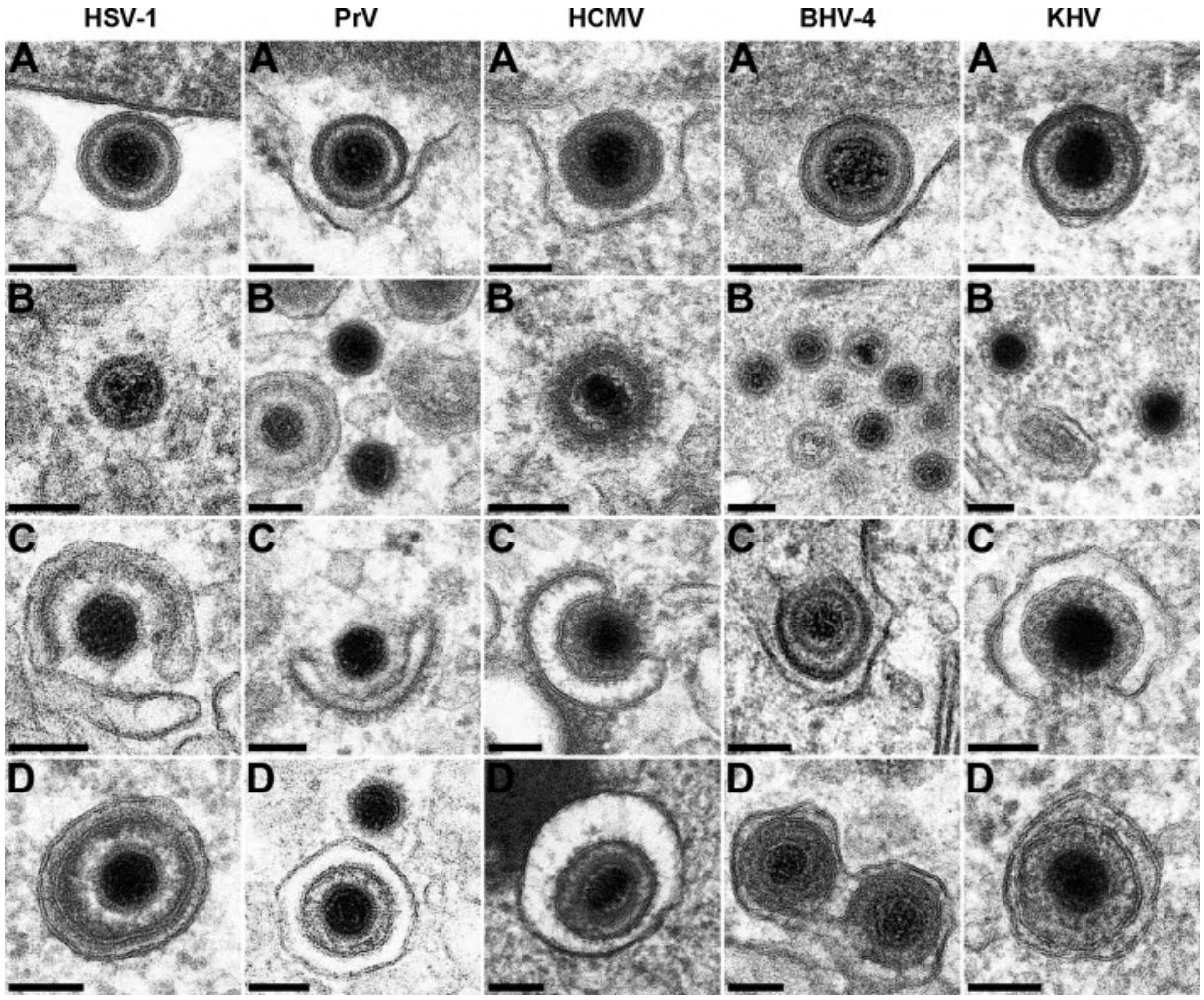

Figure 5. Acquisition process of herpesvirus envelope. (A) Primary enveloped virions in the perinuclear space. The electron-dense sharply bordered layer of tegument underlying the envelope and the absence of envelope glycoprotein spikes is noteworthy. (B) After translocation into the cytosol, capsids of HSV-1, PrV and BoHV-4 appear "naked", whereas those of HCMV and KHV are covered with a visible layer of "inner" tegument. (C) Secondary envelopment and (D) presence of enveloped virions within a cellular vesicle during transport to the plasma membrane. The same stages can be observed for members of the *Herpesviridae* family and KHV, a member of the *Alloherpesviridae* family. HSV-1: Herpes simplex type 1; PrV: Pseudorabies virus; HCMV: Human cytomegalovirus; BHV-4: Bovin herpesvirus 4; KHV: Koi herpesvirus. Bars represent 100 nm. Reproduced from Mettenleiter et al. (2009).

(ii) reach the golgi apparatus through connexions between the latter and the ER (intra-cisternal model). Independently of these models, the enveloped virions are released by exocytosis (Darlington & Moss, 1968; Johnson & Spear, 1982). Recently, a new model was described for BoHV-1 where capsids present in the nucleus are able to reach the cytoplasm trough enlarged nuclear pore (Wild *et al.*, 2005). The capsids, once in the cytoplasm, bud with golgi-derived vesicles before egress from the host cell by exocytosis.

A recent study by electron microscopy on the morphogenesis of different herpesviruses belonging to the *Herpesviridae* and *Alloherpesviridae* families, concludes that the nucleocapsids follow the envelopment-deenvelopment model before being released in the extracellular space by exocytosis (Figure 5) (Mettenleiter *et al.*, 2009).

Latent infection

Latency is observed in all members of the *Herpesviridae*. It consists in the virus maintenance in the host cell without production of viral particles. The mechanisms that induce latency are still poorly understood (Roizman & Pellet, 2007). Latency is supposed to occur when the virus infected specific cell types. The virus can then persist in the host even after the onset of an adaptive immune response able to clear cells supporting a replicative infection. Only few viral genes are expressed during latency. During latency, the genome is maintained as non integrated episome in the nucleus. When the latent infected cells divide (if they do so), the viral episome is replicated with the cellular genomic DNA. Copies of this episome are then distributed between daughter cells. The latent infection can be interrupted by exogenous stimulus and switched to lytic infection. Latency has been studied mainly in the family *Herpesviridae*. Regulation of latency seems to be mediated mainly by transcripts (LATs for latency associated transcripts) in alphaherpesviruses (Jones, 2003) while in beta- and gammaherpesviruses latency proteins are expressed (Ballestas & Kaye, 2001; Cardin *et al.*, 2009; Lee *et al.*, 1999).

Recent studies described the presence of microRNAs (miRNA) in the genome of different herpesviruses of the *Herpesviridae* family (Pfeffer *et al.*, 2005). Ever since, several studies demonstrated miRNA productions amongst the latency transcripts (alphaherpesvirus LATs). They seem to play an important role in cooperation with the beta- and gammaherpesvirus proteins during the viral biological cycle and essentially during the latency where they can modulate cell apoptosis and immune pathways, as well as the viral lytic cycle (Burnside *et al.*, 2006; Cai *et al.*, 2005; Lu *et al.*, 2008; Umbach *et al.*, 2008; Wang *et al.*, 2008).

Classification of the order *Herpesvirales*

The International Committee on Taxonomy of Viruses (ICTV) has classified the order *Herpesvirales* according to viruses encoding the putative ATPase subunit of the terminase (a complex that is responsible for packaging virus DNA into progeny capsids) (Davison, 1992; 2002; Waltzek *et al.*, 2009). This protein is specific to herpesviruses; however, it is also conserved to a lesser degree in the T4-like bacteriophages of the family *Myoviridae* (Davison *et al.*, 2009). The *Herpesvirales* order is subdivided in three families: the *Herpesviridae*, the *Alloherpesviridae* and the *Malacoherpesviridae* (Davison *et al.*, 2009; Roizmann *et al.*, 1992).

The *Herpesviridae* family

The family *Herpesviridae* is highly studied and is divided in three sub-families: *Alpha-*, *Beta-*, and *Gammaherpesvirinae* (Davison *et al.*, 2009; Roizman & Pellet, 2007). It regroups herpesviruses infecting reptiles, birds and mammals, including humans.

The alphaherpesviruses have a variable host range, a relatively short reproduction cycle, a rapid spread in culture, an efficient destruction of infected cells, and a capacity to establish latent infection in sensory neurons. As example, this subfamily contains the human herpesvirus 1 (HHV-1 or HSV-1) and 3 (HHV-3 or VZV), belonging to the genera *Simplexvirus* and *Varicellovirus*, respectively.

In contrast to alphaherpesviruses, betaherpesviruses have a restricted host range. The reproductive cycle is relatively long, and the infection progresses slowly in cell culture. Infected cells frequently become enlarged (cytomegalia). Their latency is established mainly in secretary glands. As example, this subfamily contains the human herpesvirus 5 (HHV-5 or HCMV) and the murid herpesvirus 1 (MuHV-1 or MCHV), belonging to the genera *Cytomegalovirus* and the *Muromegalovirus*, respectively.

Gammaherpesviruses have usually a host range restricted to the family or the order of their natural host. *In vitro*, all members replicate in lymphoblastoid cells, and some also cause lytic infections in some types of epithelioid and fibroblastic cells. Viruses in this group are usually specific for either T or B lymphocytes. Latent virus is frequently demonstrated in lymphoid tissue. As example, this subfamily contains the human herpesvirus 4 (HHV-4 or EBV) and 8 (HHV-8 or KSHV), belonging to the genera *Lymphocryptovirus* and *Rhadinovirus*, respectively.

The *Malacoherpesviridae* family

Until recently, this family consisted in a single virus (Davison *et al.*, 2005): the *Ostreid herpesvirus 1* (OsHV-1) infecting the Japanese oyster (*Crassostrea gigas*). Its genome contains 207 kb and is composed of two unique regions (U_L and U_S; 168 kb and 3 kb, respectively), each flanked by an inverted repeat (TR_L/IR_L and TR_S/IR_S of 7 kb and 10 kb, respectively). The presence of 124 ORFs are

Virus name (abbreviation)	Clade	Common name (abbreviation)	Host(s)	Disease
Anguillid HV 1 (AngHV1)	1	HV anguillae (HVA)	Japanese eel *Anguilla japonica* and European eel *A. Anguilla*	Haemorrhages of skin, fins, gills, liver
Cyprinid HV 1 (CyHV1)	1	HV cyprini, carp pox HV, carp HV(CHV)	Common carp *Cyprinus carpio*	High losses in fry- exophthalmia haemorrhages, survivors have papilloma
Cyprinid HV 2 (CyHV2)	1	Goldfish hematopoietic necrosis virus (GFHNV)	Goldfish *Carassius auratus*	High mortality at all ages. Necrosis of hematopoietic tissue, spleen, pancreas, intestine
Cyprinid HV 3 (CyHV3)	1	Koi HV (KHV), carp nephritis and gill necrosis virus (CNGV)	Common carp	Gill inflammation, hyperplasia, and necrosis, hematopoietic tissue necrosis. High mortality at all ages
Ictalurid HV 1 (IcHV1)	2	Channel catfish virus (CCV), Channel catfish herpesvirus	Channel catfish *Ictalurus punctatus*	Kidney, liver and intestinal necrosis, haemorrhages, high mortality in young subjects
Ictalurid HV 2 (IcHV2)	2	Ictalurus melas HV (IcmHV)	Black bullhead *Ameiurus melas*	Kidney necrosis, haemorrhages, high mortality at all ages
Acipenserid HV 1 (AciHV1)	2	White sturgeon HV 1	White sturgeon *Acipenser transmontanus*	diffuse dermatitis, high losses in juveniles
Acipenserid HV 2 (AciHV2)	2	White sturgeon HV 2	White sturgeon	Epithelial hyperplasia
Salmonid HV 1 (SalHV1)	2	HV salmonis (HPV) Steelhead herpesvirus (SHV)	Rainbow trout *Oncorhynchus mykiss*	Mild disease associated with low losses at 10 °C. Adults: female shed virus in ovarian fluid. Asymptomatic infection
Salmonid HV 2 (SalHV2)	2	Oncorhynchus masou virus (OMV)	Cherry salmon *O. masou*, coho salmon *O. kisutch*, sockeye salmon *O. nerka*, coho salmon *O. keta*, rainbow trout,	Viremia, external haemorrhages exophthalmia, hepatic necrosis. High mortality in young subjects. Survivors have oral papilloma. Infected female shed virus in ovarian fluid
Salmonid HV 3 (SalHV3)	2	Epizootic epitheliotropic disease virus (EEDV)	Lake trout *Salvelinus namaycush*, lake trout × brook trout *S. fontinalis* hybrids	Epithelial hyperplasia, hypertrophy, haemorrhages on eye and jaw. High mortality in juveniles at 6–15 °C
Gadid herpesvirus 1 (GaHV1)	2	Atlantic cod herpesvirus (ACHV)	Atlantic cod *Gadus morhua*	Hypertrophy of cells in gills. High mortality in adults.
Ranid HV 1 (RaHV1)	2	Lucké tumor HV (LTHV)	Leopard frog *Rana pipiens*	Renal adenocarcinoma
Ranid HV 2 (RaHV2)	2	Frog virus 4 (FV-4)	Leopard frog	No known disease
Pilchard HV	2		Australian pilchard *Sardinops sagax*	Gill inflammation associated with epithelial hyperplasia and hypertrophy. High mortality
Tilapia HV	Possible *Herpesviridae*	Tilapia larvae encephalitis virus (TLEV)	Blue tilapia *Oreochromis aureus*	Encephalitis in larvae. High mortality
Percid HV 1 (PeHV1)		HV vitreum, walleye HV	Walleye *Stizostedion vitreum*	Diffuse epidermal hyperplasia

Table 1. Herpesviruses of fish and amphibians (adapted from Hallon *et al.* 2011).

described whose 12 are duplicated in inverted repeats. Interestingly, among all these genes, 38 belong to 12 families of related genes (Davison *et al.*, 2005). Recently, a neurotropic herpesvirus infecting the gastropod abalone (*Haliotis* spp) was described (Savin *et al.*, 2010). Based on the homology existing between Abalone Herpesvirus (AbHV) and OsHV-1, it has been proposed to include the AbHV-1 in the *Malacoherpesviridae* family (Savin *et al.*, 2010). Despite the lack of similarity with the capsid proteins encoded by other herpesviruses, electron microscopy analysis demonstrates that OsHV-1and AbHV-1 have a capsid morphology comparable to that of HHV-1 and IcHV-1 (Davison *et al.*, 2005; Savin *et al.*, 2010).

The *Alloherpesviridae* family

The *Alloherpesviridae* encompasses viruses infecting fish and amphibians. So far, this family regroups 13 viruses infecting teleostei fish, 2 viruses of chondrostei fish and 2 viruses infecting amphibians (Hanson *et al.*, 2011) (Table 1). Phylogenetic studies based on the DNA polymerase and the terminase genes led the subdivision of the *Alloherpesviridae* family into two clades: the first clade comprises large linear dsDNA viruses (245-295 kb) as Anguillid and Cyprinid herpesviruses; the second clade comprises viruses with smaller genome (134-235 kb) as Ictalurid, Salmonid, Acipenserid and Ranid herpesviruses (Davison & Stow, 2005; Waltzek *et al.*, 2009). The genomes of several *Alloherpesviridae* have been sequenced: *Ictalurid herpesvirus 1* (IcHV-1), *Cyprinid herpesvirus 3* (CyHV-3), *Anguillid herpesvirus 1* (AngHV-1); the *Ranid herpesvirus 1* (RaHV-1) and *2* (RaHV-2). Based on these sequences, 12 conserved genes have been identified in the *Alloherpesviridae* family (Aoki *et al.*, 2007; van Beurden *et al.*, 2010).

Even though *Alloherpesviridae* are distantly related to *Herpesviridae*, there are similarities in the way they infect, replicate and persist in the host (Table 1). (i) They display a high level of host specificity, causing disease in only one species or in closely related members of the same genus. (ii) Some alloherpesviruses have been evaluated for long-term latent infections (persistence of viral DNA in survivors without production of infectious particles). Latency has been demonstrated in CyHV-1, CyHV-3, SalHV-2 and IcHV-1 (Hanson *et al.*, 2011). Much of our knowledge on the biology of *Alloherpesviridae* is derived from research on two models of infection: IcHV-1 for clade 2 and CyHV-3 for clade 1. CyHV-3 being the subject of this thesis, the remaining part of this introduction has been devoted to this virus.

References

Ackermann, M. (2004). Herpesviruses: a brief overview. *Methods Mol Biol* **256**, 199-219.

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.

Ballestas, M. E. & Kaye, K. M. (2001). Kaposi's sarcoma-associated herpesvirus latency-associated nuclear antigen 1 mediates episome persistence through cis-acting terminal repeat (TR) sequence and specifically binds TR DNA. *J Virol* **75**, 3250-3258.

Browne, H., Bell, S., Minson, T. & Wilson, D. W. (1996). An endoplasmic reticulum-retained herpes simplex virus glycoprotein H is absent from secreted virions: evidence for reenvelopment during egress. *J Virol* **70**, 4311-4316.

Burnside, J., Bernberg, E., Anderson, A., Lu, C., Meyers, B. C., Green, P. J., Jain, N., Isaacs, G. & Morgan, R. W. (2006). Marek's disease virus encodes MicroRNAs that map to meq and the latency-associated transcript. *J Virol* **80**, 8778-8786.

Cai, X., Lu, S., Zhang, Z., Gonzalez, C. M., Damania, B. & Cullen, B. R. (2005). Kaposi's sarcoma-associated herpesvirus expresses an array of viral microRNAs in latently infected cells. *Proc Natl Acad Sci U S A* **102**, 5570-5575.

Cardin, R. D., Schaefer, G. C., Allen, J. R., Davis-Poynter, N. J. & Farrell, H. E. (2009). The M33 chemokine receptor homolog of murine cytomegalovirus exhibits a differential tissue-specific role during in vivo replication and latency. *J Virol* **83**, 7590-7601.

Darlington, R. W. & Moss, L. H., 3rd (1968). Herpesvirus envelopment. *J Virol* **2**, 48-55.

Davison, A. J. (1984). Structure of the genome termini of varicella-zoster virus. *J Gen Virol* **65 (Pt 11)**, 1969-1977.

Davison, A. J. (1992). Channel catfish virus: a new type of herpesvirus. *Virology* **186**, 9-14.

Davison, A. J. (2002). Evolution of the herpesviruses. *Vet Microbiol* **86**, 69-88.

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.

Davison, A. J. & Stow, N. D. (2005). New genes from old: redeployment of dUTPase by herpesviruses. *J Virol* **79**, 12880-12892.

Davison, A. J., Trus, B. L., Cheng, N., Steven, A. C., Watson, M. S., Cunningham, C., Le Deuff, R. M. & Renault, T. (2005). A novel class of herpesvirus with bivalve hosts. *J Gen Virol* **86**, 41-53.

Dimmock, N. J., Easton, A. J. & Leppard, K. N. (2007). Towards a definition of a virus. In *Introduction to modern virology*, 6 edn, pp. 3 - 17. Edited by N. J. Dimmock, A. J. Easton & K. N. Leppard. Oxford: Wiley-Blackwel.

Flint, S., Racaniello, V. R., Enquist, L. W., Skalka, A. M. & Krug, R. M. (2000). *Virology : molecular biology, pathogenesis, and control*. Washington, D. C: ASM press.

Garber, D. A., Beverley, S. M. & Coen, D. M. (1993). Demonstration of circularization of herpes simplex virus DNA following infection using pulsed field gel electrophoresis. *Virology* **197**, 459-462.

Granzow, H., Klupp, B. G., Fuchs, W., Veits, J., Osterrieder, N. & Mettenleiter, T. C. (2001). Egress of alphaherpesviruses: comparative ultrastructural study. *J Virol* **75**, 3675-3684.

Hanson, L., Dishon, A. & Kotler, M. (2011). Herpesviruses that infect fish. *Viruses* **3**, 2160-2191.

Honess, R. W. & Roizman, B. (1974). Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. *J Virol* **14**, 8-19.

Honess, R. W. & Roizman, B. (1975). Regulation of herpesvirus macromolecular synthesis: sequential transition of polypeptide synthesis requires functional viral polypeptides. *Proc Natl Acad Sci U S A* **72**, 1276-1280.

Jacob, R. J., Morse, L. S. & Roizman, B. (1979). Anatomy of herpes simplex virus DNA. XII. Accumulation of head-to-tail concatemers in nuclei of infected cells and their role in the generation of the four isomeric arrangements of viral DNA. *J Virol* **29**, 448-457.

Johnson, D. C. & Spear, P. G. (1982). Monensin inhibits the processing of herpes simplex virus glycoproteins, their transport to the cell surface, and the egress of virions from infected cells. *J Virol* **43**, 1102-1112.

Jones, C. (2003). Herpes simplex virus type 1 and bovine herpesvirus 1 latency. *Clin Microbiol Rev* **16**, 79-95.

Jones, P. C. & Roizman, B. (1979). Regulation of herpesvirus macromolecular synthesis. VIII. The transcription program consists of three phases during which both extent of transcription and accumulation of RNA in the cytoplasm are regulated. *J Virol* **31**, 299-314.

King, A., Lefkowitz, E., Adams, M. & Carstens, E. (2012). Order of presentation of virus taxonomic descriptions. In *Virus Taxonomy : Ninth Report of the International Committee on Taxonomy of Viruses*, pp. 23-36. Edited by A. King, E. Lefkowitz, M. Adams & E. Carstens. London: Elsevier.

Lee, M. A., Diamond, M. E. & Yates, J. L. (1999). Genetic evidence that EBNA-1 is needed for efficient, stable latent infection by Epstein-Barr virus. *J Virol* **73**, 2974-2982.

Lu, F., Weidmer, A., Liu, C. G., Volinia, S., Croce, C. M. & Lieberman, P. M. (2008). Epstein-Barr virus-induced miR-155 attenuates NF-kappaB signaling and stabilizes latent virus persistence. *J Virol* **82**, 10436-10443.

Masse, M. J., Jons, A., Dijkstra, J. M., Mettenleiter, T. C. & Flamand, A. (1999). Glycoproteins gM and gN of pseudorabies virus are dispensable for viral penetration and propagation in the nervous systems of adult mice. *J Virol* **73**, 10503-10507.

McGeoch, D. J., Davison, A. J., Dolan, A., Gatherer, D. & Sevilla-Reyes, E. E. (2008). Molecular evolution of the Herpesvirales. In *Origin and evolution of viruses*, 2 edn, pp. 447-476. Edited by E. Domingo, C. R. Parrish & J. J. Holland. Amsterdam: Elsevier.

Mettenleiter, T. C. (2004). Budding events in herpesvirus morphogenesis. *Virus Res* **106**, 167-180.

Mettenleiter, T. C., Klupp, B. G. & Granzow, H. (2009). Herpesvirus assembly: an update. *Virus Res* **143**, 222-234.

Pfeffer, S., Sewer, A., Lagos-Quintana, M., Sheridan, R., Sander, C., Grasser, F. A., van Dyk, L. F., Ho, C. K., Shuman, S., Chien, M., Russo, J. J., Ju, J., Randall, G., Lindenbach, B. D., Rice, C. M., Simon, V., Ho, D. D., Zavolan, M. & Tuschl, T. (2005). Identification of microRNAs of the herpesvirus family. *Nat Methods* **2**, 269-276.

Roizman, B. & Pellet, P. E. (2007). The Family: Herpesviridae a brief introduction. In *Fields virology*, 5 edn, pp. 2479-2499. Edited by D. M. Knipe & P. M. Howley. Philadelphia: Lippincott Williams and Wilkins.

Roizmann, B., Desrosiers, R. C., Fleckenstein, B., Lopez, C., Minson, A. C. & Studdert, M. J. (1992). The family Herpesviridae: an update. The Herpesvirus Study Group of the International Committee on Taxonomy of Viruses. *Arch Virol* **123**, 425-449.

Savin, K. W., Cocks, B. G., Wong, F., Sawbridge, T., Cogan, N., Savage, D. & Warner, S. (2010). A neurotropic herpesvirus infecting the gastropod, abalone, shares ancestry with oyster herpesvirus and a herpesvirus associated with the amphioxus genome. *Virol J* **7**, 308.

Smith, J. D. (1980). An additional role for the outer nuclear membrane in the morphogenesis of herpes simplex virus. *Intervirology* **13**, 312-316.

Sodeik, B., Ebersold, M. W. & Helenius, A. (1997). Microtubule-mediated transport of incoming herpes simplex virus 1 capsids to the nucleus. *J Cell Biol* **136**, 1007-1021.

Spear, P. G. (2004). Herpes simplex virus: receptors and ligands for cell entry. *Cell Microbiol* **6**, 401-410.

Umbach, J. L., Kramer, M. F., Jurak, I., Karnowski, H. W., Coen, D. M. & Cullen, B. R. (2008). MicroRNAs expressed by herpes simplex virus 1 during latent infection regulate viral mRNAs. *Nature* **454**, 780-783.

van Beurden, S. J., Bossers, A., Voorbergen-Laarman, M. H., Haenen, O. L., Peters, S., Abma-Henkens, M. H., Peeters, B. P., Rottier, P. J. & Engelsma, M. Y. (2010). Complete genome sequence and taxonomic position of anguillid herpesvirus 1. *J Gen Virol* **91**, 880-887.

Wagner, E. K., Petroski, M. D., Pande, N. T., Lieu, P. T. & Rice, M. (1998). Analysis of factors influencing kinetics of herpes simplex virus transcription utilizing recombinant virus. *Methods* **16**, 105-116.

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.

Wang, F. Z., Weber, F., Croce, C., Liu, C. G., Liao, X. & Pellett, P. E. (2008). Human cytomegalovirus infection alters the expression of cellular microRNA species that affect its replication. *J Virol* **82**, 9065-9074.

Wild, P., Engels, M., Senn, C., Tobler, K., Ziegler, U., Schraner, E. M., Loepfe, E., Ackermann, M., Mueller, M. & Walther, P. (2005). Impairment of nuclear pores in bovine herpesvirus 1-infected MDBK cells. *J Virol* **79**, 1071-1083.

Introduction

2nd chapter:

Cyprinid herpesvirus 3: an interesting virus for applied and fundamental research

Emerging Infectious Diseases (2010)16, 1835-1843

B. Michel, G. Fournier, F. Lieffrig, B. Costes and A. Vanderplasschen

Note: Most of the text of this introduction has been copy-pasted from a recent review on CyHV-3. This introduction has been updated with the data published after the publication of the review. To avoid confusion of the readers, the data generated during the present thesis were not included in this introduction.

Abstract

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 suggest that CyHV-3 is an interesting object for fundamental research: CyHV-3 has the largest genome amongst the order *Herpesvirales*, and serves as an extreme model for mutagenesis of large DNA viruses. The effect of temperature on viral replication suggests that the body temperature of its poikilotherm host could regulate the outcome of the infection (replicative versus non replicative). In this review, we summarize the recent advances in CyHV-3 fundamental and applied research.

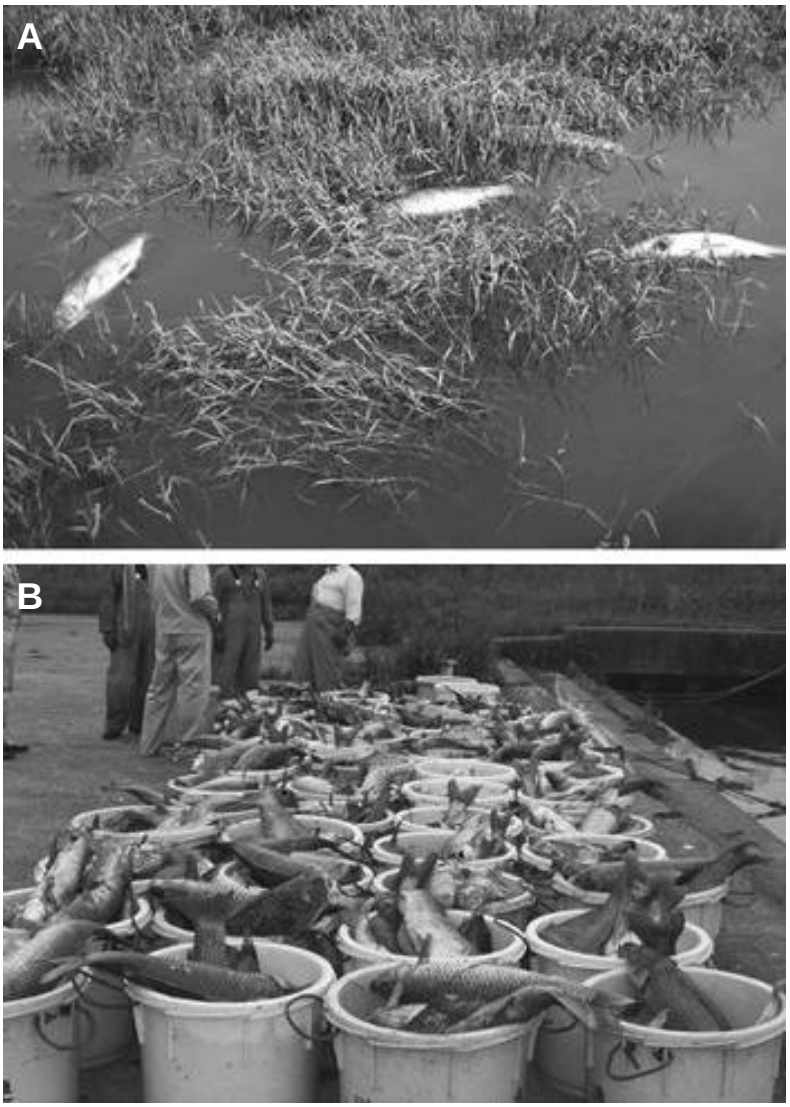

Figure 1. Mass mortality of common carp caused by CyHV-3 disease in Lake Biwa, Japan, in 2004. (A) The death of wild common carp was observed throughout the lake. (B) More than 100,000 dead carp were collected from this lake in 2004 alone. It is estimated that 2–3 times as many carp died but were not collected from the lake. Reproduced with permission from Matsui et al. (2008).

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 (Figure 1)(Haenen, 2004; Matsui *et al.*, 2008). The observed rapid worldwide spread of the disease has been attributed to the international fish trade and koi shows that occur around the world (Hedrick, 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, 2000). Later, the virus was also known as carp interstitial nephritis and gill necrosis virus (CNGV), because of the associated lesions (Ronen *et al.*, 2003). 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 that of previously described cyprinid herpesviruses (Waltzek *et al.*, 2005).

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 object. In the present review, we summarize recent advances made in CyHV-3 applied and fundamental research.

Characterization of CyHV-3

Viral classification

CyHV-3 is a member of the newly designated *Alloherpesviridae* family of the order *Herpesvirales* (Figure 2A) (Davison, 2002; Waltzek *et al.*, 2009). The 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) some 450 million years ago (MYA) (Davison, 2002). The *Alloherpesviridae* family appears to be subdivided into two clades according to phylogenetic analysis of specific genes (the DNA polymerase and the terminase genes) (Figure 2B) (Waltzek *et al.*, 2009). The first clade comprises anguillid and cyprinid herpesviruses that possess the largest genomes in the order *Herpesvirales* (245–295 kilobase pairs [kb]). The second clade comprises ictalurid, salmonid, acipenserid, and ranid herpesviruses with smaller DNA genomes (134–235 kb) (Hanson *et al.*, 2011).

Structural characterization

The CyHV-3 structure is typical of the order *Herpesvirales*. An icosahedral capsid contains the genome, which consists of a single linear, double-stranded DNA (dsDNA) molecule. The capsid is

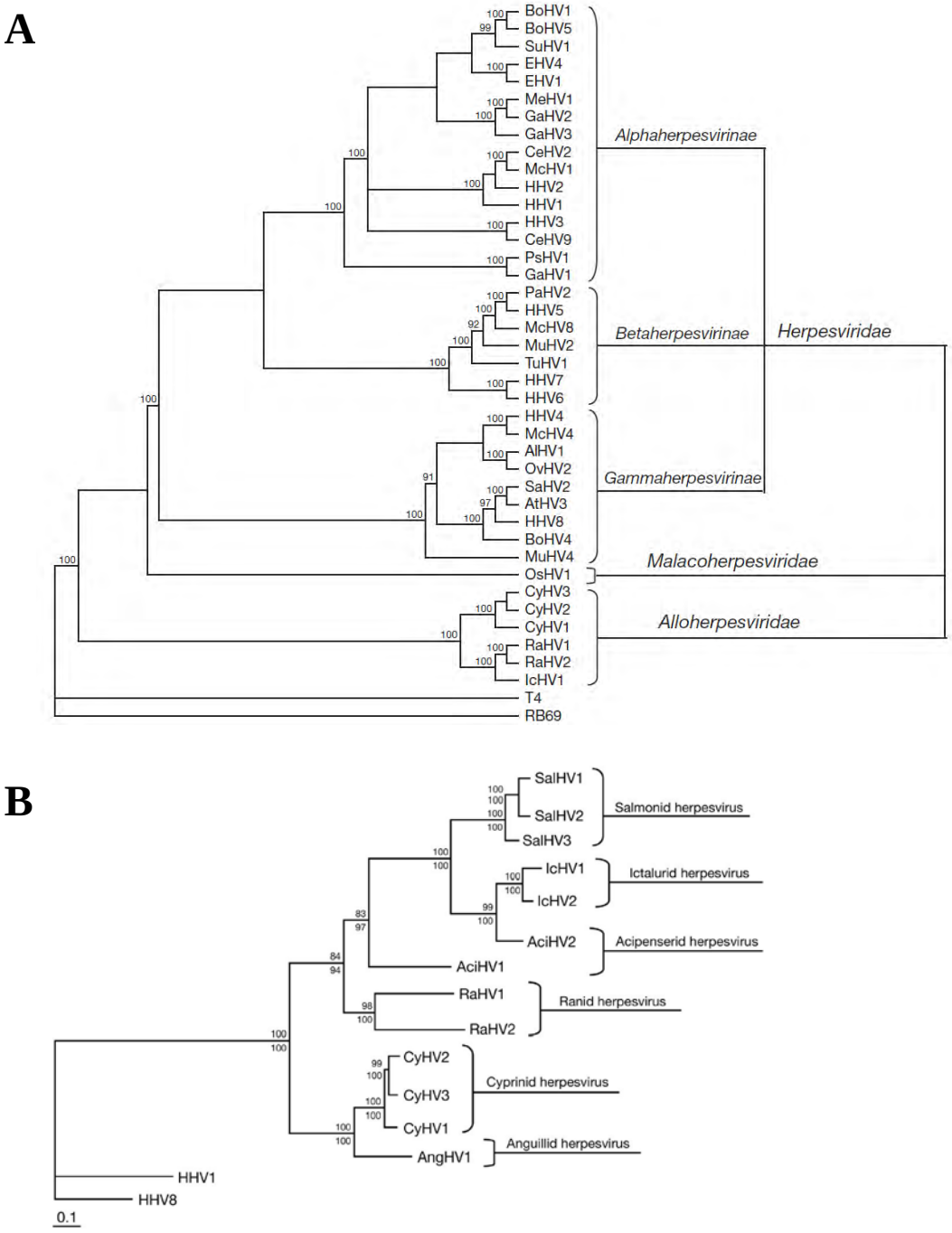

Figure 2. (A) Cladogram depicting relationships among viruses in the order *Herpesvirales*, based on the conserved regions of the terminase gene. The Bayesian maximum likelihood tree was rooted using bacteriophages T4 and RB69. Numbers at each node represent the posterior probabilities (values > 90 shown) 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. AlHV-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: saimiriine herpesvirus 2; SuHV-1: suid herpesvirus 1; TuHV-1: tupaiid herpesvirus 1. Reproduced with permission from Waltzek et al. (2009).

covered by a proteinaceous matrix called the tegument, which is surrounded by a lipid envelope derived from host cell *trans*-golgi membrane (Figure 3) (Mettenleiter *et al.*, 2009; Miyazaki *et al.*, 2008). The envelope contains viral glycoproteins (Hedrick, 2000). The entire CyHV-3 particle has a diameter of approximately 170–200 nm (Hedrick, 2000; Miyazaki *et al.*, 2008; Neukirch & Kunz, 2001).

Molecular characterization

Viral genome. 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) (Figure 4)(Aoki *et al.*, 2007). The genome size is similar to that of CyHV-1 (Waltzek *et al.*, 2005), but is larger than that of the other members of the order *Herpesvirales*, which generally range from 125 to 240 kb. The GC content of the genome is relatively high approaching 59.2% (Aoki *et al.*, 2007).

The CyHV-3 genome encodes 156 potential protein-coding open reading frames (ORFs) including eight ORFs encoded by the repeat regions. These eight ORFs are consequently present as two copies in the genome (Aoki *et al.*, 2007). Among them, 3 genes (ORF1, -3 and -6) are potential immediate early genes (Dr Aoki, personal communication) and one is coding for a tumor necrosis factor receptor (TNFR; ORF4) (Aoki *et al.*, 2007). The terminal repeats are supposed to be involved in the capsid packaging of a single copy of the genome coming from the concatemer during the viral replication. Five families of related genes have been described in the CyHV-3 genome: the ORF2, TNFR, ORF22, ORF25, and RING families. The ORF25 family consists of six ORFs (ORF25, -26, -27, -65, -148, and -149) encoding related, potential membrane glycoproteins. The expression products of four of the sequences were detected in mature virions (ORF25, -65, -148, and -149) (Michel *et al.*, 2010). CyHV-3 encodes several genes that could be involved in immune evasion processes, such as ORF16, which codes for a potential G-protein coupled receptor (GPCR); ORF134, which codes for an IL-10 homologue; and ORF12, which codes for a TNFR homologue.

Within the *Alloherpesviridae* family, anguillid herpesvirus 1 (AngHV-1) is the closest relative of CyHV-3 sequenced to date (Doszpoly *et al.*, 2011; van Beurden *et al.*, 2010). The two viruses possess 40 ORFs exhibiting similarity. It is likely that the sequencing of CyHV-1 and -2 should reveal even more CyHV-3 gene homologues. The putative products of most ORFs in the CyHV-3 genome lack obvious relatives in other organisms. Indeed, 110 ORFs fall into this class. Six ORFs encode proteins whose closest relatives are found in virus families such as the *Poxviridae* and *Iridoviridae* (Aoki *et al.*, 2007; Waltzek *et al.*, 2005). For example, CyHV-3 genes such as B22R (ORF139), thymidylate kinase ([TmpK] ORF140), thymidine kinase ([TK] ORF55), and the subunits of ribonucleotide reductase (ORF23 and -141) appear to have evolved from poxvirus genes (Aoki *et al.*, 2007). Interestingly, neither TmpK nor B22R has been identified previously in a member of the order *Herpesvirales*.

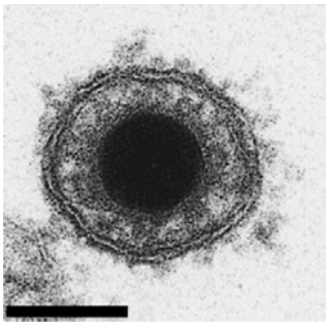

Figure 3. Electron microscopy examination of CyHV-3 virion. Bar represents 100 nm. Adapted with permission from Mettenleiter et al. (2009).

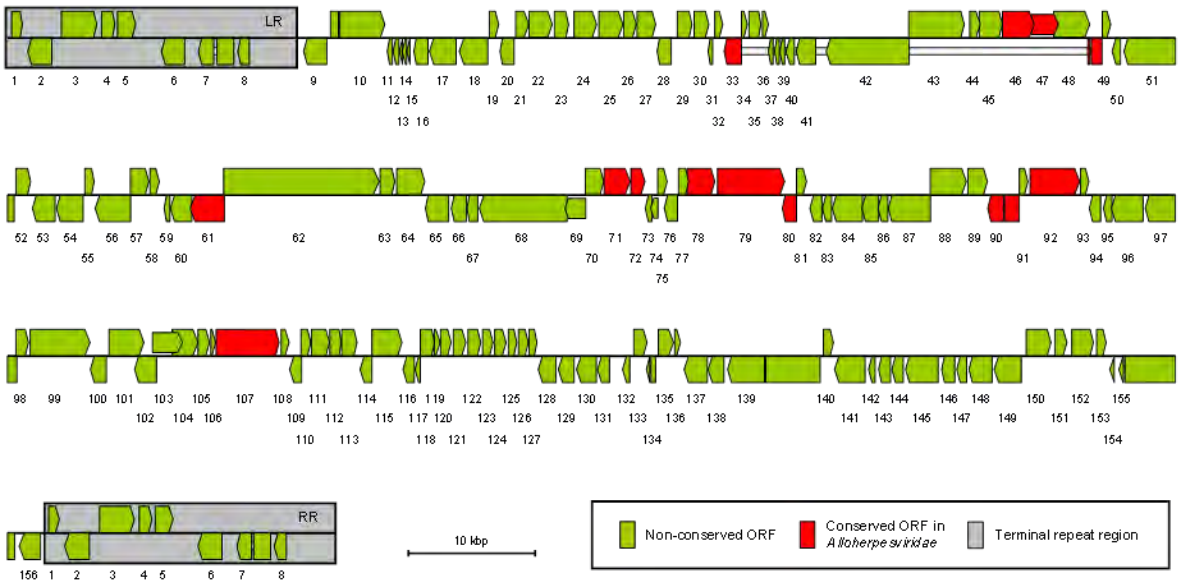

Figure 4. Genomic organisation of the CyHV-3. Potential ORF and their orientations are shown by coloured arrows and numbered from 1 to 156. Introns are depicted as narrow bars. Left (LR) and right (RR) repeats are represented by grey rectangles. Red arrows represent conserved ORF among the *Alloherpesviridae* CyHV-3, IcHV-1, AngHV-1, RaHV-1 and -2. Reproduced from Aoki et al. (2007) and van Beurden et al. (2010).

Cell lines	Cytopathic effect
Cyprinus carpio brain cells (CCB)	Yes (Neukirch & Kunz, 2001,15)
Cyprinus carpio gill cells (CCG)	Yes (Neukirch & Kunz, 2001)
Epithelioma papulosum cyprinid cells (EPC)	No (Hedrick *et al.*, 2000, Ronen *et al.*, 2003), Davidovich *et al.*, 2007, Oh *et al.*, 2001) / Yes (Neukirch & Kunz, 2001)
Koi fin cells (KFC, KF-1)	Yes (Hedrick *et al.*, 2000, Ronen *et al.*, 2003), Davidovich *et al.*, 2007, Pikarsky *et al.*, 2004)
Carp fin cells (CFC, CaF-2)	Yes (Neukirch & Kunz, 2001)
Fathead minnow cells (FHM)	No (Hedrick *et al.*, 2000, Davidovich *et al.*, 2007) / Yes (Oh *et al.*, 2001)
Chinook salmon embryo cells (CHSE-214)	No (Oh *et al.*, 2001)
Rainbow trout gonad cells (RTG-2)	No (Oh *et al.*, 2001)
Glodfish fin cells (Au)	Yes (Davidovich *et al.*, 2007)
Channel catfish ovary cells (CCO)	No (Davidovich *et al.*, 2007)
Silver carp fin cells (Tol/FL)	Yes (Davidovich *et al.*, 2007)
Koi caudal fin cells (KCF-1)	Yes (Dong *et al.*, 2011)

Table 1. CyHV-3 susceptible cell lines

Three strains of CyHV-3, isolated in Israel (CyHV-3 I), Japan (CyHV-3 J), and the USA (CyHV-3 U), have been fully sequenced (Aoki *et al.*, 2007). Despite their distant geographical origin, these strains exhibit highly homologous sequences. A low diversity of sequences among strains seems to be a characteristic of the CyHV-3 species. Despite this low diversity, molecular markers allowing for discrimination among nine genotypes (seven European and two Asian) have been identified (Kurita *et al.*, 2009). Analyses of variable number of tandem repeat (VNTR) proved to be useful to investigate CyHV-3 genetic diversity. These analyses revealed a relatively slow genetic evolution of CyHV-3 *in vitro* (Avarre *et al.*, 2011). However, prolonged CyHV-3 cultivation *in vitro* leads to the spontaneous attenuation of the virus (Ronen *et al.*, 2003).

Viral structural proteome. The structural proteome of CyHV-3 was characterized recently using liquid chromatography tandem mass spectrometry (Michel *et al.*, 2010). Forty structural proteins, comprising three capsid, 13 envelope, two tegument, and 22 unclassified proteins, were described. The genome of CyHV-3 possesses 30 potential transmembrane protein-coding ORFs (Aoki *et al.*, 2007). One cannot exclude that some low abundant envelope proteins have been overlooked during proteome analysis With the exception of ORF81, which encodes a type 3 membrane protein expressed on the CyHV-3 envelope (Michel *et al.*, 2010; Rosenkranz *et al.*, 2008), none of the CyHV-3 structural proteins have been studied to date. ORF81 is thought to be one of the most immunogenic (major) membrane proteins of CyHV-3 (Rosenkranz *et al.*, 2008). A recent study of the structural proteome of AngHV-1 revealed that its viral particle contains a number of proteins comparable to CyHV-3 (van Beurden *et al.*, 2011).

In vitro replication

CyHV-3 is widely cultivated in cell lines derived from koi fin (KFC), *Cyprinus carpio* brain (CCB), and *Cyprinus carpio* gill (Table 1) (Davidovich *et al.*, 2007; Hedrick, 2000; Neukirch & Kunz, 2001; Oh, 2001; Pikarsky *et al.*, 2004; Ronen *et al.*, 2003). Other cell lines have been tested but few showed cytopathic effect following CyHV-3 infection (Table 1). For cell lines that did not support viral replication, it is not known whether these cell lines are nevertheless sensitive to the infection.

The CyHV-3 replication cycle was studied recently by electron microscopy (Mettenleiter *et al.*, 2009). The morphological stages observed suggest that it replicates in a manner similar to that of the family *Herpesviridae* that utilizes an envelopment–deenvelopment mechanism to acquire the viral envelope (Mettenleiter *et al.*, 2009; Miwa *et al.*, 2007; Miyazaki *et al.*, 2008). Following this theory, capsids leave the nucleus by a first budding event at the inner nuclear membrane resulting in the formation of primary enveloped virions in the perinuclear space. The primary envelope then fuses with the outer leaflet of the nuclear membrane thereby releasing nucleocapsids into the cytoplasm. Final envelopment occurs by budding into *trans*-golgi vesicles.

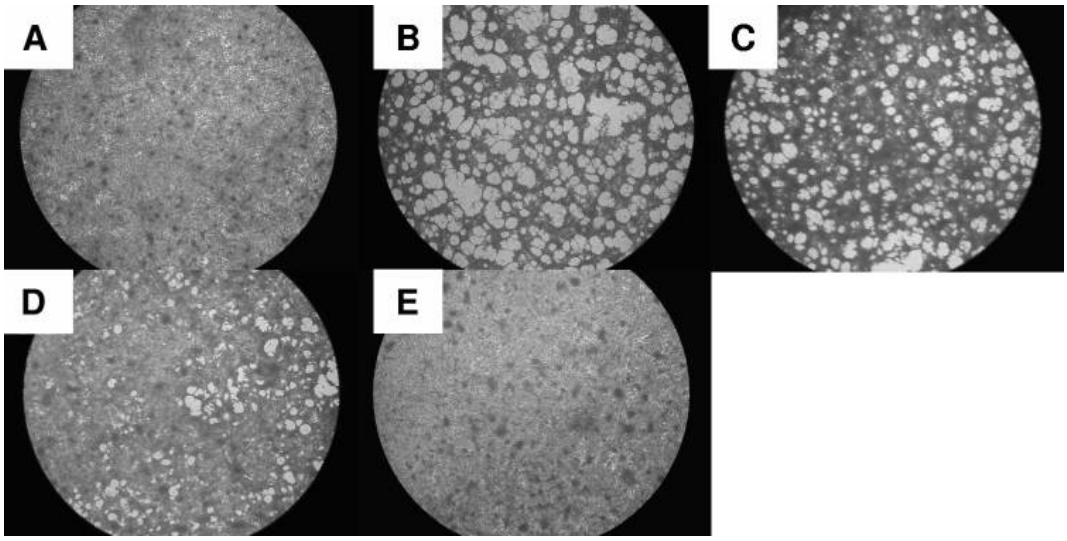

Figure 5. Effect of temperature on CyHV-3 replication. CCB cells were infected with CyHV-3. Following infection, the cells were either kept at 22 °C (B) or shifted to 30 °C. At 24 hours post-infection (hpi) (C) or 48 hpi (D), the cells were returned to 22 °C. At 9 days post-infection (dpi), the cells were fixed, stained, and photographed. (A) Noninfected control. (E) Infected cells kept at 30 °C after infection. Magnification: 20x. Reproduced with permission from Dishon et al. (2007).

As mentioned above, CyHV-3 glycoproteins have little or no similarity with those of members of the family *Herpesviridae*. Further studies are required to identify the CyHV-3 glycoproteins involved in entry and egress.

Because fish are poikilotherms and because CyHV-3 only affects fish when the water temperature is 18–28 °C, the effect of temperature on CyHV-3 replication growth *in vitro* has been investigated. Replication in cell culture is restricted by temperature, with optimal viral growth at 15–25 °C. Viral propagation and viral gene transcription are turned off by moving cells to a non-permissive temperature of 30 °C (Dishon *et al.*, 2007). Despite the absence of detectable viral replication, infected cells maintained for 30 days at 30 °C preserve infectious virus, as demonstrated by viral replication when the cells are returned to permissive temperatures (Figure 5) (Dishon *et al.*, 2007). These results suggest that CyHV-3 can persist asymptomatically for long periods in the fish body when the temperature prevents viral replication, with a burst of new infection upon exposure to permissive temperatures.

Resistance to environmental factors

CyHV-3 virions remain infectious in environmental water for at least 4 hours at 23-25 °C (Perelberg *et al.*, 2005; Perelberg, 2003). A study performed by Shimizu *et al.* (2006) demonstrated an anti-CyHV-3 activity for different bacterial strains present in water and in the sediment (Shimizu *et al.*, 2006). The CyHV-3 is quickly inactivated by UV irradiation and temperature above 50 °C (Kasai *et al.*, 2005a). The following disinfectants are also effective to inactivate the virus: iodophor (200 mg/l), benzalkonium chloride (60 mg/l), ethyl alcohol (40%) and sodium hypochlorite (400 mg/l) (Kasai *et al.*, 2005b) but also Virkon (1%) (Vetoquinol Canada Inc.).

Disease caused by CyHV-3

Epidemiological history

In 1998, the first mass mortalities of common and koi carp were reported in Israel and the USA (Ariav, 1999; Bretzinger, 1999; Hedrick, 2000). However, analyses of samples from archives determined that the virus had been present in wild common carp since 1996 in the UK (Walster, 1999). Soon after the first report, outbreaks of CyHV-3 were identified in different European (Body *et al.*, 2000; Bretzinger, 1999; Hedrick, 2000; Marek *et al.*, 2010; Walster, 1999), Asian (Bondad-Reantaso *et al.*, 2007; Cheng *et al.*, 2011; Choi *et al.*, 2004; Dong *et al.*, 2011; Gomez *et al.*, 2011; Oh, 2001; Sano, 2004; Sunarto *et al.*, 2011; Tu *et al.*, 2004), and African countries(Haenen & Engelsma, 2004). Currently, CyHV-3 has been identified all around the world with the exception of South America, Australia, and northern Africa (Haenen & Engelsma, 2004; Pokorova, 2005). This rapid spread of CyHV-3 is thought to be linked to the largely unregulated international koi trade and the numerous fish meeting occurring around the world. These kinds of exhibition are held without

Fish species	Inoculated fish			Mortality in carp during cohabitation
	DNA	Protein	Clinical signs	
Carassius auratus (goldfish)	Yes (*23*)	Yes (*23*)	No (*21*)/Yes (*23*)	No (*21*)/Yes (*22*)
Ctenopharyngodon idella (grass carp)			No (*21*)	No (*21*)/Yes (*22*)
Carassius carassius (crucian carp)				No (*22*)
Hypophthalmichthys molitrix (silver carp)			No (*21*)	No (*21, 22*)
Aristichtys nobilis (bighead carp)				No (*22*)
Bidyanus bidyanus (silver perch)			No (*21*)	No (*21*)
Oreochromis niloticus (Nile tilapia)			No (*21*)	No (*21*)
Tinca tinca (tench)				Yes (*22*)
Silurus glanis (sheatfish)				No (*22*)
Vimba vimba (vimba)				No (*22*)
Acipenser ruthenus (sterlet)				No (*22*)
Acipenser gueldenstaedtii (Russian sturgeon)				No (*22*)
Acipenser oxyrinchus (Atlantic sturgeon)				No (*22*)

Table 2. Effect of CyHV-3 infection in different fish species

previous health examinations or health certificates enhancing the transmission from fish to fish and quick dissemination. In particular, the fish meeting of New York in 1998 is suspected to be the origin point for the first outbreak in Israel and in the USA (Hedrick, 2000).

CyHV-3 has caused severe financial and economic losses in both koi and common carp culture industries worldwide (Haenen & Engelsma, 2004). In 2003, Perelberg *et al.* (2003) reported that Israeli farms have lost around US$3 million per year since 1998 due to the CyHV-3 (Perelberg, 2003). In 2004, Waltzek and Hedrick highlighted an economic loss totaling US$5.5 million for Indonesia and approximately US$2.5 million for Japan (Waltzek & Hedrick, 2004).

Host range

Common and koi carp are the only species known to suffer from CyHV-3 infection (Bretzinger, 1999; Perelberg, 2003). Numerous fish species, including cyprinid and noncyprinid species, were tested for their ability to carry CyHV-3 asymptomatically and to spread it to naïve carp (Table 2) (Bergmann *et al.*, 2007; Haenen & Hedrick, 2006; Perelberg, 2003). CyHV-3 DNA was recovered from only two other fish species: goldfish and crucian carp. Cohabitation experiments suggest that goldfish, grass carp, and tench can carry CyHV-3 asymptomatically and spread it to naïve common carp (Table 2). Interestingly, hybrids of koi x goldfish and koi x crucian carp die from CyHV-3 infection (Bergmann *et al.*, 2010b).

Susceptible stages

CyHV-3 affects carp at all ages, but younger fish (1–3 months, equivalent to 2.5–6 g) appear to be more sensitive to the disease than mature fish (1 year, equivalent to 230 g) (Oh, 2001; Perelberg, 2003). Recently, the susceptibility of young carp to CyHV-3 infection was analyzed by experimental infection (Ito *et al.*, 2007). Most of the infected juveniles (>13 days post-hatching) died from the disease, while the larvae (three days post-hatching) were not susceptible to CyHV-3.

Pathogenesis

Several authors have postulated that the gills might be the portal of entry for CyHV-3 in carp (Dishon *et al.*, 2005; Gilad *et al.*, 2004; Ilouze *et al.*, 2006; Miyazaki *et al.*, 2008; Pikarsky *et al.*, 2004). This hypothesis relied on several observations. First, the gills have been demonstrated to act as the portal of entry for many fish pathogens (Roberts, 2001). Second, fish expressing CyHV-3 disease have gill lesions, which explains why the virus was initially called *Carp interstitial nephritis and gill necrosis* virus (Hedrick, 2000; Miyazaki *et al.*, 2008; Perelberg, 2003; Pikarsky *et al.*, 2004; Ronen *et al.*, 2003). Third, the gills (like virtually all tissues) were shown by PCR to contain the viral genome at an early stage of infection (Gilad *et al.*, 2004; Pikarsky *et al.*, 2004). However, no data demonstrating the role of the gills as the portal of entry of CyHV-3 are available.

It has also been postulated that the virus spreads very rapidly in infected fish, based on detection of CyHV-3 DNA in fish tissues (Gilad *et al.*, 2004). Indeed, as early as 24 hours post-infection, CyHV-3 DNA was recovered from almost all internal tissue (including liver, kidney, gut, spleen, and brain) (Gilad *et al.*, 2004) where viral replication occurs at later stages of infection and causes lesions. One hypothesis regarding the rapid and systemic dissemination observed by PCR is that CyHV-3 targets blood cells as a secondary site of infection. Viral replication in organs such as the gills, skin, and gut at the later stages of infection represents sources of viral excretion into the environment. Following natural infection under permissive temperatures (18–28 °C), the highest mortality rate occurs 8–12 days post-infection (dpi) (Gilad *et al.*, 2004; Perelberg, 2003) suggest that death is due to loss of the osmoregulatory functions of the gills, kidneys, and gut.

All members of the family *Herpesviridae* exhibit two distinct phases in their life cycle: lytic replication and latency. Latency is characterized by maintenance of the viral genome as a non-integrated episome and the expression of a limited number of viral genes and microRNAs. Upon reactivation, latency is replaced by lytic replication. So far, latency has not been firmly demonstrated in CyHV-3. However, there is a growing list of observations that support the existence of a latent phase. CyHV-3 DNA was detected by real-time TaqMan PCR 65 dpi in clinically healthy fish (Gilad *et al.*, 2004). Furthermore, the virus persisted in a wild population of common carp for at least two years after the initial outbreak (Uchii *et al.*, 2009). Moreover, St-Hilaire *et al.* (2005) demonstrated the possibility of a temperature-dependent reactivation of CyHV-3 lytic infection several months after initial exposure to the virus (St-Hilaire *et al.*, 2005). This study suggests that the temperature of the water could control the outcome of the infection (replicative/nonreplicative). The latter results were confirmed recently by an independent study (Eide *et al.*, 2011). The latter study suggested that leukocytes could be a site of latency.

Transmission

Transmission of CyHV-3 is likely to occur through mobility of infected carp (supporting and replicative or a latent infection). Importantly, it has been demonstrated that some fish species could act as an asymptotic reservoir of the virus and could transmit the infection to naïve carp (El-Matbouli *et al.*, 2007b; Haenen & Hedrick, 2006). Horizontal transmission of CyHV-3 has been demonstrated via feces (Dishon *et al.*, 2005) and secretion of viral particles into water (Perelberg, 2003). Several studies demonstrated the presence of infectious viral particles in filtrating invertebrate organisms such as mollusks or crustaceans (Kielpinski *et al.*, 2010); while CyHV-3 DNA have been detected in planktons (Minamoto *et al.*, 2011) or sediments (Honjo *et al.*, 2011).

Clinical signs

The first symptoms appear at 2–3 dpi. The fish exhibit appetite loss and lethargy; they lie at the bottom of the tank with the dorsal fin folded. Depending on the stage of the infection, the skin

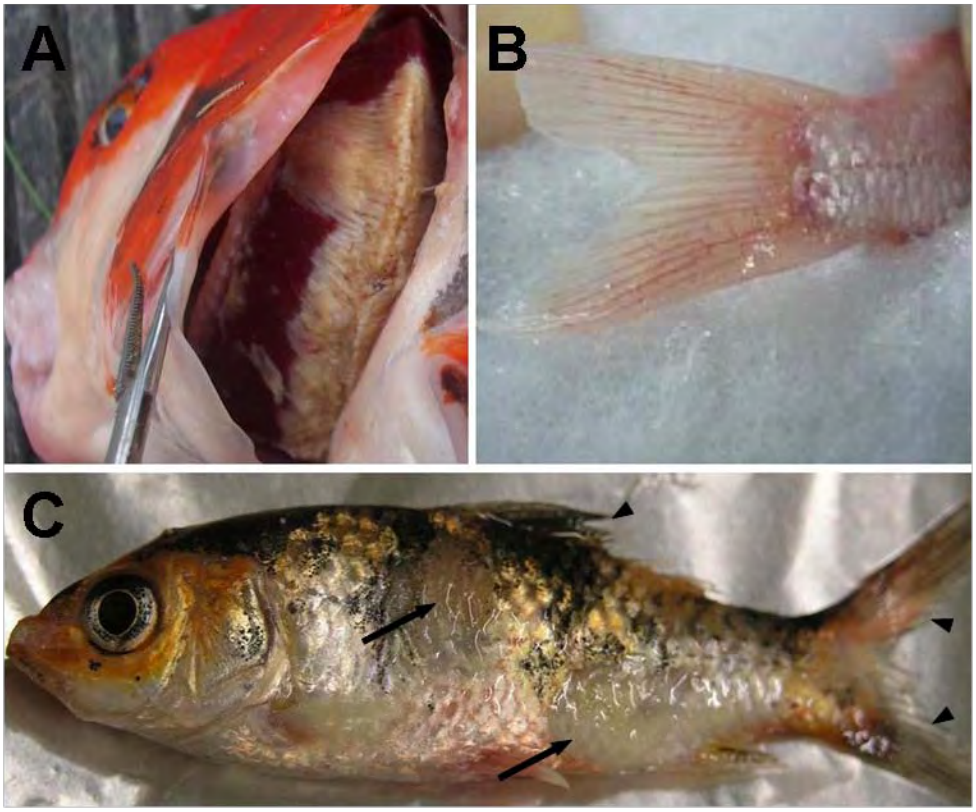

Figure 6. Some of the clinical signs observed during CyHV-3 infection. (A) Severe gill necrosis. (B) Hyperemia at the base of the caudal fin. (C) Herpetic skin lesions on the body (arrows) and fin erosion (arrowheads).

exhibits different clinical signs, such as hyperemia, particularly at the base of the fins and on the abdomen; mucus hypersecretion; and herpetic lesions (Figure 6). The gills are frequently subject to necrosis; together with mucus hypersecretion, this leads to suffocation of the fish. Bilateral enophthalmia is occasionally observed in the later stages of infection. Some of the fish exhibit neurological clinical signs in the final stage of the disease; they become disoriented and lose equilibrium (Bretzinger, 1999; Hedrick, 2000; Hutoran *et al.*, 2005; Perelberg, 2003; Walster, 1999).

Histopathology

In CyHV-3–infected fish, prominent pathological changes are observed in the gill, skin, kidney, liver, spleen, gastrointestinal system, and brain (Hedrick, 2000; Miyazaki *et al.*, 2008; Perelberg, 2003; Pikarsky *et al.*, 2004). Histopathological changes appear in the gills as early as 2 dpi and involve the epithelial cells of the gill filaments. These cells exhibit hyperplasia, hypertrophy, and/or nuclear degeneration (Hedrick, 2000; Miyazaki *et al.*, 2008; Perelberg, 2003; Pikarsky *et al.*, 2004). Severe inflammation leads to the fusion of respiratory epithelial cells with cells of the neighbouring lamellae, resulting in lamellar fusion (Miyazaki *et al.*, 2008; Pikarsky *et al.*, 2004). In the kidney, a weak peritubular inflammatory infiltrate is evident as early as 2 dpi and increases with time, along with blood vessel congestion and degeneration of the tubular epithelium in many nephrons (Pikarsky *et al.*, 2004). In the spleen and liver, splenocytes and hepatocytes, respectively, are the most obviously infected cells (Miyazaki *et al.*, 2008). In brain of fish that exhibited neurological symptoms, congestion of capillaries and small veins are apparent in the valvula cerebelli and medulla oblongata involving oedematous dissociation of nerve fibers (Miyazaki *et al.*, 2008).

Diagnosis of CyHV-3

Viral isolation

The first diagnostic method for the CyHV-3 was based on isolation of viral particles from infected fish tissue in cell cultures (Hedrick, 2000). Cell cultures (CCB and KFC) were infected with tissue extracts at 23 °C and observed for appearance of cytopathic effects (CPE), then supernatant containing virions was used to infect naïve fish and to reproduce the disease (Hedrick, 2000). Viral isolation is time-consuming but is still the best effective method to detect infectious particles in the mortality phase of the disease. However, this method is useless to detect asymptomatic carrier as survivor of an outbreak.

Polymerase chain reaction

PCR molecular techniques offer a rapid and sensitive diagnostic method that overcomes the laborious isolation of viral particles. The first PCR methods directed against the CyHV-3 were developed independently by Gray *et al.* (2002) and Gilad *et al.* (2002). They amplify DNA located in

non coding regions (Gilad *et al.*, 2002; Gray, 2002). Few years later, Bercovier *et al.* (2005) developed a PCR directed against the TK gene (ORF55) (Bercovier *et al.*, 2005). El-Matbouli *et al.* (2007) developed a nested PCR assay specific to the major capsid protein gene (ORF92) (El-Matbouli *et al.*, 2007a). This highly specific assay, based on 2-round amplification step performed on the same DNA fragment, diminishes the possibility of false positive results. The sensitivity of the nested PCR can be compared to the sensitivity of the PCR raised against the TK gene, with a detection level between 5 and 10 DNA copies (Bergmann *et al.*, 2010a). A real-time TaqMan PCR assay was developed by Gilad *et al.* (2004). This technique was shown to detect the CyHV-3 DNA at 1 dpi in almost all tissue (Gilad *et al.*, 2004). This method can detect traces of DNA as long as 64 dpi in the gills, kidney and brain tissues (Gilad *et al.*, 2004).

Loop-mediated isothermal amplification

To overcome the requirement of a thermal cycler and the use of expensive consumables in PCR method, some laboratories developed the loop-mediated isothermal amplification (LAMP) technique (Gunimaladevi *et al.*, 2004; Soliman & El-Matbouli, 2009). This method consists to amplify DNA with high specificity and sensitivity under isothermal conditions. LAMP is a rapid (60 min), simple and inexpensive method, with the same sensitivity as the PCR assays (Soliman & El-Matbouli, 2005). This technique is very useful when a rapid diagnostic is required as it can be performed almost everywhere with a single water bath at 65 °C. Positive results can be read rapidly by adding SYBR green I stain in the final reaction mix. Very recently, a LAMP method was coupled to a direct binding assay (Gunimaladevi *et al.*, 2004; Soliman & El-Matbouli, 2009). This method consists to concentrate, purify and coat CyHV-3 viral particles in the LAMP reaction tube. This method can be performed outside the laboratory but is less sensitive than PCR (Bergmann *et al.*, 2010a).

Enzyme-linked immunosorbent assay

ELISA is a useful diagnostic method for the detection of CyHV-3 specific antibodies (Adkison, 2005; St-Hilaire *et al.*, 2009). The limit of this method is the delay for the production of antibodies directed against CyHV-3. Consequently, ELISA is especially useful to the diagnosis of fish who survived on infection. ELISA has also been used to detect specific viral proteins using a rabbit anti-CyHV-3 polyserum (Pikarsky *et al.*, 2004). Using this method, CyHV-3 proteins have been detected in fish dropping at 6-7 dpi (Dishon *et al.*, 2005).

Recently an enzyme immunoassay has been commercialized by an Israeli company (KoVax). This kit allows the quantitative detection of CyHV-3 antigens in stool or in tissue of infected fish.

Sero-neutralization

This diagnostic method consists to quantify specific neutralizing antibodies of a carp raised against the CyHV-3. Used as a non-invasive method, sero-neutralization, like ELISA, required facilities but avoid the cross reaction problems (Bergmann *et al.*, 2010a).

Detection of CyHV-3 in environmental water

Since 2006, several studies described the detection of CyHV-3 in environmental water. The method used is based on the detection of viral DNA by real-time TaqMan PCR (Gilad *et al.*, 2004; Haramoto *et al.*, 2007; Minamoto *et al.*, 2009). This method was applied on water of a Japanese river and allowed the detection of CyHV-3 DNA during an outbreak but also 3 months later (Minamoto *et al.*, 2009). A similar but quantitative approach was developed using an external standard virus (Honjo *et al.*, 2010). Detection of CyHV-3 in water samples is laborious and time-consuming. It requires preliminary concentration and purification of viral particles from water samples before analysis. Also, it implies the persitence of viral particles for long period in environmental water without being degraded. It has been postulated that CyHV-3 viral particles could adsorb to solids to be protected from UV irradiations and degrading enzymes (Matsui *et al.*, 2008; Shimizu *et al.*, 2006). This attachment could also increase the accumulation of viral particles in carrier organisms such as shellfish (Matsui *et al.*, 2008).

Immune response against CyHV-3

Immunity in ectothermic vertebrates depends upon the temperature of the environment. In carp, at temperatures below 14 °C, adaptive immunity is switched off, while the innate immune response remains functional (Bly & Clem, 1992). As mentioned above, the host temperature also has an effect on CyHV-3 replication, which can occur only at 18–28 °C. In carp that are infected and maintained at 24 °C, antibody titers begin to rise at around 10 dpi and reach a plateau between 20 and 40 dpi (Perelberg *et al.*, 2008). In the absence of antigenic re-exposure, the specific antibodies gradually decrease over six months to a level slightly above or comparable to that of naïve fish. While protection against CyHV-3 is proportional to the titer of specific antibodies during primary infection, immunized fish—even those in which antibodies are no longer detectable—are resistant to a lethal challenge, probably due to the subsequent rapid response of B and T memory cells upon antigen restimulation (Perelberg *et al.*, 2008).

Prophylaxis and control of CyHV-3

Three main approaches are currently being developed to control CyHV-3: (i) management and commercial measures to enhance the international market of certified CyHV-3 free carp and to favor the eradication of CyHV-3; (ii) selection of carp that are resistant to CyHV-3; and (iii) development of

safe and efficacious vaccines. Scientific publications addressing the latter two approaches are reviewed below.

Selection of CyHV-3 resistant carp

The hypothesis that resistance of carp to CyHV-3 might be affected by host genetic factors received support (Shapira *et al.*, 2005). Shapira *et al.* investigated differential resistance to CyHV-3 (survival rates ranging from 8% to 60%) by crossbreeding sensitive domesticate strains and a relatively resistant wild strain of carp. Recently, high heritability of resistance to CyHV-3 disease was demonstrated in *Cyprinus carpio* (Ødegård *et al.*, 2010). Further supporting the role of host genetic factors in CyHV-3 resistance, major histocompatibility class II genes were recently shown to affect carp resistance (Rakus *et al.*, 2009). Similarly, single nucleotide polymorphisms (SNP) markers correlated to carp resistance were identified amongst 14 genes implicated in the anti-viral immune response (Kongchum *et al.*, 2011).

Vaccination of carp against CyHV-3

Soon after the characterization of CyHV-3, a protocol was developed to induce a protective adaptive immune response in carp. This approach is based on the fact that CyHV-3 induces fatal infections only when the water temperature is between 18 °C and 28 °C.

According to this protocol, healthy naïve fish are exposed to CyHV-3 infected fish for 3–5 days at permissive temperature (22 °C–23 °C) and then transferred to ponds with a water temperature of approximately 30 °C (nonpermissive temperature) for 30 days. Following this procedure, 60% of the fish become resistant to further challenge with CyHV-3 (Ronen *et al.*, 2003). Despite its ingenuity, this method has several disadvantages: (i) increasing the water temperature to 30 °C makes the fish more susceptible to secondary infection by other pathogens and requires a large amount of energy in places where the water is naturally cool; (ii) the protection is observed in only 60% of the fish; (iii) carp that are “vaccinated” using this protocol have been exposed to wild-type virulent CyHV-3 and could therefore represent a potential source of CyHV-3 outbreaks if they later come into contact with naïve carp.

Attenuated live vaccine appears to be the most appropriate vaccine for mass vaccination of carp. Attenuated vaccine candidates have been produced by successive passages in cell culture (Ronen *et al.*, 2003). The vaccine strain candidate was further attenuated by UV irradiation to increase the mutation rate of the viral genome (Perelberg *et al.*, 2008; Ronen *et al.*, 2003). A vaccine strain obtained by this process has been commercialized by an Israeli company (KoVax) and has been shown to confer protection against a virulent challenge. This vaccine is only available in Israel and exhibits two main disadvantages: (i) The molecular basis for the reduced virulence is unknown, and, consequently, reversions to a pathogenic phenotype cannot be excluded. (ii) Under certain conditions,

it is possible that the attenuated strain retained residual virulence that could be lethal for a portion of the vaccinated subjects (Perelberg *et al.*, 2008).

An inactivated vaccine candidate was described by Yasumoto *et al.* (2006). It consists of formalin-inactivated CyHV-3 trapped within a liposomal compartment. This vaccine can be used for oral immunization in fish food. Immunization of carp with this inactivated vaccine results in 70% protection efficacy (Yasumoto *et al.*, 2006).

Conclusion

Because CyHV-3 causes several financial and economical losses in both koi and common carp culture industries worldwide, it is an important subject for applied science. Safe and efficacious vaccines adapted to mass vaccination of carp and efficient diagnostic methods need to be developed. Several aspects of CyHV-3 make it an interesting fundamental science subject, including its large genome, the relationship between CyHV-3 infectivity and temperature, and the low similarity between CyHV-3 genes and the genes of other members of the order *Herpesvirales* that have been studied to date. Further studies are required to identify the roles of CyHV-3 genes in viral entry, egress, and pathogenesis.

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References

Adkison, M. A., Gilad, O., and Hedrick, R. P. (2005). An enzyme linked immunosorbent assay (ELISA) for detection of antibodies to the koi herpesvirus (KHV) in the serum of koi Cyprinus carpio. *Fish Pathol* **40**, 53-62.

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.

Ariav, R., Tinman, S., Paperna, I., Bejerano, I. (1999). First report of newly emerging viral disease of Cyprinus carpio species in Israel. In *the EAFP 9th International Conference*. Rhodes, Greece.

Avarre, J. C., Madeira, J. P., Santika, A., Zainun, Z., Baud, M., Cabon, J., Caruso, D., Castric, J., Bigarre, L., Engelsma, M. & Maskur, M. (2011). Investigation of Cyprinid herpesvirus-3 genetic diversity by a multi-locus variable number of tandem repeats analysis. *J Virol Methods* **173**, 320-327.

Bercovier, H., Fishman, Y., Nahary, R., Sinai, S., Zlotkin, A., Eyngor, M., Gilad, O., Eldar, A. & Hedrick, R. P. (2005). Cloning of the koi herpesvirus (KHV) gene encoding thymidine kinase and its use for a highly sensitive PCR based diagnosis. *BMC Microbiol* **5**, 13.

Bergmann, S. M., Riechardt, M., Fichtner, D., Lee, P. & Kempter, J. (2010a). Investigation on the diagnostic sensitivity of molecular tools used for detection of koi herpesvirus. *Journal of Virological Methods* **163**, 229-233.

Bergmann, S. M., Sadowski, J., Kiepiski, M., Bartomiejczyk, M., Fichtner, D., Riebe, R., Lenk, M. & Kempter, J. (2010b). Susceptibility of koi x crucian carp and koi x goldfish hybrids to koi herpesvirus (KHV) and the development of KHV disease (KHVD). *Journal of Fish Diseases* **33**, 267-272.

Bergmann, S. M., Stumpf, P., Schütze, H., Fichtner, D., Sadowski, J. & Kempter, J. (2007). Similarities and heterogenicity of koi herpes virus (KHV) genome detected in ornamental fish without clinical signs. *Aquaculture* **272**, S245-S245.

Bly, J. E. & Clem, W. (1992). Temperature and teleost immune functions. *Fish & Shellfish Immunology* **2**, 159-171.

Body, A., Lieffrig, F., Charlier, G. & Collard, A. (2000). Isolation of Virus-like particles from Koi (Cyprinus carpio) suffering gill necrosis. *Bull Eur Assoc Fish Pathol* **20**, 87-88.

Bondad-Reantaso, M. G., Sunarto, A. & Subasinghe, R. P. (2007). Managing the koi herpesvirus disease outbreak in Indonesia and the lessons learned. *Dev Biol (Basel)* **129**, 21-28.

Bretzinger, A., Fischer-Scherl, T., Oumouma, R., Hoffmann, R., Truyen, U. (1999). Mass mortalities in koi, Cyprinus carpio, associated with gill and skin disease. *Bull Eur Assoc Fish Pathol* **19**, 182-185.

Cheng, L., Chen, C. Y., Tsai, M. A., Wang, P. C., Hsu, J. P., Chern, R. S. & Chen, S. C. (2011). Koi herpesvirus epizootic in cultured carp and koi, Cyprinus carpio L., in Taiwan. *J Fish Dis* **34**, 547-554.

Choi, D. L., Sohn, S. G., Bang, J. D., Do, J. W. & Park, M. S. (2004). Ultrastructural identification of a herpes-like virus infection in common carp Cyprinus carpio in Korea. *Dis Aquat Organ* **61**, 165-168.

Davidovich, M., Dishon, A., Ilouze, M. & Kotler, M. (2007). Susceptibility of cyprinid cultured cells to cyprinid herpesvirus 3. *Arch Virol* **152**, 1541-1546.

Davison, A. J. (2002). Evolution of the herpesviruses. *Vet Microbiol* **86**, 69-88.

Dishon, A., Davidovich, M., Ilouze, M. & Kotler, M. (2007). Persistence of cyprinid herpesvirus 3 in infected cultured carp cells. *J Virol* **81**, 4828-4836.

Dishon, A., Perelberg, A., Bishara-Shieban, J., Ilouze, M., Davidovich, M., Werker, S. & Kotler, M. (2005). Detection of carp interstitial nephritis and gill necrosis virus in fish droppings. *Appl Environ Microbiol* **71**, 7285-7291.

Dong, C., Weng, S., Li, W., Li, X., Yi, Y., Liang, Q. & He, J. (2011). Characterization of a new cell line from caudal fin of koi, Cyprinus carpio koi, and first isolation of cyprinid herpesvirus 3 in China. *Virus Research* **161**, 140-149.

Doszpoly, A., Somogyi, V., LaPatra, S. E. & Benko, M. (2011). Partial genome characterization of acipenserid herpesvirus 2: taxonomical proposal for the demarcation of three subfamilies in Alloherpesviridae. *Arch Virol* **156**, 2291-2296.

Eide, K. E., Miller-Morgan, T., Heidel, J. R., Kent, M. L., Bildfell, R. J., Lapatra, S., Watson, G. & Jin, L. (2011). Investigation of koi herpesvirus latency in koi. *J Virol* **85**, 4954-4962.

El-Matbouli, M., Rucker, U. & Soliman, H. (2007a). Detection of Cyprinid herpesvirus-3 (CyHV-3) DNA in infected fish tissues by nested polymerase chain reaction, pp. 23-28.

El-Matbouli, M., Saleh, M. & Soliman, H. (2007b). Detection of cyprinid herpesvirus type 3 in goldfish cohabiting with CyHV-3-infected koi carp (Cyprinus carpio koi). *Vet Rec* **161**, 792-793.

Gilad, O., Yun, S., Andree, K. B., Adkison, M. A., Zlotkin, A., Bercovier, H., Eldar, A. & Hedrick, R. P. (2002). Initial characteristics of koi herpesvirus and development of a polymerase chain reaction assay to detect the virus in koi, Cyprinus carpio koi. *Dis Aquat Organ* **48**, 101-108.

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.

Gomez, D. K., Joh, S. J., Jang, H., Shin, S. P., Choresca Jr, C. H., Han, J. E., Kim, J. H., Jun, J. W. & Park, S. C. (2011). Detection of koi herpesvirus (KHV) from koi (Cyprinus carpio koi) broodstock in South Korea. *Aquaculture* **311**, 42-47.

Gray, W. L., Mullis, L., LaPatra, S. E., Groff, J. M., and Goodwin, A. (2002). Detection of koi herpesvirus DNA in tissues of infected fish. *J Fish Dis* **25**, 171-178.

Gunimaladevi, I., Kono, T., Venugopal, M. N. & Sakai, M. (2004). Detection of koi herpesvirus in common carp, Cyprinus carpio L., by loop-mediated isothermal amplification. *J Fish Dis* **27**, 583-589.

Haenen, O. L. & Engelsma, M. (2004). Global distribution of KHV with particular reference to Europe.

Haenen, O. L. & Hedrick, R. P. (2006). Koi herpesvirus workshop *Bull Eur Assoc Fish Pathol* **26**.

Haenen, O. L. M., Way, K., Bergmann, S.M., and Ariel, E. (2004). The emergence of koi herpesvirus and its significance to European aquaculture. *Bull Eur Assoc Fish Pathol* **24**, 293-307.

Hanson, L., Dishon, A. & Kotler, M. (2011). Herpesviruses that infect fish. *Viruses* **3**, 2160-2191.

Haramoto, E., Kitajima, M., Katayama, H. & Ohgaki, S. (2007). Detection of koi herpesvirus DNA in river water in Japan. *J Fish Dis* **30**, 59-61.

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.

Honjo, M. N., Minamoto, T. & Kawabata, Z. (2011). Reservoirs of Cyprinid herpesvirus 3 (CyHV-3) DNA in sediments of natural lakes and ponds. *Vet Microbiol*.

Honjo, M. N., Minamoto, T., Matsui, K., Uchii, K., Yamanaka, H., Suzuki, A. A., Kohmatsu, Y., Iida, T. & Kawabata, Z. (2010). Quantification of cyprinid herpesvirus 3 in environmental water by using an external standard virus. *Appl Environ Microbiol* **76**, 161-168.

Hutoran, M., Ronen, A., Perelberg, A., Ilouze, M., Dishon, A., Bejerano, I., Chen, N. & Kotler, M. (2005). Description of an as yet unclassified DNA virus from diseased Cyprinus carpio species. *J Virol* **79**, 1983-1991.

Ilouze, M., Dishon, A., Kahan, T. & Kotler, M. (2006). Cyprinid herpes virus-3 (CyHV-3) bears genes of genetically distant large DNA viruses. **580**, 4473-4478.

Ito, T., Sano, M., Kurita, J., Yuasa, K. & Iida, T. (2007). Carp larvae are not susceptible to Koi Herpesvirus. *Fish Pathol* **42**, 107-109.

Kasai, H., Muto, Y. & Yoshimizu, M. (2005a). Virucidal effects of ultraviolet, heat treatment and disinfectants against Koi Herpesvirus (KHV). *Fish Pathol* **40**, 137-138.

Kasai, H., Muto, Y. & Yoshimizu, M. (2005b). Virucidal effects of ultraviolet, heat treatment and disinfectants against koi herpesvirus (KHV). *Fish Pathology* **40**, 137-138.

Kielpinski, M., Kempter, J., Panicz, R., Sadowski, J., Schütze, H., Ohlemeyer, S. & Bergmann, S. M. (2010). Detection of KHV in Freshwater Mussels and Crustaceans from Ponds with KHV History in Common Carp (Cyprinus carpio). *The Israeli Journal of Aquaculture* **62**, 28-37.

Kongchum, P., Sandel, E., Lutzky, S., Hallerman, E. M., Hulata, G., David, L. & Palti, Y. (2011). Association between IL-10a single nucleotide polymorphisms and resistance to cyprinid herpesvirus-3 infection in common carp (Cyprinus carpio). *Aquaculture* **315**, 417-421.

Kurita, J., Yuasa, K., Ito, T., Sano, M., Hedrick, R. P., Engelsma, M. Y., Haenen, O. L. M., Sunarto, A., Kholidin, E. B., Chou, H.-Y., Tung, M.-C., Pe, ntilde, a, L. d. l., Lio-Po, G., Tu, C., Way, K. & Iida, T. (2009). Molecular Epidemiology of Koi Herpesvirus. *Fish Pathology* **44**, 59-66.

Marek, A., Schachner, O., Bilic, I. & Hess, M. (2010). Characterization of Austrian koi herpesvirus samples based on the ORF40 region. *Dis Aquat Organ* **88**, 267-270.

Matsui, K., Honjo, M., Yukihiro, K., Uchii, K., Yonekura, R. & Kawabata, Z. (2008). Detection and significance of koi herpesvirus (KHV) in freshwater environments. *Freshwat Biol* **53**, 1262-1272.

Mettenleiter, T. C., Klupp, B. G. & Granzow, H. (2009). Herpesvirus assembly: an update. *Virus Res* **143**, 222-234.

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.

Minamoto, T., Honjo, M. N., Uchii, K., Yamanaka, H., Suzuki, A. A., Kohmatsu, Y., Iida, T. & Kawabata, Z. (2009). Detection of cyprinid herpesvirus 3 DNA in river water during and after an outbreak. *Vet Microbiol* **135**, 261-266.

Minamoto, T., Honjo, M. N., Yamanaka, H., Tanaka, N., Itayama, T. & Kawabata, Z. (2011). Detection of cyprinid herpesvirus-3 DNA in lake plankton. *Res Vet Sci* **90**, 530-532.

Miwa, S., Ito, T. & Sano, M. (2007). Morphogenesis of koi herpesvirus observed by electron microscopy. *J Fish Dis* **30**, 715-722.

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.

Neukirch, M. & Kunz, U. (2001). Isolation and preliminary characterization of several viruses from koi (Cyprinus carpio) suffering gill necrosis and mortality. *Bull Eur Assoc Fish Pathol* **21**, 125-135.

Ødegård, J., Olesena, I., Dixonb, P., Jeneyc, Z., Nielsena, H.-M., Wayb, K., Joinerb, C., Jeneyc, G., Ardóc, L., Rónyaic, A. & Gjerdea, B. (2010). Genetic analysis of common carp (Cyprinus carpio) strains. II: Resistance to koi herpesvirus and Aeromonas hydrophila and their relationship with pond survival *Aquaculture* **304**, 7-13.

Oh, M. J., Jung, S.J., Choi, T.J., Kim, H.R., Rajendran, K.V., Kim, Y.J., Park, M.A., Chun, S.K. (2001). A viral disease occurring in cultured carp Cyprinus carpio in Korea. *Fish pathology* **36**, 147-151.

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.

Perelberg, A., Ronen, A., Hutoran, M., Smith, Y. & Kotler, M. (2005). Protection of cultured Cyprinus carpio against a lethal viral disease by an attenuated virus vaccine. *Vaccine* **23**, 3396-3403.

Perelberg, A., Smirnov, M., Hutoran, M., Diamant, A., Bejerano, Y., Kotler, M. (2003). Epidemiological description of a new viral disease afflicting cultured Cyprinus Carpio in Israel. *The Israeli Journal of Aquaculture* **55**, 5-12.

Pikarsky, E., Ronen, A., Abramowitz, J., Levavi-Sivan, B., Hutoran, M., Shapira, Y., Steinitz, M., Perelberg, A., Soffer, D. & Kotler, M. (2004). Pathogenesis of acute viral disease induced in fish by carp interstitial nephritis and gill necrosis virus. *J Virol* **78**, 9544-9551.

Pokorova, D., Vesely, T., Piackova, V., Reschova, S., Hulova, J. (2005). Current knowledge on koi herpesvirus (KHV): a review. *Vet Med-Czech* **5**, 139-147.

Rakus, K. L., Wiegertjes, G. F., Jurecka, P., Walker, P. D., Pilarczyk, A. & Irnazarow, I. (2009). Major histocompatibility (MH) class II B gene polymorphism influences disease resistance of common carp (Cyprinus carpio L.). *Aquaculture* **288**, 44-50.

Roberts, R. J. (2001). *Fish pathology*. London: W. B. Saunders.

Ronen, A., Perelberg, A., Abramowitz, J., Hutoran, M., Tinman, S., Bejerano, I., Steinitz, M. & Kotler, M. (2003). Efficient vaccine against the virus causing a lethal disease in cultured Cyprinus carpio. *Vaccine* **21**, 4677-4684.

Rosenkranz, D., Klupp, B. G., Teifke, J. P., Granzow, H., Fichtner, D., Mettenleiter, T. C. & Fuchs, W. (2008). Identification of envelope protein pORF81 of koi herpesvirus. *J Gen Virol* **89**, 896-900.

Sano, M., Ito, T., Kurita,J., Yanai, T., Watanabe, N., Satoshi, M., and Iida, T. (2004). First detection of koi herpes virus in cultured common carp Cyprinus carpio in Japan. *Fish Pathol* **39**, 165-168.

Shapira, Y., Magen, Y., Zak, T., Kotler, M., Hulata, G. & Levavi-Sivan, B. (2005). Differential resistance to koi herpes virus (KHV)/carp interstitial nephritis and gill necrosis virus (CNGV) among common carp (Cyprinus carpio L.) strains and crossbreds. *Aquaculture* **245**, 1-11.

Shimizu, T., Yoshida, N., Kasai, H. & Yoshimizu, M. (2006). Survival of koi herpesvirus (KHV) in environmental water. *Fish Pathol* **41**, 153-157.

Soliman, H. & El-Matbouli, M. (2005). An inexpensive and rapid diagnostic method of Koi Herpesvirus (KHV) infection by loop-mediated isothermal amplification. *Virol J* **2**, 83.

Soliman, H. & El-Matbouli, M. (2009). Immunocapture and direct binding loop mediated isothermal amplification simplify molecular diagnosis of Cyprinid herpsvirus-3. *J Virol Methods*.

St-Hilaire, S., Beevers, N., Joiner, C., Hedrick, R. P. & Way, K. (2009). Antibody response of two populations of common carp, Cyprinus carpio L., exposed to koi herpesvirus. *J Fish Dis* **32**, 311-320.

St-Hilaire, S., Beevers, N., Way, K., Le Deuff, R. M., Martin, P. & Joiner, C. (2005). Reactivation of koi herpesvirus infections in common carp Cyprinus carpio. *Dis Aquat Organ* **67**, 15-23.

Sunarto, A., McColl, K. A., Crane, M. S., Sumiati, T., Hyatt, A. D., Barnes, A. C. & Walker, P. J. (2011). Isolation and characterization of koi herpesvirus (KHV) from Indonesia: identification of a new genetic lineage. *J Fish Dis* **34**, 87-101.

Tu, C., Weng, M. C., Shiau, J. R. & Lin, S. Y. (2004). Detection of koi herpesvirus in koi Cyprinus carpio in Taiwan. *Fish Pathology* **39**, 109-110.

Uchii, K., Matsui, K., Iida, T. & Kawabata, Z. (2009). Distribution of the introduced cyprinid herpesvirus 3 in a wild population of common carp, Cyprinus carpio L. *J Fish Dis*.

van Beurden, S. J., Bossers, A., Voorbergen-Laarman, M. H., Haenen, O. L., Peters, S., Abma-Henkens, M. H., Peeters, B. P., Rottier, P. J. & Engelsma, M. Y. (2010). Complete genome sequence and taxonomic position of anguillid herpesvirus 1. *J Gen Virol* **91**, 880-887.

van Beurden, S. J., Leroy, B., Wattiez, R., Haenen, O. L., Boeren, S., Vervoort, J. J., Peeters, B. P., Rottier, P. J., Engelsma, M. Y. & Vanderplasschen, A. F. (2011). Identification and localization of the structural proteins of anguillid herpesvirus 1. *Vet Res* **42**, 105.

Walster (1999). Clinical observations of severe mortalities in koi carp, Cyprinus carpio, with gill disease. *Fish Veterinary Journal* **3**, 54-58.

Waltzek, T. B. & Hedrick, R. P. (2004). Koi Herpesvirus update *California veterinarian* **July-August 2004**.

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.

Waltzek, T. B., Kelley, G. O., Stone, D. M., Way, K., Hanson, L., Fukuda, H., Hirono, I., Aoki, T., Davison, A. J. & Hedrick, R. P. (2005). Koi herpesvirus represents a third cyprinid herpesvirus (CyHV-3) in the family Herpesviridae. *J Gen Virol* **86**, 1659-1667.

Yasumoto, S., Kuzuya, Y., Yasuda, M., Yoshimura, T. & Miyazaki, T. (2006). Oral Immunization of Common Carp with a Liposome Vaccine Fusing Koi Herpesvirus Antigen. *Fish Pathology* **41**, 141-145.

Objectives

Common carp (*Cyprinus carpio carpio*), which is cultivated for human consumption, is a one of the most important freshwater fish species in aquaculture. Its colourful subspecies, koi (*Cyprinus carpio koi)* is grown for personal pleasure and competitive exhibitions. Both subspecies are economically important. In the late 1990s, a highly contagious and lethal disease began to cause severe economic losses in these two carp industries worldwide. The causative agent of the disease was initially called koi herpesvirus (KHV) then recently renamed cyprinid herpesvirus 3 (CyHV-3) based on the homology of its genome with previously described cyprinid herpesviruses. CyHV-3 is a member of the newly described family *Alloherpesviridae* which includes herpesviruses that infect fish and amphibians. Because of its economic importance and its numerous original biological properties, CyHV-3 became rapidly an attractive subject for applied and fundamental research.

The goal of this thesis was to identify the portal(s) of entry of CyHV-3 in carp. This information is crucial both for fundamental and applied research on CyHV-3. Indeed, the identification of the portal of entry of a viral infection is essential to understand the pathogenesis and the epidemiology of the infection. It is also crucial for the development of efficacious vaccines.

One of the best methods to provide insights into the viral portal of entry is the use of a recombinant expressing luciferase as a reporter gene and noninvasive whole-body imaging of living animals (IVIS which stands for *In Vivo Imaging System*). The objective of this thesis was to identify CyHV-3 portal(s) of entry using this approach.

As described in the introduction, prolonged CyHV-3 cultivation *in vitro* leads to the spontaneous attenuation of the virus, making the production of CyHV-3 recombinants by classical homologous recombination in eukaryotic cells difficult. To circumvent this problem, the initial goal of this thesis was to clone the entire genome of a pathogenic CyHV-3 strain as a bacterial artificial chromosome (BAC) and to test the usefulness of the BAC clone to generate recombinants. This objective was completed successfully. The results obtained are presented in the first chapter of the experimental section.

In the second chapter, we took profit of the cloning of the CyHV-3 genome as a BAC to produce a recombinant strain encoding a firefly luciferase (LUC) expression cassette inserted in an intergenic region. The CyHV-3 LUC recombinant produced was then used to identify by IVIS the portal of entry of CyHV-3 in carp after inoculation by immersion in water containing the virus. All the results obtained demonstrate that the skin, and not the gills, is the major portal of entry for CyHV-3 in carp.

Fish skin is a complex limiting structure providing mechanical, chemical and immune protection against injury and pathogenic microorganisms. Its mucus layer confers an innate immune protection against pathogen entry. 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. However, despite the abundance of studies on fish skin

immunity and skin bacterial infection, there are little 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. In the third chapter, we used the luciferase CyHV-3 recombinant strain produced and bioluminescence imaging to investigate the roles of epidermal mucus as an innate immune barrier against CyHV-3 entry. Our results demonstrate that the mucus of the skin inhibits CyHV-3 binding to epidermal cells and contains soluble molecules able to neutralize CyHV-3 infectivity.

The data of the two previous chapters demonstrate that the skin is the major portal of entry after inoculation of carp by immersion in water containing CyHV-3. While this model of infection mimics some natural conditions in which infection takes place, other epidemiological conditions could favour entry of virus through the digestive tract. Consequently, in the fourth and last chapter, we investigated the role of the carp digestive tract as a viral portal of entry using bioluminescence imaging. We found that feeding carp with infectious materials induces CyHV-3 entry through infection of the pharyngeal periodontal mucosa.

In conclusion, this study demonstrated that according to epidemiological conditions, CyHV-3 can enter carp either through infection of the skin (immersion in infectious water) or through infection of the pharyngeal periodontal mucosa (feeding on infectious materials). The existence of these two portal of entry adapted to different epidemiological conditions most probably contributes to the high contagious nature of the virus.

Experimental section

Fundamental and applied researches in virology require the production of viral recombinants. As described in the introduction, prolonged CyHV-3 cultivation *in vitro* leads to the spontaneous attenuation of the virus, making the production of CyHV-3 recombinants by classical homologous recombination in eukaryotic cells difficult. Recently, the manipulation of large herpesvirus genomes has been facilitated by the use of bacterial artificial chromosome (BAC). These vectors allow the stable maintenance and efficient mutagenesis of the viral genome in *Escherichia coli*, followed by the reconstitution of progeny virions by the transfection of permissive eukaryotic cells with the BAC vectors. The 235-kb genome of human cytomegalovirus was before the publication of the present work the largest herpesvirus genome which has been BAC cloned. BAC cloning is an obvious approach to avoid the problems in the production of CyHV-3 recombinants described above. Consequently, the initial goal of this thesis was to clone the entire genome of a pathogenic CyHV-3 strain and to test the usefulness of the BAC clone to generate recombinants.

The results of the present study demonstrate that despite its large size and its abundant repetitive sequence content, the CyHV-3 genome can be cloned as an infectious BAC that can be used to produce CyHV-3 recombinants using prokaryotic recombination technologies.

Experimental section

1st chapter:

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*

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**Author contribution: G. Fournier demonstrated the stability of the KHV FL BAC in bacteria, the possibility to reconstitute infectious particle by transfection of the BAC into permissive cells, and the potential of the BAC to produce recombinants using prokaryotic recombination technologies.*

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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*[▽]

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Koi herpesvirus (KHV) is the causative agent of a lethal disease in koi and common carp. In the present study, we describe the cloning of the KHV genome as a stable and infectious bacterial artificial chromosome (BAC) clone that can be used to produce KHV recombinant strains. This goal was achieved by the insertion of a *loxP*-flanked BAC cassette into the thymidine kinase (TK) locus. This insertion led to a BAC plasmid that was stably maintained in bacteria and was able to regenerate virions when permissive cells were transfected with the plasmid. Reconstituted virions free of the BAC cassette but carrying a disrupted TK locus (the FL BAC-excised strain) were produced by the transfection of Cre recombinase-expressing cells with the BAC. Similarly, virions with a wild-type revertant TK sequence (the FL BAC revertant strain) were produced by the cotransfection of cells with the BAC and a DNA fragment encoding the wild-type TK sequence. Reconstituted recombinant viruses were compared to the wild-type parental virus in vitro and in vivo. The FL BAC revertant strain and the FL BAC-excised strain replicated comparably to the parental FL strain. The FL BAC revertant strain induced KHV infection in koi carp that was indistinguishable from that induced by the parental strain, while the FL BAC-excised strain exhibited a partially attenuated phenotype. Finally, the usefulness of the KHV BAC for recombination studies was demonstrated by the production of an ORF16-deleted strain by using prokaryotic recombination technology. The availability of the KHV BAC is an important advance that will allow the study of viral genes involved in KHV pathogenesis, as well as the production of attenuated recombinant candidate vaccines.

Common carp (*Cyprinus carpio carpio*) is the most widely cultivated fish for human consumption mainly in Asia, Europe, and the Middle East (3). In contrast, the koi (*Cyprinus carpio koi*) subspecies is cultivated as an expensive, beautiful, and colorful pet fish for personal pleasure or competitive showing, especially in Japan but also worldwide (3). Recently, koi herpesvirus (KHV) was identified as the cause of mass mortality among koi and common carp in Israel, the United States, and Germany (7, 21, 22). The intensive culture of common carp, koi shows, and international trading have unfortunately contributed to the rapid global spread of highly contagious and extremely virulent KHV disease (19, 27, 35). Since its emergence, KHV has caused severe financial and economic losses in both koi and common carp culture industries worldwide (20, 34).

The genome of KHV comprises a linear double-stranded DNA sequence of ~295 kb (2, 24), similar to that of cyprinid herpesvirus 1 (41) but larger than those of other *Herpesviridae* members, which generally range from 125 to 240 kb. The sequence of the KHV 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 other double-stranded DNA viruses, such as other herpesviruses, poxviruses, iridoviruses, and other large DNA viruses (24, 41).

Since the first isolation of KHV, an increasing number of studies have been devoted to the virus. They have reported data related to viral gene content (2, 4, 13, 14, 24–26, 41), pathogenesis (12, 13, 33, 38), epidemiology (24, 32), the diagnosis of KHV infection (1, 4, 14, 15, 17, 18, 37), and control (31, 34). However, no information on the roles of individual KHV genes in the biology of KHV infection or in pathogenesis has been published to date. Two reasons can explain this lacuna. Firstly, the KHV genome sequence has been published only very recently (2). Secondly, prolonged KHV cultivation in vitro leads to the spontaneous attenuation of the virus, making the production of KHV recombinants by classical homologous recombination in eukaryotic cells difficult (34).

Recently, the manipulation of large herpesvirus genomes has been facilitated by the use of bacterial artificial chromosome (BAC) vectors (6, 40). These vectors allow the stable maintenance and efficient mutagenesis of the viral genome in *Escherichia coli*, followed by the reconstitution of progeny virions by the transfection of permissive eukaryotic cells with the

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BAC plasmid. Several herpesviruses have been successfully propagated as infectious BAC clones. The 235-kb genome of human cytomegalovirus is to date the largest herpesvirus genome which has been BAC cloned (6).

BAC cloning is an obvious approach to avoid the problems in the production of KHV recombinants described above. However, the large size of the KHV genome and its abundant repetitive sequence content (2) are two intrinsic features of KHV that may render its BAC cloning difficult.

In the present study, we describe for the first time the cloning of the KHV genome as a stable and infectious BAC clone. Several recombinant strains were derived from the BAC clone by using homologous recombination in eukaryotic cells and prokaryotic recombination technology. The comparison of these recombinant strains in vivo revealed that thymidine kinase (TK) gene disruption led to the partial attenuation of KHV and that the deletion of ORF16, encoding a putative G protein-coupled receptor (GPCR), did not affect KHV virulence. The availability of the KHV BAC is an important advance that will allow the study of viral genes involved in KHV pathogenesis, as well as the production of attenuated recombinant candidate vaccines.

MATERIALS AND METHODS

Cells and virus. *Cyprinus carpio* brain (CCB) cells (30) were cultured in minimum essential medium (Invitrogen) containing 4.5 g of glucose (D-glucose monohydrate; Merck)/liter and 10% fetal calf serum (FCS). Cells were cultured at 25°C in a humid atmosphere containing 5% CO_2. The KHV FL strain was isolated from a kidney of a fish that died from KHV infection (CER, Marloie, Belgium). FL stands for François Lieffrig, who isolated the strain.

BAC cloning of KHV. Firstly, a 1,137-bp DNA fragment corresponding to the TK open reading frame (ORF; ORF55) and ORF56 of the KHV genome was amplified by PCR using KHV FL DNA as a template. The following primers were used for the amplification: the forward primer TKfw (5′-ATGGCTATGC TGGAACTGGTG-3′) and the reverse primer TKrev (5′-CTCAACAGGGAA GAGTGGCG-3′), corresponding to nucleotides 1 to 21 of the KHV TK ORF and nucleotides 279 to 297 of ORF56 (GenBank accession no. for the KHV genome, DQ177346), respectively. The amplification product was sequenced and TA cloned into the pGEM-T Easy vector (Promega), resulting in pGEMT-TK (Fig. 1A). A BAC cassette was released by PmeI digestion of the pBeloBAC-Modified-EGFPNeo vector (11) and then ligated into the RsrII site of the pGEMT-TK vector, resulting in the pGEMT-TKBAC vector (Fig. 1A), in which the BAC cassette is flanked by KHV sequences. These KHV homologous sequences were exploited to produce the KHV FL BAC strain by homologous recombination in eukaryotic cells (Fig. 1B). Briefly, freshly seeded CCB cells were infected with KHV at a multiplicity of infection (MOI) of 0.5 PFU/cell. After an incubation period of 2 h, cells were transfected with circular pGEMT-TKBAC by using Lipofectamine Plus (Invitrogen). Four days postinfection (pi), cell supernatant was harvested and inoculated onto confluent CCB cell monolayers (10^6 cells per 9.5 cm^2) in the presence of G418 (final concentration of 500 μg/ml). This step was repeated three times, leading to infected cultures containing predominantly the KHV FL BAC recombinant strain. This viral preparation was inoculated onto freshly seeded CCB cells at a MOI of 1 PFU/cell. The circularized form of the viral BAC recombinant genome was extracted 20 h pi as described previously (29), and 2 μg of DNA was introduced into *E. coli* DH10B cells (Invitrogen) by electroporation (at 2,250 V, 132 Ω, and 40 μF) as described elsewhere (36). Electroporated cells were plated immediately onto solid-Luria-Bertani medium plates supplemented with chloramphenicol (17 μg/ml). Note that it is crucial at this stage to avoid liquid preculture in order to avoid the preferential growing of bacteria containing incomplete KHV BAC plasmids.

Reconstitution of infectious virus from the KHV FL BAC plasmid. Permissive CCB cells were transfected with the FL BAC plasmid by using Lipofectamine Plus (Invitrogen) in order to produce the FL BAC recovered strain. To produce a wild-type revertant strain derived from the BAC, CCB cells were cotransfected with the FL BAC plasmid and the pGEMT-TK vector (molar ratio, 1:75). Seven days posttransfection, viral plaques negative for enhanced green fluorescent protein (EGFP) expression were picked and enriched by three successive rounds of plaque purification. Similarly, to reconstitute virions with the BAC cassette excised from the viral genome, CCB cells were cotransfected with the FL BAC plasmid and the pEFIN3-NLS-Cre vector, encoding Cre recombinase fused to a nuclear localization signal (16) (molar ratio, 1:70).

Southern blotting. Southern blot analysis was performed as described previously (28). Several probes were used. The TK probe was produced by PCR using the TKfw and TKrev primers described above and the KHV FL genome as a template. The terminal repeat (TR) probe corresponded to nucleotides 3817 to 4228 of the left TR and nucleotides 276494 to 276905 of the right TR of the KHV genome. The BAC probe was released from the pBeloBACModified-EGFPNeo vector by PmeI digestion. The ORF16 probe was produced by PCR using ORF16fw and ORF16rev primers corresponding to nucleotides 1 to 50 and 1027 to 1077 of KHV ORF16, respectively.

Indirect immunofluorescence staining. CCB cells were fixed and permeabilized with acetone-ethanol (50:50, vol/vol) for 10 min at −20°C. Immunofluorescence staining (incubation and washes) was performed in phosphate-buffered saline containing 10% FCS. Samples were incubated at 25°C for 45 min with mouse monoclonal antibody 8G12 raised against an unidentified KHV antigen expressed in the nuclei of infected cells. After three washes, samples were incubated at 25°C for 30 min with Alexa Fluor 568-conjugated goat anti-mouse immunoglobulin G (heavy and light chains [GAM 568; 2 μg/μl; Molecular Probes]) as the secondary conjugate.

Microscopy analysis. Epifluorescence microscopy analysis was performed with a DMIRBE microscope (Leica) equipped with a DC 300F charge-coupled device camera (Leica) as described previously (39).

Multistep growth curves. Triplicate cultures of CCB cells were infected at a MOI of 0.5 PFU/cell. After an incubation period of 2 h, cells were washed with phosphate-buffered saline and then overlaid with Dulbecco's modified essential medium (Invitrogen) containing 4.5 g of glucose/liter and 10% FCS. The supernatants of infected cultures were harvested at successive intervals after infection, and the amount of infectious virus was determined by plaque assays with CCB cells as described previously (9).

Production of the KHV FL BAC recombinant plasmid by *galK* positive selection of bacteria. A KHV FL BAC recombinant plasmid with the deletion of ORF16 (encoding a putative GPCR) was produced using *galK* positive selection of bacteria as previously described (42). The recombination fragment consisted of a galactokinase gene (*galK*) flanked by 50-bp sequences corresponding to the beginning and the end of KHV ORF16. This fragment was produced by PCR using the p*galK* vector (42) as a template, the forward primer 16galfw (5′-ATGAAACCTCTGGGTCTT TTTGTTTCTGTGCTCGGGCTGCTTGCCCTGTCCCTGTTGACAATTAATC ATCGGCA-3′), and the reverse primer 16galrev (5′-TCATAGGACGCCATCGG TTGAGTTCGCTGCGGCTGCGACTCCCAGTCCTCTCAGCACTGTCCTGC TCCTT-3′). Primer 16galfw consisted of nucleotides 1 to 50 of KHV ORF16 and nucleotides 1 to 24 of the p*galK* vector (42). The reverse primer 16galrev consisted of nucleotides 1027 to 1077 of KHV ORF16 and nucleotides 1212 to 1231 of the p*galK* vector (42).

Induction of KHV disease in fish. Specific-pathogen-free koi carp, with an average weight of 7 g, were kept in 60-liter tanks at 24°C. Several groups of fish, each comprising 10 carp (with the exception of mock-infected groups, which consisted of 13 carp), were kept in separate tanks. Koi carp were infected by intraperitoneal (IP) injection with 0.1 ml containing 3×10^2 PFU. The viral inoculums were titrated before inoculation and back titrated after inoculation to ensure that the doses were equivalent among groups. The control group (mock infected) was injected with culture medium under the same conditions. Fishes were examined daily for clinical signs of KHV disease, and dead fishes were removed. The animal study was accredited by the local ethics committee of the University of Liège (Belgium).

Detection of KHV genome by PCR. DNA was extracted from tissues of fish by using the QIAamp DNA mini kit (Qiagen). PCR amplification was performed using 25 ng of total DNA as a template and the TKfw-TKrev and ORF16fw-ORF16rev primer pairs described above.

RESULTS

Cloning of the KHV genome in *E. coli*. The goal of the present study was to clone the genome of KHV as a stable and infectious BAC plasmid. When we started this project, very few sequences from KHV were available. The TK locus was one of the few KHV genes to have been sequenced. This locus was selected for the insertion of the BAC cassette, as TKs encoded

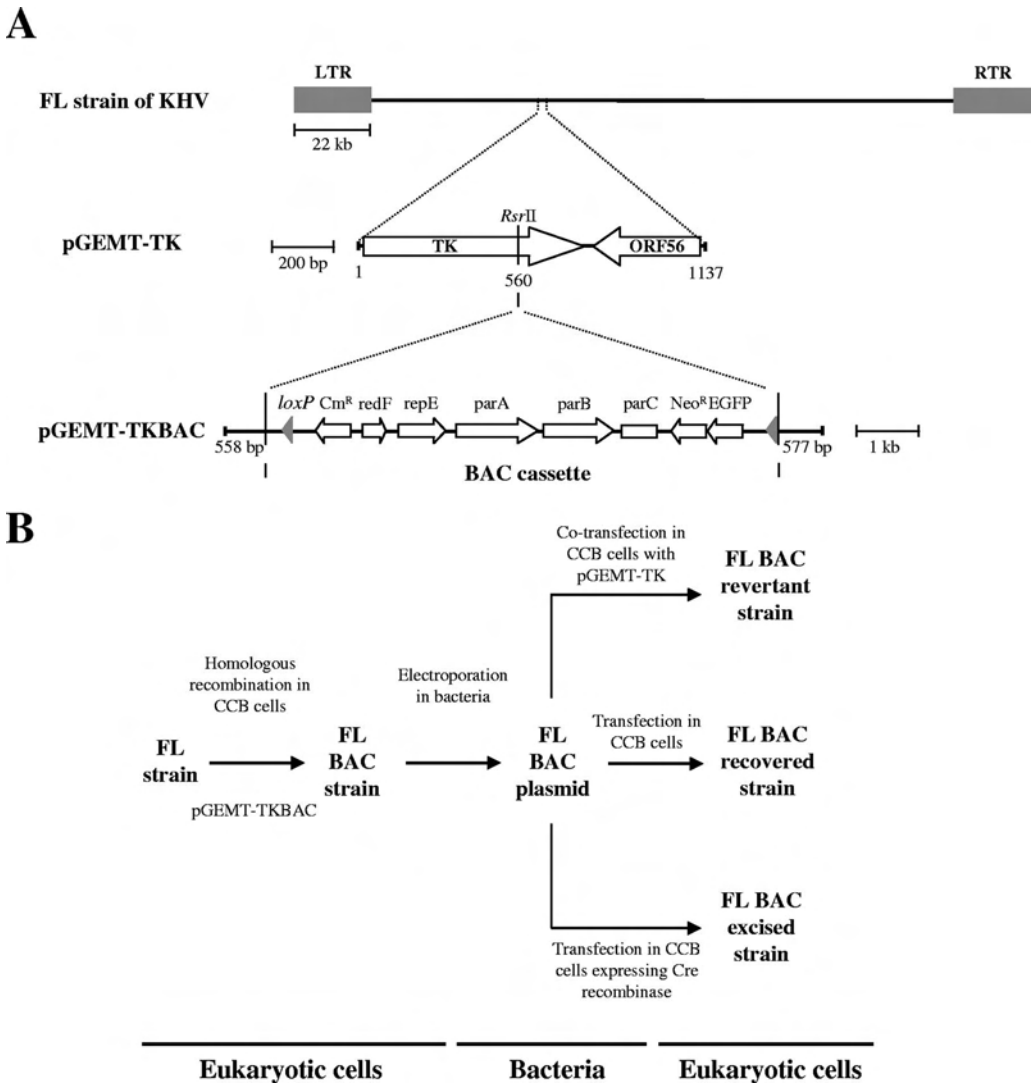

FIG. 1. Schematic representation of the strategy used to produce the infectious KHV FL BAC plasmid. (A) The genome of the KHV FL strain, flanked by two TRs (the left TR [LTR] and the right TR [RTR]), is shown at the top. A *loxP*-flanked BAC cassette was inserted into the RsrII sites of the TK ORF of the pGEMT-TK vector, resulting in pGEMT-TKBAC. (B) Flow chart of steps performed to produce the KHV FL BAC plasmid, to control its infectivity, and to demonstrate the possibility of removing the *loxP*-flanked BAC cassette from the genome of reconstituted virus or to produce a wild-type revertant strain derived from the FL BAC plasmid.

by herpesviruses and poxviruses have been shown previously to be dispensable for viral growth in vitro (8a, 30a).

The strategy depicted in Fig. 1 was used for the BAC cloning of KHV. This approach required as a first step the production of a recombinant strain called KHV FL BAC. The molecular structure of this strain was confirmed by a combined SacI restriction endonuclease and Southern blotting approach (Fig. 2). In the parental FL strain, the TK ORF was contained in a DNA fragment of approximately 5.2 kb. In the FL BAC strain, as a consequence of the BAC cassette insertion into the TK locus, the TK sequence was distributed into two fragments of approximately 5.3 and 9.1 kb (Fig. 2). Sequencing of the regions used to target homologous recombination confirmed that the FL BAC strain had the correct molecular structure (data not shown).

Next, we tried to clone circular intermediates of the FL BAC genome into bacteria by classical approaches that we have used successfully in the past for the BAC cloning of other herpesviruses (9, 11, 16). Surprisingly, despite the screening of more than 500 independent clones, we were unable to select a single clone carrying the FL BAC genome. The BAC plasmids obtained generated heterogeneous restriction profiles with only a few bands corresponding to the expected restriction profile (data not shown). Due to the large size of the KHV genome, one may postulate that bacteria carrying incomplete KHV BAC plasmids may have a selective advantage over bacteria carrying a full-length KHV BAC clone and, consequently, that the liquid culture of transformed bacteria (performed before the plating of the bacteria onto solid medium) may lead to the selection of bacteria with BAC plasmids comprising only part of the KHV genome. To test this hypothesis, *E. coli* cells were plated immediately onto solid Luria-Bertani medium after

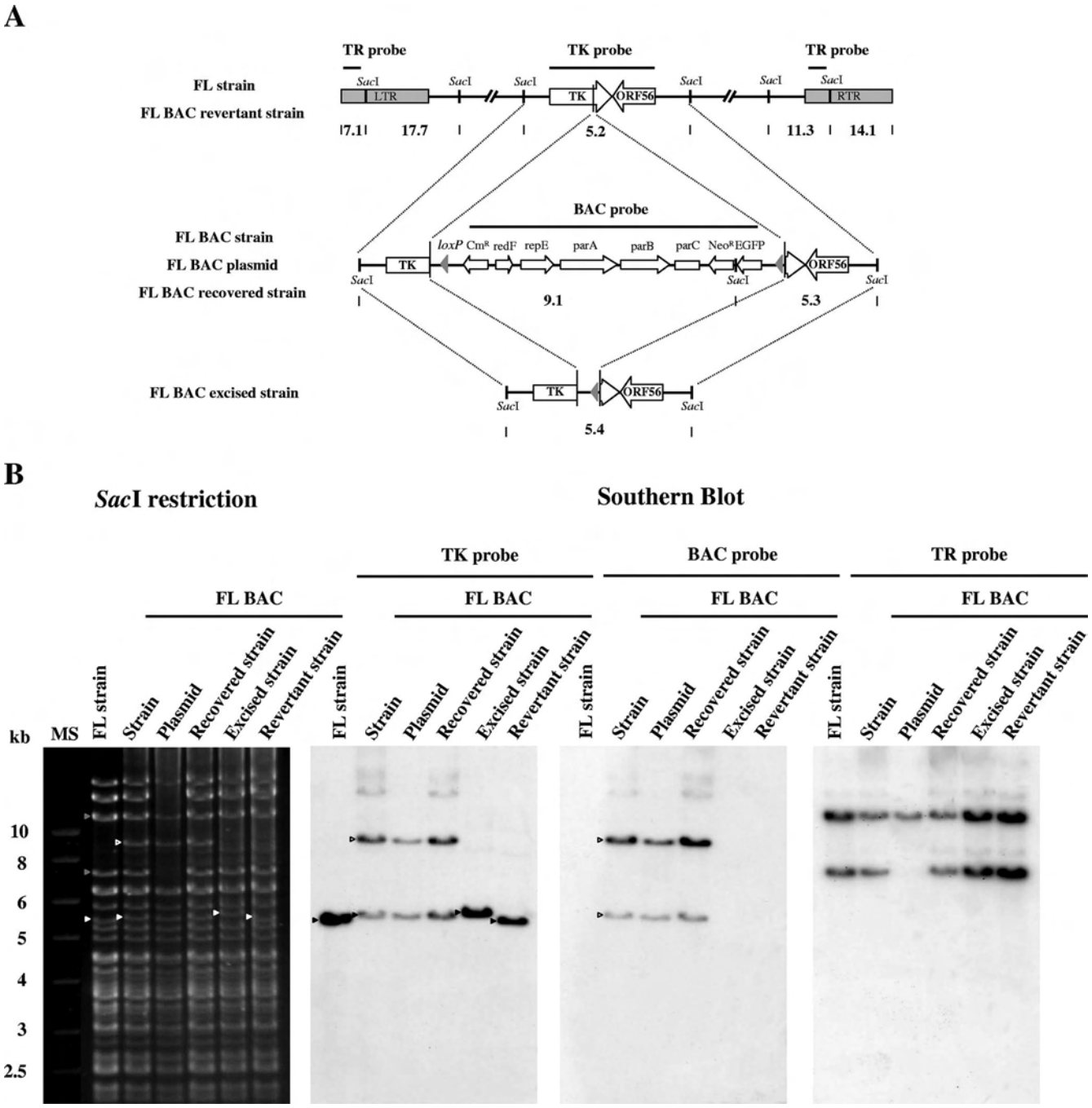

FIG. 2. Structural analysis of the KHV FL BAC plasmid and derived strains. (A) Schematic representation of some of the fragments generated by SacI enzymatic restriction. The genomes of the KHV FL and KHV FL BAC revertant strains are shown at the top. TK, BAC, and TR probes are indicated by bold horizontal lines. Fragment sizes in kilobases are indicated. Note that this cartoon is not drawn to scale. LTR, left TR; RTR, right TR. (B) The KHV FL BAC plasmid and the genomes of the KHV FL, FL BAC, FL BAC recovered, FL BAC-excised, and FL BAC revertant strains were analyzed by SacI restriction (agarose gel, first panel) and further tested by Southern blotting using probes corresponding to the TK ORF (second panel), the BAC cassette (third panel), or the TRs (fourth panel). Black and white arrowheads and open arrowheads indicate restriction fragments containing the TK ORF and the BAC cassette, respectively. Gray arrowheads indicate restriction fragments hybridizing with the TR probe. Marker sizes (MS) are indicated on the left.

electroporation. This approach led to BAC plasmids comprising most of the restriction fragments found in the FL BAC strain genome. However, only 1 to 2% of the clones exhibited a restriction profile comparable to that of the FL BAC strain genome. One of these correct clones was characterized by a combined SacI restriction endonuclease and Southern blotting approach (Fig. 2). Due to the circularization of the genome in bacteria, the two bands encompassing the extremities of the

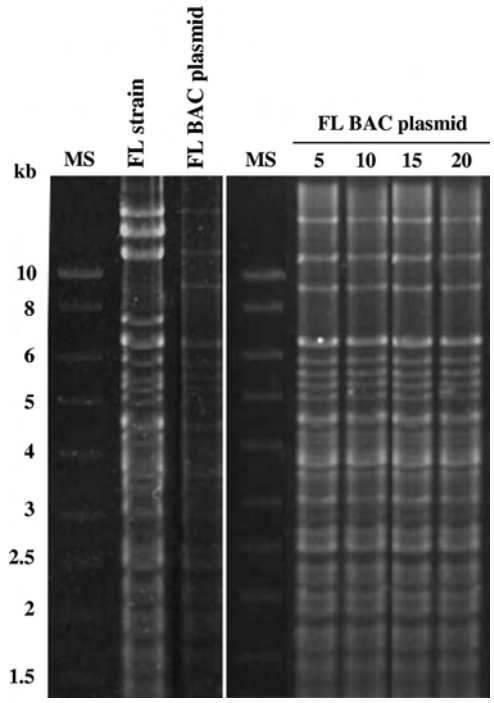

FIG. 3. Stability of the FL BAC plasmid in *E. coli*. DH10B cells containing the FL BAC plasmid were passaged every day at a ratio of 1:100 (vol/vol) for 20 consecutive days. On the indicated days, BAC DNA from the culture was prepared. Finally, BAC DNA samples collected at various intervals were compared with parental FL strain and FL BAC strain DNA by SacI digestion and agarose gel electrophoresis. Marker sizes (MS) are indicated on the left.

left and the right terminal repeats (7.1 and 14.1 kb) present in the FL and FL BAC strains were missing in the FL BAC plasmid (Fig. 2). However, the restriction profile of the FL BAC plasmid did not reveal the expected fused fragment of 21.2 kb (Fig. 2). This observation suggested that the BAC contains a single copy of the terminal repeat. To test this hypothesis, SacI profiles were analyzed by Southern blotting using the TR probe. This analysis revealed the presence of two bands (7.1 and 11.3 kb) in the FL and FL BAC strain profiles and only a single band (11.3 kb) in the FL BAC plasmid profile (Fig. 2). These results demonstrate that the FL BAC plasmid contains only a single copy of the terminal repeat.

Stability of the KHV genome in *E. coli*. BAC plasmids are usually propagated in bacteria carrying a *recA* mutation that minimizes recombination. However, the large size and the complex structure of the KHV genome may lead to relative instability of the FL BAC plasmid (25). To assess the stability of the KHV genome as a BAC, bacteria containing the FL BAC plasmid were serially cultured for 20 consecutive days (about 130 generations). After various periods of culture, the BAC plasmids were isolated and characterized by SacI endonuclease digestion (Fig. 3). No difference among plasmids grown for various periods of time was observed, demonstrating a high level of stability of the KHV genome in *E. coli*.

Reconstitution of infectious virus from the FL BAC plasmid. The usefulness of a herpesvirus BAC clone requires the ability to reconstitute infectious particles from the BAC plasmid. Consequently, we tested whether infectious particles could be produced by the transfection of CCB cells with the FL BAC plasmid (Fig. 1B). Six days posttransfection, viral syncytia expressing EGFP were detected. SacI restriction analysis of the DNA of reconstituted virus (the FL BAC recovered strain) revealed a restriction profile identical to the pattern observed for the FL BAC strain (Fig. 2). These data demonstrate that the BAC is able to regenerate the entire genome and infectious particles even if it includes only a single terminal repeat. To excise the BAC cassette from the genomes of reconstituted virions, CCB cells were cotransfected with the FL BAC plasmid and a Cre recombinase-expressing vector (Fig. 1B). The deletion of the BAC cassette was monitored by the disappearance of EGFP fluorescence and by a combined restriction endonuclease and Southern blotting approach (Fig. 2). The *cre-loxP*-mediated excision of the BAC cassette left a sequence of 172 bp in the TK ORF, leading to a SacI restriction fragment slightly larger than the corresponding wild-type fragment (Fig. 2). The 172-bp sequence consists of one *loxP* site (34 bp) and the sequences of the BAC cassette upstream (126 bp) and downstream (12 bp) of the *loxP* sites. Due to this insertion of a 172-bp foreign sequence into the TK ORF, the FL BAC-excised strain expressed a truncated form of TK corresponding to the first 185 amino acids (aa) of the wild-type protein (217 residues). Finally, to generate a revertant strain, CCB cells were cotransfected with the FL BAC plasmid and the pGEMT-TK vector (Fig. 1B). A revertant recombinant was selected on the basis of the nonexpression of EGFP. Restriction analysis revealed a profile identical to the pattern observed for the parental wild-type FL strain (Fig. 2).

Additional characterization of FL BAC-derived strains in cell cultures was performed. Firstly, microscopic examination of immunostained viral syncytia did not reveal differences among recombinants (Fig. 4A). Secondly, in order to investigate the putative effects of the recombination processes on viral growth in vitro, all recombinant strains were compared using a multistep growth assay (Fig. 4B). All viruses tested exhibited similar growth curves ($P \leq 0.05$), leading to the conclusion that TK disruption does not affect KHV replication in vitro and that the KHV genome can support a large insertion (of at least 9.2 kb) despite its large size.

Production of an FL BAC recombinant plasmid by mutagenesis in bacteria with *galK* positive selection. The usefulness of a BAC clone for recombination studies relies on the possibility to use it for the production of recombinants by prokaryotic mutagenesis methods. To test the usefulness of the FL BAC clone, we produced a KHV recombinant strain with the deletion of ORF16 (encoding a putative GPCR) by using *galK* positive selection of bacteria. The molecular structure of the recombinant plasmid was confirmed by a combined SacI restriction endonuclease and Southern blotting approach (Fig. 5). In the parental FL strain and in the KHV FL BAC plasmid, ORF16 was contained in a DNA fragment of approximately 4.8 kb, whereas in the KHV FL BAC ΔORF16 plasmid, the corresponding fragment had a size of approximately 5 kb due to the deletion of most of ORF16 and the insertion of the *galK* cassette. This band encompassing the *galK* cassette is slightly visible on the Southern blot due to the short ORF16 nucleotide sequence left after deletion. Next, to reconstitute virions and to excise the BAC cassette from the genome of the recombinant plasmid, CCB cells were cotransfected with the KHV FL BAC

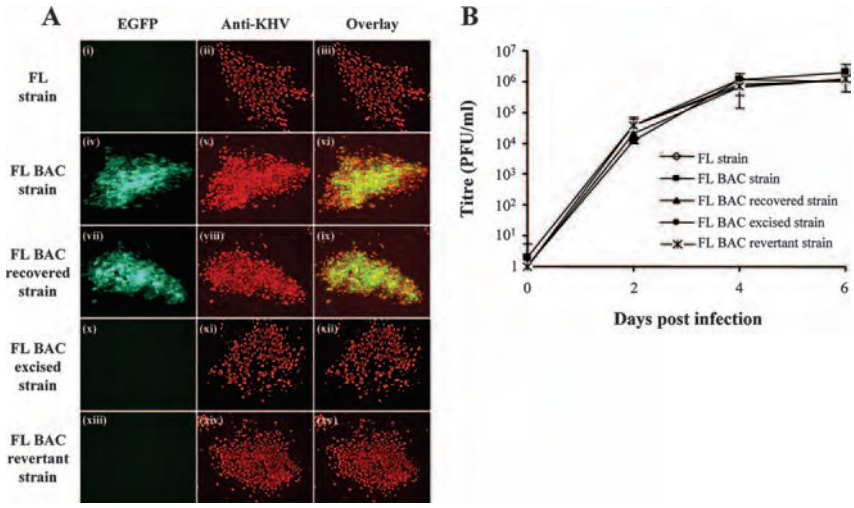

FIG. 4. Characterization of KHV strains derived from the FL BAC plasmid. (A) Epifluorescence analysis of KHV syncytia. CCB cells were infected (MOI of 0.1 PFU/cell) with FL, FL BAC, FL BAC recovered, FL BAC-excised, and FL BAC revertant strains and were overlaid with Dulbecco's modified essential medium containing 10% FCS and 0.6% (wt/vol) carboxymethyl cellulose (Sigma) to obtain isolated syncytia. Seven days pi, syncytia were revealed by indirect immunofluorescent staining using monoclonal antibody 8G12 and GAM 568 as the primary and secondary antibodies, respectively. The three horizontal panels in each set represent analyses of the same syncytium. Panels i, iv, vii, x, and xiii and panels ii, v, viii, xi, and xiv were analyzed for EGFP and GAM 568 fluorescent emissions, respectively. The merged EGFP and Alexa signals are shown in panels iii, vi, ix, xii, and xv. The side of each panel corresponds to 10 μm of the specimen. (B) Replication kinetics of KHV recombinant strains were compared with those of the parental KHV FL strain as described in Materials and Methods. The data presented are the means ± standard errors of triplicate measurements.

ΔORF16 plasmid and a Cre recombinase-expressing plasmid. The deletion of the BAC cassette was monitored by the disappearance of EGFP fluorescence (data not shown) and by a combined restriction endonuclease and Southern blotting approach (Fig. 5). As described earlier, the *cre-loxP*-mediated deletion of the BAC cassette leaves a sequence of 172 bp disrupting the TK ORF. Consequently, the FL BAC ΔORF16-excised strain has a disrupted TK locus and a deletion of ORF16.

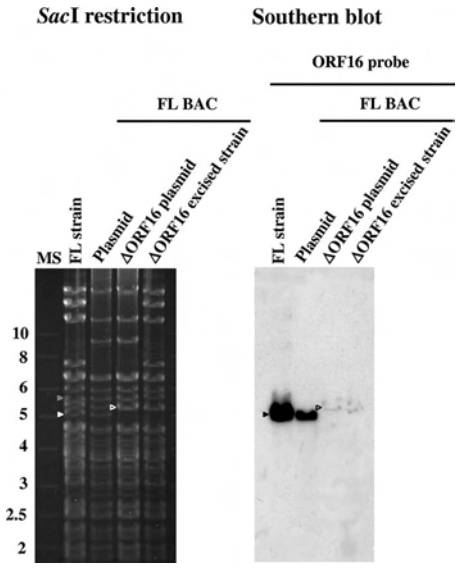

FIG. 5. Structural analysis of the KHV FL BAC *galK* recombinant plasmid. The KHV FL BAC plasmid, the derived FL BAC ΔORF16 plasmid, and the genome of the KHV FL BAC ΔORF16-excised strain were analyzed by SacI restriction (agarose gel) and further tested by Southern blotting using a probe corresponding to ORF16. The KHV FL strain was used as a control. White and black arrowheads and open arrowheads indicate restriction fragments containing ORF16 and the *galK* cassette, respectively. The gray arrowhead indicates a restriction fragment containing the TK ORF. Marker sizes (MS) in kilobases are indicated on the left.

Pathogenicities of FL BAC-derived strains in koi carp. The strains derived from the FL BAC plasmid described above facilitated the testing of the effect of TK disruption (in FL BAC-excised and FL BAC revertant strains) and the effect of TK disruption and ORF16 deletion (in the FL BAC ΔORF16-excised strain) on the virulence of KHV. To address the effect of TK disruption on KHV virulence, the parental FL, FL BAC excision, and FL BAC revertant strains were compared by IP inoculation of naïve koi carp (Fig. 6A). The parental FL strain induced all the clinical signs associated with KHV disease, including apathy, the folding of the dorsal fin, increased mucus secretions, suffocation, erratic swimming, and the loss of equilibrium. The FL strain induced a mortality rate of 80%. At necropsy, the discoloration of gill filaments, herpetic skin lesions, and necrotic nephritis were observed for most fishes. In comparison to the FL parental strain, the FL BAC-excised strain exhibited a partially attenuated phenotype characterized by the production of similar clinical signs and lesions but with reduced intensities. Consistent with the attenuation observed, the mortality rate of fishes infected with the FL BAC-excised strain was reduced to 40%. Importantly, the virulence of the FL BAC revertant strain was similar to that of the parental FL strain.

The effect of TK disruption and ORF16 deletion was assessed in the same way, by IP inoculation of naïve koi carp. The parental FL strain and the FL BAC-excised strain were used as controls (Fig. 6B). Fishes infected with the parental FL strain developed KHV disease as described above (80% mortality).

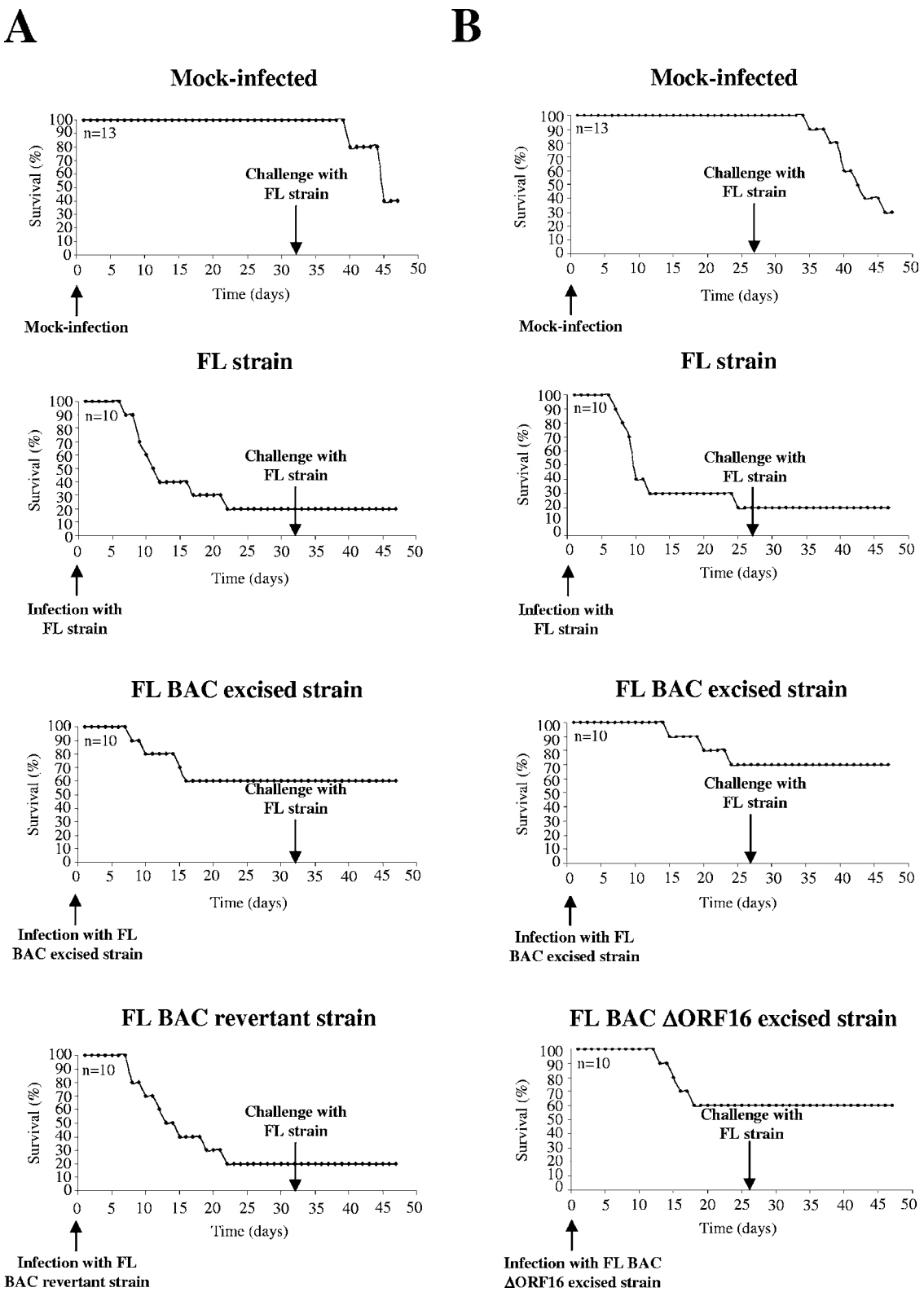

FIG. 6. Cumulative survival rates of carp infected with FL BAC plasmid-derived strains. (A) On day 0, four groups, each consisting of 10 koi carp (with the exception of mock-infected groups, consisting of 13 carp), were inoculated by IP injection with mock-infected culture medium and culture medium containing 3×10^2 PFU of FL, FL BAC-excised, and FL BAC revertant strains. On day 32 pi, surviving fishes were challenged by IP injection with the parental FL strain. (B) On day 0, four groups, each consisting of 10 koi carp (with the exception of mock-infected groups, consisting of 13 carp), were inoculated by IP injection with mock-infected culture medium and culture medium containing 3×10^2 PFU of FL, FL BAC-excised, and FL BAC ΔORF16-excised strains. On day 27 pi, surviving fishes were challenged by IP injection with the parental FL strain. Percentages of surviving carp are expressed according to days pi. The results presented are representative of three independent experiments.

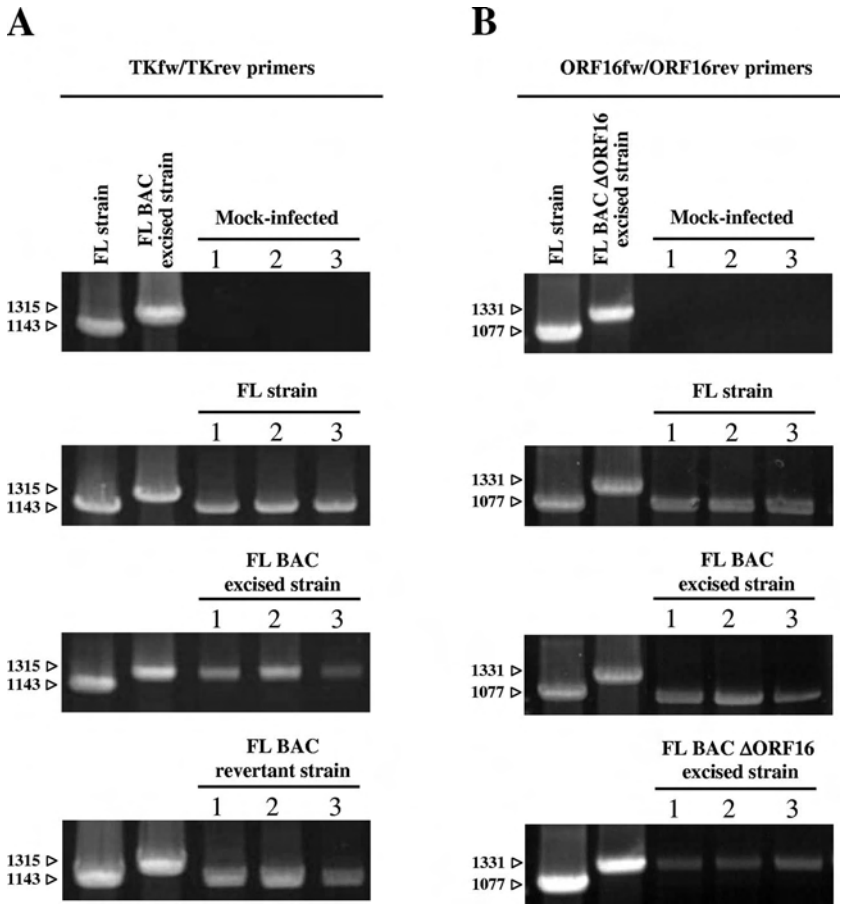

FIG. 7. PCR detection and characterization of KHV genomes recovered from infected dead carp. (A) DNA was extracted from the intestines of three mock-infected carp (selected randomly before the challenge) and from three dead carp from each of the groups infected with the FL, FL BAC-excised, and FL BAC revertant strains. PCRs were performed with the TKfw/TKrev pair of primers. FL strain DNA and FL BAC-excised strain DNA were used as controls. (B) DNA was extracted from the intestines of mock-infected carp and from dead carp infected with the FL, FL BAC-excised, and FL BAC ΔORF16-excised strains. PCRs were performed with the ORF16fw/ORF16rev pair of primers. FL strain DNA and FL BAC ΔORF16-excised strain DNA were used as controls. The images are photographs of agarose gels. Numbers on the left of each gel are marker sizes.

In comparison to the parental strain, the FL BAC excision and FL BAC ΔORF16-excised strains exhibited partially attenuated phenotypes, inducing 30 and 40% mortality, respectively. This result suggests that ORF16, encoding a putative GPCR, does not contribute to KHV virulence significantly.

To control the infection of all groups of fish with the correct viral strain and to exclude any possibility of virus spread among tanks, PCR assays were performed on three randomly selected dead fishes from each infected group and three mock-infected fishes randomly selected before the challenge (Fig. 7). PCRs performed with the TKfw/TKrev (Fig. 7A) or ORF16fw/ORF16rev (Fig. 7B) primers confirmed that all samples from infected groups contained the KHV genome, while the sizes of the amplicons excluded the possibility of viral spread among the groups of fishes.

Finally, on day 32 (Fig. 6A) or day 27 (Fig. 6B) pi, the fishes that survived the primary inoculation were challenged by IP injection with the parental FL strain. Fishes were monitored until day 47. Independently of the strain used for the primary infection, none of the fishes died after the challenge or exhibited clinical signs of disease. In contrast, the challenge of the mock-infected control group led to mortality rates of 60% (Fig. 6A) and 70% (Fig. 6B) by day 15 postchallenge.

DISCUSSION

KHV is the etiological agent of an emerging disease which is highly contagious and extremely virulent and has a high mortality rate (25). Since the discovery of KHV in 1996, an increasing number of studies have been devoted to KHV. However, to date no information on the roles of individual KHV genes in the biology of KHV infection or in pathogenesis has been published. Similarly, there is a lack of safe and efficacious attenuated recombinant vaccines for the control of KHV disease. These lacunas are a consequence of the difficulty of generating KHV recombinant viruses by classical homologous recombination in eukaryotic cells. This problem can be circumvented by the use of BAC cloning technology (6, 40).

In the present study, we describe for the first time the cloning of the KHV genome as a stable and infectious BAC clone. The KHV BAC clone had several interesting features: (i) it was stable when propagated in bacteria, even over long periods

of culture corresponding to approximately 130 generations; (ii) it was infectious, as demonstrated by its ability to generate infectious virions after the transfection of permissive cells; (iii) the BAC cassette could be excised from the genome of reconstituted virus; (iv) the insertion of a large DNA sequence into the KHV genome did not affect the ability of KHV to replicate in vitro; (v) importantly, the replication of the FL BAC revertant strain was comparable to that of the FL strain, and the FL BAC revertant strain induced KHV disease in koi carp that was indistinguishable from that induced by the virulent parental strain; and (vi) finally, the usefulness of the KHV BAC clone for recombination studies was demonstrated by the production of an ORF16-deleted strain by prokaryotic recombination technology.

Even if the primary goal of the present study was not to investigate the role of KHV TK in pathogenesis, the recombinants derived from the FL BAC clone allowed us to do so. The FL BAC-excised strain encoding a truncated form of TK exhibited a partially attenuated phenotype in carp (Fig. 6). Four hypotheses may explain the partial attenuation observed. A first hypothesis may be the existence of a KHV enzyme that may partially compensate for TK gene deletion. Viral and cellular TKs have been classified into two types which differ in several respects (5). Type I TKs have higher molecular masses, typically around 40 kDa, and are active as homodimers. This subfamily contains herpesvirus TKs (with the exception of KHV TK) and also human mitochondrial TK. The herpes simplex virus type 1 TK is the viral prototype of this group. It is a multifunctional enzyme that possesses kinase activities normally performed by three separate cellular enzymes. It phosphorylates deoxythymidine and deoxyuridine, as does human TK, and deoxycytidine, as does human deoxycytidine kinase, and acts as a thymidylate kinase, as does human TMP kinase (TMPK) (8). TKs of type II include those from *Poxviridae* such as vaccinia virus and variola virus, as well as the human cytosolic TK. Type II TKs have smaller polypeptide chains than type I TKs, being ~25 kDa, but form homotetramers. Moreover, type II TKs have much narrower substrate specificities than type I TKs and phosphorylate only deoxyuridine and/or deoxythymidine. Based on the relatively small size and the nucleotide binding motif of KHV TK, it can be postulated that this TK belongs to type II (10). In poxviruses, the narrower substrate specificities of type II TKs are compensated for by a TMPK gene. Interestingly, the recent sequencing of the KHV genome has revealed the presence of a TMPK ORF (ORF140) (2). It is attractive to speculate that the encoded enzyme may at least partially compensate for the deletion of the KHV TK gene. In support of this hypothesis, it has been shown previously that the replacement of the herpes simplex virus type 1 TK ORF by a human TMPK gene renders the recombinant virus partially competent for replication in mouse sensory ganglia and reactivation from latency upon explant (8). Further studies are required to determine KHV TK and TMPK enzymatic activities and to determine how these enzymes contribute to the pathogenesis in the natural host.

Secondly, the partial attenuation observed with the FL BAC-excised strain may result from residual TK activity expressed by the truncated protein encoded by the FL BAC-excised strain. This hypothesis is very unlikely. Indeed, several studies of herpesviruses and poxviruses have demonstrated previously that the C-terminal region of TK is essential for its activity (23). For example, it has been demonstrated previously that the last 10 residues of the 607-aa-long Epstein-Barr virus TK are essential for its activity (23). In comparison to Epstein-Barr virus TK, KHV TK is rather small, consisting of only 217 aa, among which only the first 185 residues are expressed by the FL BAC-excised strain. A third hypothesis to explain the partial attenuation observed with the FL BAC-excised strain may be that the removal of KHV TK function readily results in a partial-attenuation phenotype in the absence of functional complementation from another virus gene. Finally, a fourth hypothesis may be that host TK may partially replace the eliminated KHV TK.

The usefulness of the KHV BAC clone for recombination studies was demonstrated by the production of an ORF16-deleted strain by using prokaryotic recombination technology. In vivo, the strain induced a mortality rate comparable to that induced by the FL BAC-excised strain, suggesting that ORF16 does not contribute significantly to KHV virulence under the conditions used (Fig. 6B).

In conclusion, this study is the first to report the BAC cloning of a herpesvirus genome as large as that of KHV. The availability of a KHV BAC is an important advance that will allow the study of viral genes involved in KHV pathogenesis, as well as the production of safe and efficacious multiattenuated recombinant candidate vaccines to control KHV infection.

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REFERENCES

1. **Adkison, M. A., O. Gilad, and R. P. Hedrick.** 2005. An enzyme linked immunosorbent assay (ELISA) for detection of antibodies to the koi herpesvirus (KHV) in the serum of koi Cyprinus carpio. Fish Pathol. **40:**53–62.
2. **Aoki, T., I. Hirono, K. Kurokawa, H. Fukuda, R. Nahary, A. Eldar, A. J. Davison, T. B. Waltzek, H. Bercovier, and R. P. Hedrick.** 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.
3. **Balon, E. K.** 1995. Origin and domestication of the wild carp, Cyprinus carpio: from Roman gourmets to the swimming flowers. Aquaculture **129:** 3–48.
4. **Bercovier, H., Y. Fishman, R. Nahary, S. Sinai, A. Zlotkin, M. Eyngor, O. Gilad, A. Eldar, and R. P. Hedrick.** 2005. Cloning of the koi herpesvirus (KHV) gene encoding thymidine kinase and its use for a highly sensitive PCR based diagnosis. BMC Microbiol. **5:**13.
5. **Black, M. E., and D. E. Hruby.** 1990. Quaternary structure of vaccinia virus thymidine kinase. Biochem. Biophys. Res. Commun. **169:**1080–1086.
6. **Borst, E. M., G. Hahn, U. H. Koszinowski, and M. Messerle.** 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.
7. **Bretzinger, A., T. Fischer-Scherl, R. Oumouma, R. Hoffmann, and U. Truyen.** 1999. Mass mortalities in koi, Cyprinus carpio, associated with gill and skin disease. Bull. Eur. Assoc. Fish Pathol. **19:**182–185.
8. **Chen, S. H., W. J. Cook, K. L. Grove, and D. M. Coen.** 1998. Human thymidine kinase can functionally replace herpes simplex virus type 1 thymidine kinase for viral replication in mouse sensory ganglia and reactivation from latency upon explant. J. Virol. **72:**6710–6715.

8a. **Coen, D. M., M. Kosz-Vnenchak, J. G. Jacobson, D. A. Leib, C. L. Bogard, P. A. Schaffer, K. L. Tyler, and D. M. Knipe.** 1989. Thymidine kinase-negative herpes simplex virus mutants establish latency in mouse trigeminal ganglia but do not reactivate. Proc. Natl. Acad. Sci. USA **86:**4736–4740.

9. **Costes, B., M. Thirion, B. Dewals, J. Mast, M. Ackermann, N. Markine-Goriaynoff, L. Gillet, and A. Vanderplasschen.** 2006. Felid herpesvirus 1

glycoprotein G is a structural protein that mediates the binding of chemokines on the viral envelope. Microbes Infect. **8:**2657–2667.

10. **Coupar, B. E., S. G. Goldie, A. D. Hyatt, and J. A. Pallister.** 2005. Identification of a Bohle iridovirus thymidine kinase gene and demonstration of activity using vaccinia virus. Arch. Virol. **150:**1797–1812.
11. **Dewals, B., C. Boudry, L. Gillet, N. Markine-Goriaynoff, L. de Leval, D. M. Haig, and A. Vanderplasschen.** 2006. Cloning of the genome of Alcelaphine herpesvirus 1 as an infectious and pathogenic bacterial artificial chromosome. J. Gen. Virol. **87:**509–517.
12. **Dishon, A., A. Perelberg, J. Bishara-Shieban, M. Ilouze, M. Davidovich, S. Werker, and M. Kotler.** 2005. Detection of carp interstitial nephritis and gill necrosis virus in fish droppings. Appl. Environ. Microbiol. **71:**7285–7291.
13. **Gilad, O., S. Yun, M. A. Adkison, K. Way, N. H. Willits, H. Bercovier, and R. P. Hedrick.** 2003. Molecular comparison of isolates of an emerging fish pathogen, koi herpesvirus, and the effect of water temperature on mortality of experimentally infected koi. J. Gen. Virol. **84:**2661–2667.
14. **Gilad, O., S. Yun, K. B. Andree, M. A. Adkison, A. Zlotkin, H. Bercovier, A. Eldar, and R. P. Hedrick.** 2002. Initial characteristics of koi herpesvirus and development of a polymerase chain reaction assay to detect the virus in koi, Cyprinus carpio koi. Dis. Aquat. Org. **48:**101–108.
15. **Gilad, O., S. Yun, F. J. Zagmutt-Vergara, C. M. Leutenegger, H. Bercovier, and R. P. Hedrick.** 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.
16. **Gillet, L., V. Daix, G. Donofrio, M. Wagner, U. H. Koszinowski, B. China, M. Ackermann, N. Markine-Goriaynoff, and A. Vanderplasschen.** 2005. Development of bovine herpesvirus 4 as an expression vector using bacterial artificial chromosome cloning. J. Gen. Virol. **86:**907–917.
17. **Gray, W. L., L. Mullis, S. E. LaPatra, J. M. Groff, and A. Goodwin.** 2002. Detection of koi herpesvirus DNA in tissues of infected fish. J. Fish Dis. **25:**171–178.
18. **Gunimaladevi, I., T. Kono, M. N. Venugopal, and M. Sakai.** 2004. Detection of koi herpesvirus in common carp, Cyprinus carpio L., by loop-mediated isothermal amplification. J. Fish Dis. **27:**583–589.
19. **Haenen, O. L. M., K. Way, S. M. Bergmann, and E. Ariel.** 2004. The emergence of koi herpesvirus and its significance to European aquaculture. Bull. Eur. Assoc. Fish Pathol. **24:**293–307.
20. **Hedrick, R. P.** 1996. Movement of pathogens with the international trade of live fish: problems and solutions. Rev. Sci. Tech. **15:**523–531.
21. **Hedrick, R. P., O. Gilad, S. Yun, J. Spangenberg, R. Marty, M. Nordhausen, M. Kebus, H. Bercovier, and A. Eldar.** 2000. A herpesvirus associated with mass mortality of juvenile and adult koi, a strain of common carp. J. Aquat. Anim. Health. **12:**44–55.
22. **Hedrick, R. P., R. Marty, M. Nordhausen, M. Kebus, H. Bercovier, and A. Eldar.** 1999. An herpesvirus associated with mass mortality of juvenile and adult koi *Cyprinus carpio*. Fish Health Newsl. **27**(no. 3)**:**7.
23. **Hsu, T. Y., M. W. Liu, Y. R. Chang, C. Y. Pai, M. Y. Liu, C. S. Yang, and J. Y. Chen.** 1996. Functional analysis of C-terminal deletion mutants of Epstein-Barr virus thymidine kinase. J. Gen. Virol. **77:**1893–1899.
24. **Hutoran, M., A. Ronen, A. Perelberg, M. Ilouze, A. Dishon, I. Bejerano, N. Chen, and M. Kotler.** 2005. Description of an as yet unclassified DNA virus from diseased *Cyprinus carpio* species. J. Virol. **79:**1983–1991.
25. **Ilouze, M., A. Dishon, and M. Kotler.** 2006. Characterization of a novel virus causing a lethal disease in carp and koi. Microbiol. Mol. Biol. Rev. **70:**147–156.
26. **Ishioka, T., M. Yoshizumi, S. Izumi, K. Suzuki, H. Suzuki, K. Kozawa, M. Arai, K. Nobusawa, Y. Morita, M. Kato, T. Hoshino, T. Iida, K. Kosuge, and H. Kimura.** 2005. Detection and sequence analysis of DNA polymerase and major envelope protein genes in koi herpesviruses derived from Cyprinus carpio in Gunma prefecture, Japan. Vet. Microbiol. **110:**27–33.
27. **Liu, H., X. Shi, L. Gao, and Y. Jiang.** 2002. Study on the aetiology of koi epizootic disease using the method of nested-polymerase chain reaction assay (nested-PCR). J. Huazhong Agric. Univ. **21:**414–418.
28. **Markine-Goriaynoff, N., L. Gillet, O. A. Karlsen, L. Haarr, F. Minner, P. P. Pastoret, M. Fukuda, and A. Vanderplasschen.** 2004. The core 2 beta-1,6-N-acetylglucosaminyltransferase-M encoded by bovine herpesvirus 4 is not essential for virus replication despite contributing to post-translational modifications of structural proteins. J. Gen. Virol. **85:**355–367.
29. **Morgan, R. W., J. L. Cantello, and C. H. McDermott.** 1990. Transfection of chicken embryo fibroblasts with Marek's disease virus DNA. Avian Dis. **34:**345–351.
30. **Neukirch, M., K. Böttcher, and S. Bunnajrakul.** 1999. Isolation of a virus from koi with altered gills. Bull. Eur. Assoc. Fish Pathol. **19:**221–224.

30a. **Panicali, D., and E. Paoletti.** 1982. Construction of poxviruses as cloning vectors: insertion of the thymidine kinase gene from herpes simplex virus into the DNA of infectious vaccinia virus. Proc. Natl. Acad. Sci. USA. **79:**4927–4931.

31. **Perelberg, A., A. Ronen, M. Hutoran, Y. Smith, and M. Kotler.** 2005. Protection of cultured Cyprinus carpio against a lethal viral disease by an attenuated virus vaccine. Vaccine **23:**3396–3403.
32. **Perelberg, A., M. Smirnov, M. Hutoran, A. Diamant, Y. Bejerano, and M. Kotler.** 2003. Epidemiological description of a new viral disease afflicting cultured Cyprinus carpio in Israel. Isr. J. Aquaculture **55:**5–12.
33. **Pikarsky, E., A. Ronen, J. Abramowitz, B. Levavi-Sivan, M. Hutoran, Y. Shapira, M. Steinitz, A. Perelberg, D. Soffer, and M. Kotler.** 2004. Pathogenesis of acute viral disease induced in fish by carp interstitial nephritis and gill necrosis virus. J. Virol. **78:**9544–9551.
34. **Ronen, A., A. Perelberg, J. Abramowitz, M. Hutoran, S. Tinman, I. Bejerano, M. Steinitz, and M. Kotler.** 2003. Efficient vaccine against the virus causing a lethal disease in cultured Cyprinus carpio. Vaccine **21:**4677–4684.
35. **Sano, M., T. Ito, J. Kurita, T. Yanai, N. Watanabe, M. Satoshi, and T. Iida.** 2004. First detection of koi herpesvirus in cultured common carp Cyprinus carpio in Japan. Fish Pathol. **39:**165–168.
36. **Smith, G. A., and L. W. Enquist.** 2000. A self-recombining bacterial artificial chromosome and its application for analysis of herpesvirus pathogenesis. Proc. Natl. Acad. Sci. USA **97:**4873–4878.
37. **Soliman, H., and M. El-Matbouli.** 2005. An inexpensive and rapid diagnostic method of Koi Herpesvirus (KHV) infection by loop-mediated isothermal amplification. Virol. J. **2:**83.
38. **St.-Hilaire, S., N. Beevers, K. Way, R. M. Le Deuff, P. Martin, and C. Joiner.** 2005. Reactivation of koi herpesvirus infections in common carp Cyprinus carpio. Dis. Aquat. Org. **67:**15–23.
39. **Vanderplasschen, A., and G. L. Smith.** 1997. A novel virus binding assay using confocal microscopy: demonstration that the intracellular and extracellular vaccinia virions bind to different cellular receptors. J. Virol. **71:**4032–4041.
40. **Wagner, M., Z. Ruzsics, and U. H. Koszinowski.** 2002. Herpesvirus genetics has come of age. Trends Microbiol. **10:**318–324.
41. **Waltzek, T. B., G. O. Kelley, D. M. Stone, K. Way, L. Hanson, H. Fukuda, I. Hirono, T. Aoki, A. J. Davison, and R. P. Hedrick.** 2005. Koi herpesvirus represents a third cyprinid herpesvirus (CyHV-3) in the family Herpesviridae. J. Gen. Virol. **86:**1659–1667.
42. **Warming, S., N. Costantino, D. L. Court, N. A. Jenkins, and N. G. Copeland.** 2005. Simple and highly efficient BAC recombineering using galK selection. Nucleic Acids Res. **33:**e36.

The outcome of the infection of a naive host by a virus relies on key virus-host interactions that occur early after contamination takes place. In order to be able to infect cells at the portal of entry, most viruses must first cross extracellular barriers (e.g. mucus), then interact with cellular receptors expressed on live sensitive cells. The entry of viruses in most vertebrates occurs at mucosa; while the skin with its keratinized outermost layer cannot act as a portal of entry.

Several authors postulated that the gills might be the portal of entry for CyHV-3 in carp. This hypothesis relied on several observations. First, the gills have been demonstrated to act as the portal of entry for many fish pathogens. Second, fish expressing CyHV-3 disease have gill lesions, which explain why the virus was initially called Carp interstitial nephritis and gill necrosis virus. Third, the gills (like virtually all tissues) were shown by PCR to contain the viral genome at an early stage of infection. However, no data demonstrating the role of the gills as the portal of entry of CyHV-3 are available.

In contrast the most vertebrates, the epidermis of the skin of teleost fish is a stratified squamous epithelium covering the body surface and investing the fins. Unlike its mammalian counterpart, it is living and capable of mitotic division at all levels, even at the outermost squamous layer. Consequently, potential portals of entry for CyHV-3 in carp include the skin, the mucosa covering the gills and the digestive tract.

In the present study, we investigated the portal of entry of CyHV-3 in carp using bioluminescence imaging. Taking advantage of the recent cloning of the CyHV-3 genome (see chapter 1) as a bacterial artificial chromosome (BAC), we produced a recombinant strain encoding a firefly luciferase (LUC) expression cassette inserted in an intergenic region. All the results obtained with this recombinant strain demonstrate that the skin, and not the gills, is the major portal of entry for CyHV-3 in carp.

Experimental section

2nd chapter:

The major portal of entry of koi herpesvirus in *Cyprinus carpio* is the skin

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** Author contribution: G. Fournier produced the FL BAC 136 LUC strain and contributed to identify the skin as the major portal of entry of CyHV-3 in carp after inoculation by immersion in water containing the virus.*

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The Major Portal of Entry of Koi Herpesvirus in *Cyprinus carpio* Is the Skin▽

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Koi herpesvirus (KHV), recently designated *Cyprinid herpesvirus 3*, is the causative agent of a lethal disease in koi and common carp. In the present study, we investigated the portal of entry of KHV in carp by using bioluminescence imaging. Taking advantage of the recent cloning of the KHV genome as a bacterial artificial chromosome (BAC), we produced a recombinant plasmid encoding a firefly luciferase (LUC) expression cassette inserted in the intergenic region between open reading frame (ORF) 136 and ORF 137. Two viral strains were then reconstituted from the modified plasmid, the FL BAC 136 LUC excised strain and the FL BAC 136 LUC TK revertant strain, including a disrupted and a wild-type thymidine kinase (TK) locus, respectively. In vitro, the two recombinant strains replicated comparably to the parental FL strain. The FL BAC 136 LUC TK revertant strain was shown in vitro to induce a bioluminescent signal allowing the detection of single positive cells as early as 24 h postinfection, while in vivo, it induced KHV infection in carp that was indistinguishable from that induced by the parental FL strain. To identify the KHV portal of entry, carp were analyzed by bioluminescence imaging at different times postinfection with the FL BAC 136 LUC TK revertant strain. These analyses demonstrated that the skin of the fish covering the fins and also the body is the major portal of entry for KHV in carp. Finally, to further demonstrate the role of the skin as the KHV portal of entry, we constructed an original system, nicknamed "U-tube," to perform percutaneous infection restricted to the posterior part of the fish. All the data obtained in the present study demonstrate that the skin, and not the gills, is the major portal of entry for KHV in carp.

The koi herpesvirus (KHV), recently designated *Cyprinid herpesvirus 3*, is the etiological agent of an emerging and mortal disease in common carp (*Cyprinus carpio carpio*) and koi (*Cyprinus carpio koi*) (2, 12, 13). Since its emergence in the late 1990s, this highly contagious and dreadful disease has caused severe financial and economic losses in both koi and common carp culture industries worldwide (9, 11).

The genome of KHV comprises a linear double-stranded DNA sequence of ~295 kb (1, 14), similar to that of cyprinid herpesvirus 1 (30) but larger than those of other members of the *Herpesvirales*, which generally range from 125 to 240 kb. Phylogenetic analysis of the KHV genome sequence led to its classification in the new family *Alloherpesviridae*, encompassing herpesviruses of fish and amphibians (18). The KHV genome includes 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 that resemble those of several other double-stranded DNA viruses, i.e., other herpesviruses, poxviruses, iridoviruses, and other large DNA viruses (14, 30).

Very little information is available on the roles of individual genes in the biology of KHV infection or in pathogenesis. Two facts can explain this lacuna. First, the KHV genome sequence has been published only recently (1). Second, prolonged KHV cultivation in vitro leads to spontaneous attenuation of the virus, making the production of KHV recombinants using classical homologous recombination in eukaryotic cells difficult (27). To circumvent this problem, we cloned the KHV genome as a stable and infectious bacterial artificial chromosome (BAC) that could be used to produce KHV recombinant strains (4).

Despite the lack of available KHV recombinant strains, studies have been devoted to KHV pathogenesis. Several authors have postulated that the gills might be the portal of entry for KHV in carp (6, 7, 15, 19, 22). This hypothesis relied on several observations. First, the gills have been demonstrated to act as the portal of entry for many fish pathogens (25). Second, fish expressing KHV disease have gill lesions, which explains why the virus was initially called *Carp interstitial nephritis and gill necrosis virus* (12, 19, 21, 22, 27). Third, the gills (like virtually all tissues) were shown by PCR to contain the viral genome at an early stage of infection (7, 22). However, no data demonstrating the role of the gills as the portal of entry of KHV are available.

One of the best methods to provide insights into the viral

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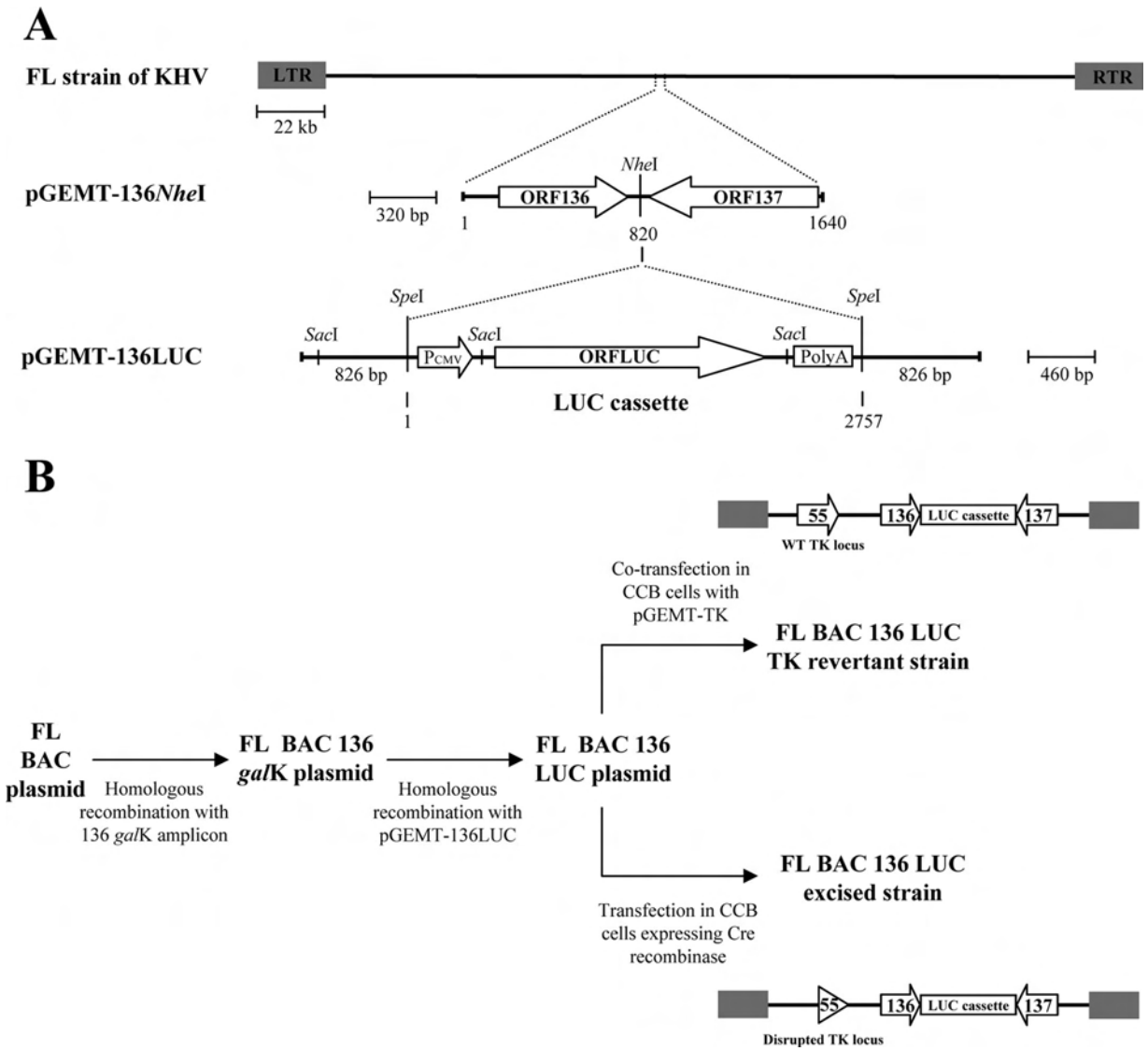

FIG. 1. Schematic representation of the strategy used to produce the FL BAC 136 LUC plasmid. (A) The genome of the KHV FL strain, flanked by two terminal repeats (LTR and RTR), is shown at the top. A LUC expression cassette, flanked by SpeI restriction sites, was inserted into the NheI site created in the intergenic region between ORF 136 and ORF 137 (pGEMT-136NheI vector), resulting in pGEMT-136LUC. (B) Flowchart of stages performed to produce the FL BAC 136 LUC plasmids to demonstrate the possibility of removing the *loxP*-flanked BAC cassette from the genome of reconstituted virus (the FL BAC 136 LUC excised strain) and to produce a TK revertant strain (the FL BAC 136 LUC TK revertant strain). WT, wild type.

portal of entry is the use of noninvasive whole-body imaging of living animals. Bioluminescence imaging, using the luciferase (LUC) reporter protein, is now widely used in small-animal models, like rodents, and also in fish (10, 16). This technique offers the advantages of using the same animal for multiple data collection over the course of the entire experiment. Moreover, D-luciferin (the substrate of luciferase) has been demonstrated to cross cell membranes and the blood-brain barrier, allowing this reporter protein to be imaged in any anatomic site (32).

In the present study, we investigated for the first time the portal of entry for KHV in carp by using bioluminescence imaging. We produced a LUC-expressing recombinant strain by intergenic insertion of a LUC expression cassette. Using this recombinant, we demonstrate that the skin of the fish, and not the gills, is the major portal of entry of KHV.

MATERIALS AND METHODS

Cells and viruses. *C. carpio* brain (CCB) cells (20) were cultured in minimum essential medium (Invitrogen) containing 4.5 g/liter glucose (D-glucose monohydrate; Merck) and 10% fetal calf serum (FCS). The cells were cultured at 25°C in a humid atmosphere containing 5% CO_2. The KHV FL strain was isolated from the kidney of a fish that died from KHV infection (CER, Marloie, Belgium) (4). The KHV FL BAC strain was described previously (4). This recombinant strain carries a BAC cassette inserted in the thymidine kinase (TK) locus.

Production of a KHV FL BAC LUC recombinant plasmid in bacteria. A KHV FL BAC LUC recombinant plasmid carrying a firefly LUC expression cassette was produced using a two-step galactokinase (*galK*) positive/negative selection in bacteria (Fig. 1) (31). The intergenic region located between open reading frame

TABLE 1. Oligonucleotides used for PCR amplification

Primer	Sequence
136galfw	5′-TATCATTGTCAAACAATAAAACTCT TACAAATGTGATTTTTGTGTGCTAT CCTGTTGACAATTAATCATCGGCA-3′
137galrev	5′-GAAAAATGAAAAATAATAAAAAAT GGTTGACACGACTCCCTGTGAAGCG TTCAGCACTGTCCTGCTCCTT-3′
136fw1	5′-TCCTGGGCAAGCCCTTCTTC-3′
137rev1	5′-AGGGCTGCATCTGCACGGG-3′
LUCfw	5′-GCAAAATTTAAGCTACAACAAGG-3′
LUCrev	5′-ATGCCCCGATTTAGAGCTTG-3′
136fw2	5′-ATGAAGGCCTCTAAACTGCTG-3′
136rev2	5′-TTAGATTTTTCTAAAGTGCAC-3′
137fw2	5′-TCAGAGGCCGGCTTCGGTC-3′
137rev2	5′-ATGGACAGCACAAACGTTAC-3′

(ORF) 136 and ORF 137 was selected for the insertion (1). The functions of these ORFs are unknown. ORF 136 and ORF 137 are right- and left-oriented ORFs, respectively. The insertion was performed between predicted polyadenylation signals of the ORFs to reduce the risk that the insertion might affect the expression of the ORFs. The KHV FL BAC plasmid described previously was used as the parental plasmid (4). In this plasmid, the BAC cassette is inserted into the TK locus.

The first recombination process (*galK* positive selection) was to insert the *galK* gene into the intergenic region of the KHV genome, resulting in the FL BAC 136 *galK* plasmid (Fig. 1). Recombination was achieved using the 136 *galK* amplicon (Fig. 1B). It consisted of the *galK* gene flanked by 50-bp sequences corresponding to the ORF 136-ORF 137 intergenic region. This amplicon was produced by PCR using the p*gal*K vector (31) as a template, the forward primer 136galfw, and the reverse primer 137galrev (Table 1). Primer 136galfw consisted of nucleotides 231761 to 231810 of the KHV genome (GenBank accession no. DQ177346; unless otherwise stated, coordinates from this accession number are used throughout this paper) and nucleotides 1 to 24 of the p*gal*K vector. Primer 137galrev consisted of nucleotides 231873 to 231922 of the KHV genome and nucleotides 1212 to 1231 of the p*gal*K vector. The 50-bp sequences of this amplicon, corresponding to the KHV genome, were used to target homologous recombination in bacteria (Fig. 1B).

The second recombination process (*galK* negative selection) was to replace the *galK* gene with a LUC expression cassette. The pGEMT-136LUC vector was used to achieve this goal (Fig. 1B). It was produced as follows (Fig. 1A). First, a 1,640-bp DNA fragment encompassing ORF 136 and ORF 137 of the KHV genome was amplified by PCR using KHV FL DNA as a template. The following primers were used for the amplification: the forward primer 136fw1 and the reverse primer 137rev1, corresponding to nucleotides 230995 to 231014 and nucleotides 232637 to 232655 of the KHV genome, respectively (Table 1). The amplified product was sequenced and TA cloned into the pGEM-T Easy vector (Promega), resulting in pGEMT-136. Next, an NheI restriction site was inserted into the intergenic region of ORF 136 and ORF 137 (between nucleotides 231835 and 231836 of the KHV genome) using a site-directed mutagenesis kit (Stratagene), resulting in pGEMT-136NheI. Finally, a LUC expression cassette, corresponding to the firefly luciferase ORF under the control of the human cytomegalovirus (HCMV) immediate-early (IE) promoter, was released by SpeI digestion of a modified pcDNA3-LUC vector (M. Bremont, INRA, France). The expression cassette was then ligated into the NheI site of the pGEMT-136NheI vector, resulting in pGEMT-136LUC, in which the LUC cassette is flanked by KHV sequences (826 bp). These KHV homologous sequences were exploited to produce the KHV FL BAC 136 LUC plasmid by homologous recombination in bacteria between the FL BAC 136 *gal*K and the pGEMT-136LUC plasmids (Fig. 1B).

Reconstitution of infectious virus from the KHV FL BAC 136 LUC plasmid. To reconstitute virions with excised BAC cassettes from the viral genome, the FL BAC 136 LUC plasmid was cotransfected (molecular ratio, 1:70) (Lipofectamine Plus; Invitrogen) in CCB cells, together with the pEFIN3-NLS-Cre vector encoding Cre recombinase fused to a nuclear localization signal (Fig. 1B) (8). The reconstituted virus, called the FL BAC 136 LUC excised strain, has a disrupted TK locus due to the sequence left by the *cre-loxP*-mediated excision of the BAC cassette. Similarly, the FL BAC 136 LUC plasmid was cotransfected into permissive CCB cells, together with the pGEMT-TK vector described previously (molecular ratio, 1:75) (4), to generate the FL BAC 136 LUC TK revertant strain with a wild-type TK sequence (Fig. 1B). Seven days posttransfection, viral plaques negative for enhanced green fluorescent protein (EGFP) expression (which had lost the BAC cassette including the EGFP gene) were picked and enriched by three successive rounds of plaque purification.

Southern blotting. Southern blot analysis was performed as described previously (4). Several probes were used. The 136-137 probe was released from the pGEMT-136 plasmid by restriction digestion. The LUC probe was produced by PCR using the forward primer LUCfw, the reverse primer LUCrev, and the pcDNA3-LUC plasmid as a template (Table 1). The TK probe was described previously (4).

Indirect immunofluorescence staining. CCB cells were fixed and permeabilized with acetone-ethanol (50:50 [vol/vol]) for 10 min at −20°C. Immunofluorescent staining (incubation and washes) was performed in PBS containing 10% FCS. Samples were incubated at 25°C for 45 min with mouse monoclonal antibody (MAb) 2F12 raised against an unidentified KHV antigen (4). After three washes, samples were incubated at 25°C for 30 min with Alexa Fluor 568 goat anti-mouse immunoglobulin G (H+L) (GAM 568; 2 μg/ml; Molecular Probes) as the secondary conjugate.

Microscopy analysis. Epifluorescence microscopy analysis was performed with a Dmirbe microscope (Leica) equipped with a DC 300F charge-coupled device (CCD) camera (Leica), as described previously (29). Confocal-microscopy analysis was performed with a TCS SP confocal microscope (Leica), as reported previously (29).

Multistep growth curves. Triplicate cultures of CCB cells were infected at a multiplicity of infection (MOI) of 0.1 PFU/cell. After an incubation period of 2 h, the cells were washed with PBS and then overlaid with Dulbecco's modified essential medium (DMEM) (Invitrogen) containing 4.5 g/liter glucose and 10% FCS. The supernatants of infected cultures were harvested at successive intervals after infection, and the amount of infectious virus was determined by plaque assay on CCB cells as described previously (5).

Transcriptional analysis. Freshly seeded CCB cells were mock infected or infected at an MOI of 1 PFU/cell. Twenty-four hours postinfection (p.i.), cytoplasmic RNA was isolated using an RNeasy Mini Kit (Qiagen) and then further purified by DNA digestion using an RNase-Free DNase Set (Qiagen). Reverse transcriptase (RT) reactions were performed on 1 μg of RNA using Superscript III Reverse Transcriptase and oligo(dT) (Invitrogen). Finally, ORF 136 and ORF 137 were amplified using the primer pairs 136fw2-136rev2 and 137fw2-137rev2, respectively (Table 1).

Bioluminescence imaging. Imaging of firefly (*Photinus pyralis*) LUC was performed using either the Biospace photon imager (Biospace Laboratory, France) or the Xenogen "in vivo imaging system" (IVIS) (Xenogen, Caliper Life Sciences). The Biospace photon imager consists of a photon-counting system based on a cooled gallium arsenide intensified-CCD (ICCD) camera. This ICCD is mounted on top of a light-tight chamber to record optical signals at a video rate of 25 Hz. For video tracking of active and unrestrained fish, a system consisting of two cameras, one recording the signal of interest and a second for video tracking the animal, was used (lens diaphragm, 91%). Awake fish were intraperitoneally injected with D-luciferin (150 mg/kg body weight) (Xenogen) and then placed in separate small tanks filled with water (stage height, 440 mm). Bioluminescence signals emitted by free-moving fish were recorded for 6 h, and then the fish were placed in bigger tanks. Seventeen hours later, the same fish were analyzed for five additional hours for bioluminescence emission.

The Xenogen IVIS consists of a CCD camera mounted on a light-tight specimen chamber, a cryogenic refrigeration unit, a camera controller, and a computer system for data analysis. For bioluminescence analysis of cell monolayers, the cell supernatant was replaced by fresh complete medium containing 150 μg/ml of D-luciferin (Xenogen). For in vivo analysis, fish were anesthetized with benzocaine (50 mg/liter of water). Ten minutes before bioluminescence analysis, D-luciferin (150 mg/kg body weight) (Xenogen) was administered by intraperitoneal injection. Each fish was analyzed lying on its left and right side. All the images presented in this study were acquired using a field view of 15 cm, a 1-min exposure time, a binning factor of 4, and an f/stop of 1. Relative intensities of transmitted light from in vivo bioluminescence were represented as a pseudocolor image ranging from violet (least intense) to red (most intense). Corresponding gray-scale photographs and color luciferase images were superimposed using LivingImage analysis software (Xenogen).

KHV inoculation of fish. Specific-pathogen-free common carp (*C. carpio carpio*) (Zodiac, Wageningen, The Netherlands) with an average weight of 13 g were kept in 60-liter tanks at 24°C. For viral inoculation mimicking natural infection, fish were kept for 2 h in 2 liters of water containing the virus (10^3 PFU/ml). At the end of the incubation period, the fish were returned to larger tanks. For viral inoculation restricted to the skin of the fish body posterior to the anterior part of the dorsal fin, we designed and constructed an original system, which we nick-

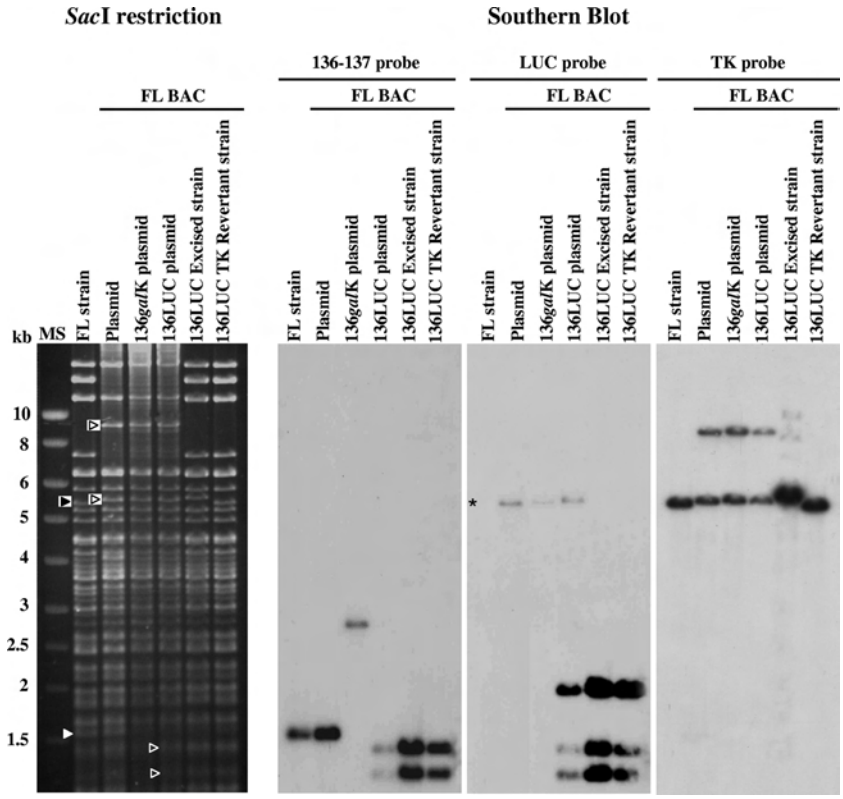

FIG. 2. Structural analysis of the FL BAC 136 LUC plasmid and derived KHV recombinant strains. The KHV FL BAC, FL BAC 136 *gal*K, and FL BAC 136 LUC plasmids and the genomes of the KHV FL, FL BAC 136 LUC excised, and FL BAC 136 LUC TK revertant strains were analyzed by SacI restriction (left; agarose gel) and further tested by Southern blotting using probes corresponding to ORFs 136 and 137 (right, 136-137 probe), to the LUC cassette (LUC probe), and to the TK ORF (TK probe). The black and the white-outlined black arrowheads indicate restriction fragments containing the TK ORF and the BAC cassette, respectively. The white and the black-outlined white arrowheads indicate restriction fragments containing ORFs 136 and 137 and the LUC cassette, respectively. The restriction fragment derived from the BAC cassette and containing the sequence of the HCMV promoter is marked with an asterisk. Marker sizes (MS) are indicated on the left.

named the "U-tube" system (see Fig. 8). It consisted of a tube in the shape of a "U" made of Plexiglas pipes (5-cm diameter). At the center of the horizontal section, an O-ring device allowed the insertion of a latex glove finger. The fish was introduced in the glove finger head first up to the beginning of the dorsal fin. Openings were created to release the mouth, the opercula, and the eyes. Both compartments (head and tail) were filled with water. To ensure and control the watertightness of the system, the water level of the head compartment was set up 5 cm higher than the tail compartment. This point was crucial to maintain the latex membrane tightly associated with the fish surface. To restrict virus inoculation through the skin, virus was added to the tail compartment (final concentration, 2×10^3 PFU/ml). Note that in case of minor leaking (not detectable by observation of the water level), the overpressure of the head compartment should prevent its contamination from the tail compartment containing the virus. Independently of the inoculation protocol, the viral inocula were titrated before inoculation and back-titrated after inoculation to ensure that the doses were equivalent among groups. The animal study was accredited by the local ethics committee of the University of Liege (Belgium).

Transmission electron microscopy. Samples were dissected and fixed in 0.1% glutaraldehyde for electron microscopy analysis. Epon blocks and sections were prepared as described elsewhere (17). Sections were analyzed using a Technai Spirit transmission electron microscope (FEI, Eindhoven, The Netherlands), and electron micrographs were taken using a bottom-mounted 4-by-4 K Eagle camera (FEI).

RESULTS

The goal of the present study was to identify the portal of entry for KHV in carp. We decided to address this question by using bioluminescence imaging. As a first step, KHV recombinant strains expressing the LUC reporter protein were produced.

Production of KHV FL recombinant strains expressing LUC reporter protein. The FL BAC plasmid described above was used as the parental background for the production of KHV recombinants expressing LUC (4). The intergenic region between ORF 136 and ORF 137 was selected for insertion of the LUC expression cassette using the two-step procedure depicted in Fig. 1 and described in Materials and Methods. The first step consisted of inserting a *galK* gene for positive selection of the resulting FL BAC 136 *gal*K plasmid. The second step consisted of replacing the *galK* gene (using negative selection against the gene) by the LUC expression cassette, resulting in the FL BAC 136 LUC plasmid (Fig. 1B). The molecular structures of these two recombinant plasmids were confirmed by a combined SacI restriction endonuclease and Southern blotting approach (Fig. 2). In the parental FL strain and in the KHV FL BAC plasmid, ORFs 136 and 137 were contained in a DNA fragment of approximately 1.5 kb, whereas in the KHV FL BAC 136 *gal*K plasmid, the corresponding fragment had a size of approximately 2.7 kb due to the insertion of the *galK* cassette (which does not contain a SacI restriction site). In the KHV FL BAC 136 LUC plasmid, as a consequence of the presence of the LUC cassette (which

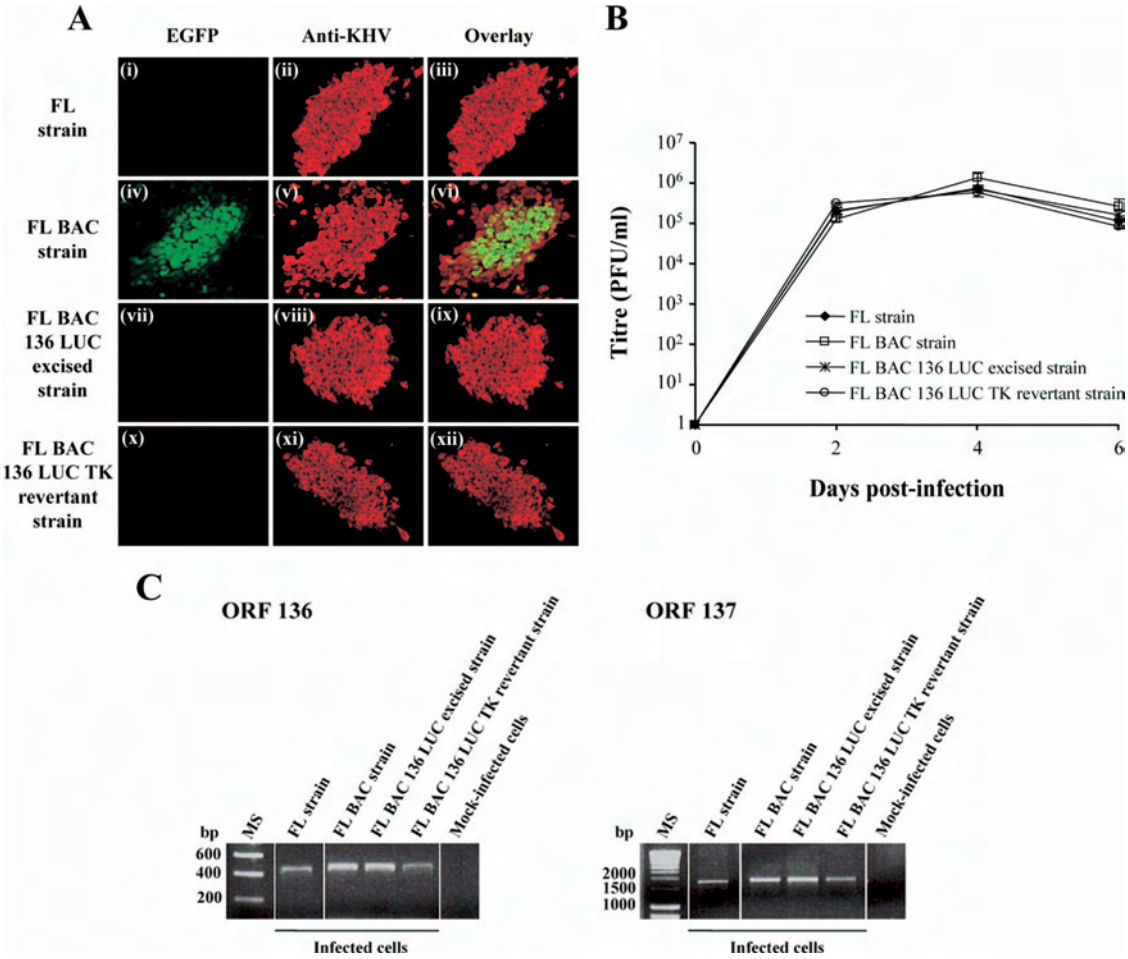

FIG. 3. Characterization of KHV recombinant strains derived from the FL BAC 136 LUC plasmid. (A) Epifluorescence analysis of KHV syncytia. CCB cells were infected (MOI, 0.001 PFU/cell) with KHV FL (i to iii), FL BAC (iv to vi), FL BAC 136 LUC excised (vii to ix), and FL BAC 136 LUC TK revertant (x to xii) strains and were overlaid with DMEM containing 10% FCS and 0.6% (wt/vol) carboxymethylcellulose (Sigma) to obtain isolated syncytia. Seven days p.i., the syncytia were revealed by indirect immunofluorescence staining using MAb 2F12 and GAM 568 as the primary and secondary antibodies, respectively. The horizontal rows represent analyses of the same syncytium. Images i, iv, vii, and x and images ii, v, viii, and xi were analyzed for EGFP and GAM 568 fluorescent emissions, respectively. The merged EGFP and Alexa signals are shown in images iii, vi, ix, and xii. The sides of each panel correspond to 250 μm. (B) The replication kinetics of KHV recombinant strains were compared with those of the parental KHV FL strain as described in Materials and Methods. The data presented are the means ± standard errors of triplicate measurements. (C) Transcriptional analyses of ORF 136 and ORF 137 from KHV recombinant strains were compared with those of the parental KHV FL strain as described in Materials and Methods. Marker sizes (MS) are indicated on the left.

contains two SacI restriction sites) (Fig. 1A), the DNA fragment was distributed in three fragments of approximately 1.2 kb, 1.4 kb, and 1.9 kb (Fig. 2, LUC probe). Moreover, due to the presence of the HCMV promoter sequence in the BAC and LUC cassettes, the fragments of the KHV FL BAC, 136 *gal*K, and 136 LUC plasmids containing the BAC cassette hybridized with the LUC probe (Fig. 2). Sequencing of the regions used to target homologous recombination confirmed that the two recombinant plasmids had the correct molecular structures (data not shown).

Next, two types of recombinant strains were reconstituted from the recombinant FL BAC 136 LUC plasmid (Fig. 1B). First, infectious particles were reconstituted by cotransfection of the FL BAC 136 LUC plasmid and a Cre recombinase-expressing plasmid. Deletion of the BAC cassette was monitored by the disappearance of EGFP fluorescence (FL BAC 136 LUC excised strain) (Fig. 3A) and by a combined restriction endonuclease and Southern blotting approach (Fig. 2). While the TK sequence was contained in a 5.2-kb fragment in the parental FL strain, due to the insertion of the BAC cassette in the TK locus, it appeared in two fragments of approximately 5.3 kb and 9.1 kb in the FL BAC, FL BAC 136 *gal*K, and FL BAC 136 LUC plasmids (Fig. 2) (4). In the FL BAC 136 LUC excised strain, the *cre-loxP*-mediated excision of the BAC cassette left a sequence of 172 bp in the TK ORF, leading to a SacI restriction fragment slightly larger than the corresponding wild-type fragment (Fig. 2). This 172 bp consisted of one *loxP* site (34 bp) and the sequences of the BAC cassette upstream (126 bp) and downstream (12 bp) of the *loxP* site. Due to this 172-bp insertion of foreign sequence into the TK ORF, the excised strain was expected to express a truncated form of TK. Second, to reconstitute virions expressing a wild-type TK se-

quence and the LUC reporter protein, the FL BAC 136 LUC plasmid was cotransfected, together with the pGEMT-TK vector (Fig. 1B). The resulting FL BAC 136 LUC TK revertant strain was selected on the nonexpression of EGFP (FL BAC 136 LUC TK revertant strain) (Fig. 3A). Restriction endonuclease and Southern blot analyses revealed that the FL BAC 136 LUC TK revertant strain possessed a wild-type TK profile and included a LUC expression cassette (Fig. 2). Sequencing of the regions encompassing the TK ORF and the LUC expression cassette confirmed that the two recombinant strains (FL BAC 136 LUC TK excised and FL BAC 136 LUC TK revertant strains) had the correct molecular structures (data not shown).

Characterization of KHV FL recombinant strains expressing LUC in cell culture. Additional characterization of the FL BAC 136 LUC excised and FL BAC 136 LUC TK revertant strains was performed in cell culture. The parental FL and FL BAC strains were used as controls. First, microscopic examination of immunostained viral syncytia did not reveal differences among the recombinants (Fig. 3A). Second, in order to investigate the putative effects of the recombination processes on viral growth in vitro, the two recombinant strains were compared to the parental strains using a multistep growth assay (Fig. 3B). All viruses tested exhibited similar growth curves ($P \leq 0.05$), leading to the conclusion that LUC insertion did not affect KHV replication in vitro. Third, using an RT-PCR approach, we controlled the process so that the insertion of the LUC expression cassette did not markedly affect the transcription of the flanking ORF 136 and ORF 137 (Fig. 3C). Transcripts of 462 bp and 1,821 bp were observed for ORF 136 and ORF 137, respectively, in infected cells. No transcript was detected in mock-infected cells. When RT was omitted from the reactions, the product seen in infected cells was not detected, indicating that the latter did not result from amplification of contaminant viral DNA (data not shown). The four strains analyzed (FL, FL BAC, FL BAC 136 LUC excised, and FL BAC 136 LUC TK revertant) led to comparable signals for both ORFs (Fig. 3C). Together, these results demonstrated that the KHV FL recombinant strains produced as described above and the parental strain exhibited similar in vitro characteristics.

In vitro expression of LUC by the FL BAC 136 LUC TK revertant strain. CCB cells were infected at MOIs ranging from 10^{-4} to 10^{-6} PFU/cell with the FL BAC 136 LUC TK revertant strain (Fig. 4A). Twenty-four hours p.i., the cells were analyzed by bioluminescence imaging. The data presented in Fig. 4A demonstrate that a bioluminescence signal was detectable as early as 24 h p.i. in infected monolayers. The time p.i. and the MOI used for the infection strongly suggested that the spots of light detected corresponded to isolated infected cells. To test this hypothesis, monolayers of cells infected at an MOI of 10^{-6} PFU/cell with the FL BAC 136 LUC TK revertant strain were analyzed at 24 h p.i. by indirect immunofluorescent staining (Fig. 4B, iv to vi). Extensive examination of the monolayers revealed only isolated cells positive for MAb 2F12 staining. The LUC expression cassette carried by the FL BAC 136 LUC TK revertant strain is driven by the HCMV IE promoter, while the as-yet-unidentified antigen recognized by MAb 2F12 could potentially be a late protein. Consequently, one could argue that while the staining with MAb 2F12 revealed isolated positive cells, the detection of an IE antigen should reveal small clusters of positive cells with MAb 2F12-positive cells in the center. To test this hypothesis, cell monolayers were infected with the KHV FL BAC strain (MOI, 10^{-6}) expressing the EGFP reporter protein under the control of the HCMV IE promoter (Fig. 4B, i to iii). Analysis of the monolayer for EGFP emission revealed only isolated positive cells (Fig. 4B, i). Together, the results presented above demonstrated that the FL BAC 136 LUC TK revertant strain induced LUC expression that allowed the detection of isolated positive cells as early as 24 h p.i. by bioluminescence imaging.

Pathogenicity of the FL BAC 136 LUC TK revertant strain in carp. In order to test whether the insertion of the LUC expression cassette into the KHV genome had led to a modification in the pathogenicity of the virus, naïve common carp were infected by bathing them in water containing the FL BAC 136 LUC TK revertant strain (Fig. 5). The parental FL strain was used as a control. Both strains induced all the clinical signs associated with KHV disease, including apathy, folding of the dorsal fin, increased mucus secretion, suffocation, erratic swimming, and loss of equilibrium. The intensities of the clinical signs were comparable in the two groups. Thirty days p.i., the FL BAC 136 LUC TK revertant strain and the parental FL strain induced mortality rates of 70% and 80%, respectively (Fig. 5). PCR assays were performed on dead fish from the group infected with the FL BAC 136 LUC TK revertant strain to exclude the possibility of contamination with the FL strain. The data confirmed the absence of contamination (data not shown).

In vivo expression of LUC by the FL BAC 136 LUC TK revertant strain at early stages of infection. The results presented above demonstrated that the FL parental strain and the FL BAC 136 LUC TK revertant strain exhibited similar in vitro and in vivo characteristics. Consequently, the latter was used to investigate the portal of entry for KHV into carp by using in vivo bioluminescence imaging.

To be accurate, bioluminescence imaging must be performed during the plateau of light emission. To determine how fast the emission of light reached the plateau after injection of D-luciferin and how long the plateau lasted before the decline, a preliminary experiment was conducted using the Biospace photon imager (Biospace Laboratory, France). The data presented in Fig. 6A demonstrated that within a few seconds after injection of D-luciferin, light emission reached a plateau that lasted for at least 6 hours. Analysis of the fish 23 h after injection of D-luciferin revealed that the animals were still emitting light. Based on this preliminary experiment, subsequent bioluminescence analyses were performed between 5 min and 30 min after injection of D-luciferin.

To investigate the portal of entry of KHV, common carp were infected by bathing them in water containing the FL BAC 136 LUC TK revertant strain. The fish were analyzed by bioluminescence imaging using the Xenogen IVIS 12, 24, 48, and 72 h p.i. (Fig. 6B). Because photon emission is attenuated exponentially through animal tissues, making detection of internal organs more challenging, each fish was analyzed lying on its right and its left side. The results of this experiment are illustrated in Fig. 6B and can be summarized as follows. Discrete luciferase activity was detected as early as 12 h p.i. (data

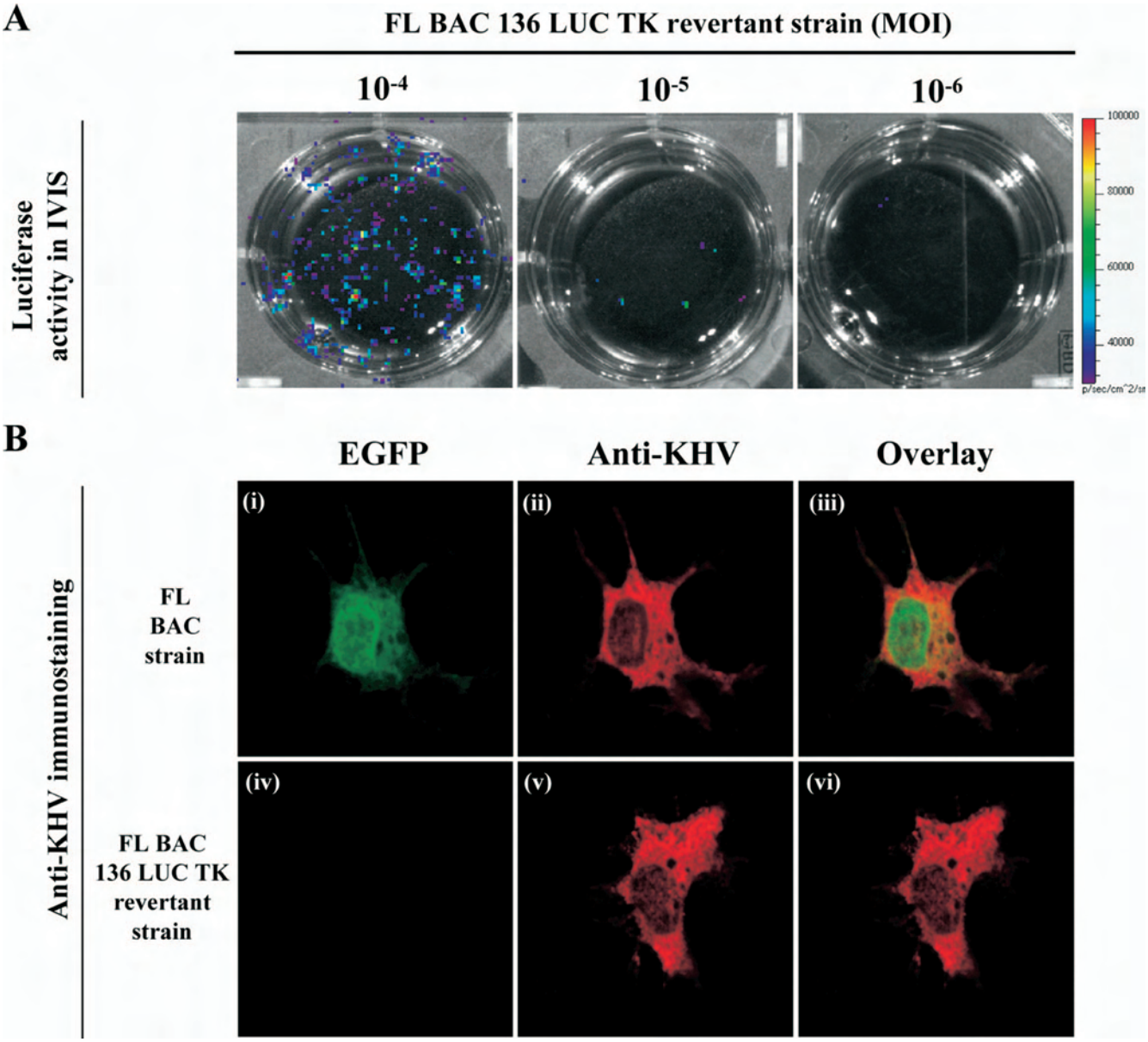

FIG. 4. In vitro expression of luciferase by the FL BAC 136 LUC TK revertant strain. CCB cells, grown on glass coverslips, were infected at the indicated MOI with FL BAC and FL BAC 136 LUC TK revertant strains and then overlaid with DMEM containing 10% FCS. Twenty-four hours p.i., the cells were analyzed by bioluminescence imaging (A) and immunofluorescent staining (B). (A) Bioluminescence imaging of cell monolayers infected with the KHV FL BAC 136 LUC TK revertant strain. The images are presented with standardized minimum and maximum threshold values for photon flux. (B) Immunofluorescent staining of infected cells. The images show cells infected at an MOI of 10^{-6} PFU/cell. Cells infected with FL BAC (i to iii) and FL BAC 136 LUC TK revertant (iv to vi) were analyzed 24 h p.i. by indirect immunofluorescent staining using MAb 2F12 and GAM 568 as the primary and secondary antibodies, respectively. The horizontal rows represent analyses of the same field of the monolayer by confocal microscopy. Images i and iv and images ii and v were analyzed for EGFP and GAM 568 fluorescent emissions, respectively. The merged EGFP and Alexa signals are shown in images iii and vi. The sides of each panel correspond to 24 μm.

not shown) in 7 out of 10 fish. At 24 h p.i., all fish expressed focal sources of light on both sides of the body (Fig. 6B). To highlight the signals detected and to use the full dynamic range of the pseudocolor scale, images collected on day 1 were presented with a relative photon flux scale adapted to each image (Fig. 6B, left). Most of the bioluminescence signals detected on day 1 increased on day 2 and day 3 (Fig. 6B, right). While rare sources of light detected on day 1 were no longer detected on days 2 and 3, new spots appeared with time. The signals were detected from various anatomic sites of the fish body, but principally on the pectoral, pelvic, dorsal, and caudal fins. Three fish out of 10 had a strong signal associated with the nostrils on day 3 p.i. Interestingly, none of the fish expressed a signal that could be associated with the gills on day 3 p.i. No source of light was detected from mock-infected carp used as negative controls (data not shown).

Because bioluminescence images are two dimensional, it is difficult to know whether signals detected arise from the skin

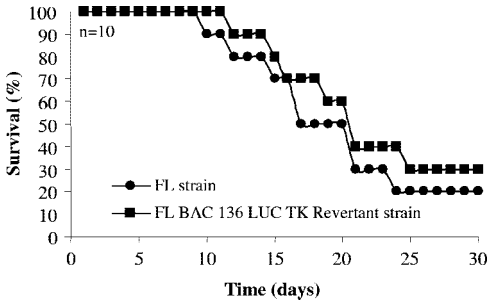

FIG. 5. Survival rates of carp infected by the FL BAC 136 LUC TK revertant strain. On day zero, two groups of fish, each consisting of 10 common carp kept in separate tanks, were infected by bathing them with FL and FL BAC 136 LUC TK revertant strains as described in Materials and Methods. The fish were examined daily for clinical signs of KHV disease, and dead fish were removed. The percentage of survival is expressed according to days p.i. The results presented are representative of three independent experiments.

surface or from superimposed internal tissues. However, the detection of signals associated with the fins suggested that the skin of the fish could be the major portal of entry for KHV. These results also argued against the role of the gills as a portal of entry. To further test these hypotheses, fish ($n = 10$) were dissected at the end of the experiment shown in Fig. 6B, as exemplified in Fig. 7A. To exclude the possibility that the operculum could hide a signal emitted by the gills, it was removed. Fins or fragments of fins were isolated from the fish body. Fragments of skin identified as positive for light emission were dissociated from the subcutaneous tissue. Finally, the abdominal wall was removed to expose internal organs. Dissected fish and isolated organs or tissues were analyzed for ex vivo bioluminescence (Fig. 7A). Analysis of the dissected fish revealed that bioluminescent signals were exclusively detected on superficial tissues, mainly on the fins but also on the skin. While none on the intact fish analyzed exhibited signals associated with the gills, 2 out of 10 dissected fish had positive gills. One of these two fish also had some signal associated with the gut (data no shown). We assumed that the low frequency of fish with positive gills and gut detected 3 days p.i. reflected internal spreading of the infection.

Next, to investigate whether LUC expression detected on the skin was associated with viral replication, a biopsy specimen of positive skin was analyzed by electron microscopy (Fig. 7B). A detailed examination of ultrathin sections revealed cells supporting viral replication in the skin epithelium. Viral capsids and enveloped particles were observed in the nuclei and the cytosol of the infected cells, respectively.

The skin is the major portal of entry for KHV into fish. The results presented above strongly suggest that the skin of the fish is the major portal of entry for KHV. To further support this conclusion, the experiment presented in Fig. 8 was performed. In this experiment, we used an original system nicknamed "U-tube" to perform percutaneous infection restricted to the posterior part of the fish (see Materials and Methods). Fish were maintained for 24 h in the system and then analyzed for bioluminescence emission. Analysis of fish maintained in the system for 24 h in the presence of the virus in the tail compartment revealed spots of light restricted to the surface of the fish exposed to the inoculum. None of the six fish analyzed expressed bioluminescent signal on the area protected from the inoculum by the latex membrane and the overpressure of the uninfected compartment (Fig. 8, half-infected fish). In contrast, bioluminescent signals were detected on the corresponding anatomic part of the fish maintained in the system in the absence of the latex diaphragm (Fig. 8, whole infected fish). Analysis of the fish 48 h p.i. (24 h after release from the U-tube system) confirmed the conclusions reached at 24 h p.i.

DISCUSSION

Several authors have postulated that the gills might be the portal of entry for KHV in carp (6, 7, 15, 19, 22). This hypothesis relied on several observations. First, the gills have been demonstrated to act as the portal of entry for many fish pathogens (25). Second, fish expressing KHV disease have gill lesions, which explains why the virus was initially called *Carp interstitial nephritis and gill necrosis virus* (12, 19, 21, 22, 27). Third, the gills (like virtually all tissues) were shown by PCR to contain the viral genome at an early stage of infection (7, 22).

In the present study, we investigated for the first time the portal of entry of KHV in carp using bioluminescence imaging. Taking advantage of the recent BAC cloning of the KHV genome (4), we produced a recombinant strain, called the FL BAC 136 LUC TK revertant strain, expressing LUC as a reporter protein. This LUC-expressing recombinant was shown to replicate comparably to the parental strain in vitro and to induce KHV disease in common carp that was indistinguishable from that induced by the parental FL strain. Bioluminescence imaging of carp infected by the natural route revealed that the major portal of entry of KHV is the skin and not the gills.

The epidermis of the skin of teleost fish is a stratified squamous epithelium covering the body surface and investing the fins. 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 and consequently are covered by the epidermis. The surface of the outermost cell layer of the epidermis is overlaid by mucus (24). The skin functions as a physical barrier that protects the fish against injury and represents, with mucus, the first line of defense against pathogens. Damage to the skin caused by rough handling or ectoparasite infestations can increase susceptibility to infection by secondary pathogens. Many fish ectoparasites are responsible for superficial abrasion of the skin; while not immediately critical, they create a portal of entry for infectious agents (26). A well-known example is the lymphocystis disease affecting many fish species from marine and freshwater environments (28). This disease is caused by an iridovirus that enters the fish body via skin abrasions produced by parasitic infestation. The fish used in the present study were derived from a specific-pathogen-free colony, and the absence of parasitic infestation was controlled just before the experiments were run. Moreover, extreme care was taken when handling the fish to avoid mucus removal and skin abrasion. Consequently, we assume that the results reported in this study reflect the infection of fish with intact and healthy skin.

Interestingly, a recent study also based on bioluminescence imaging demonstrated that the skin covering the base of the

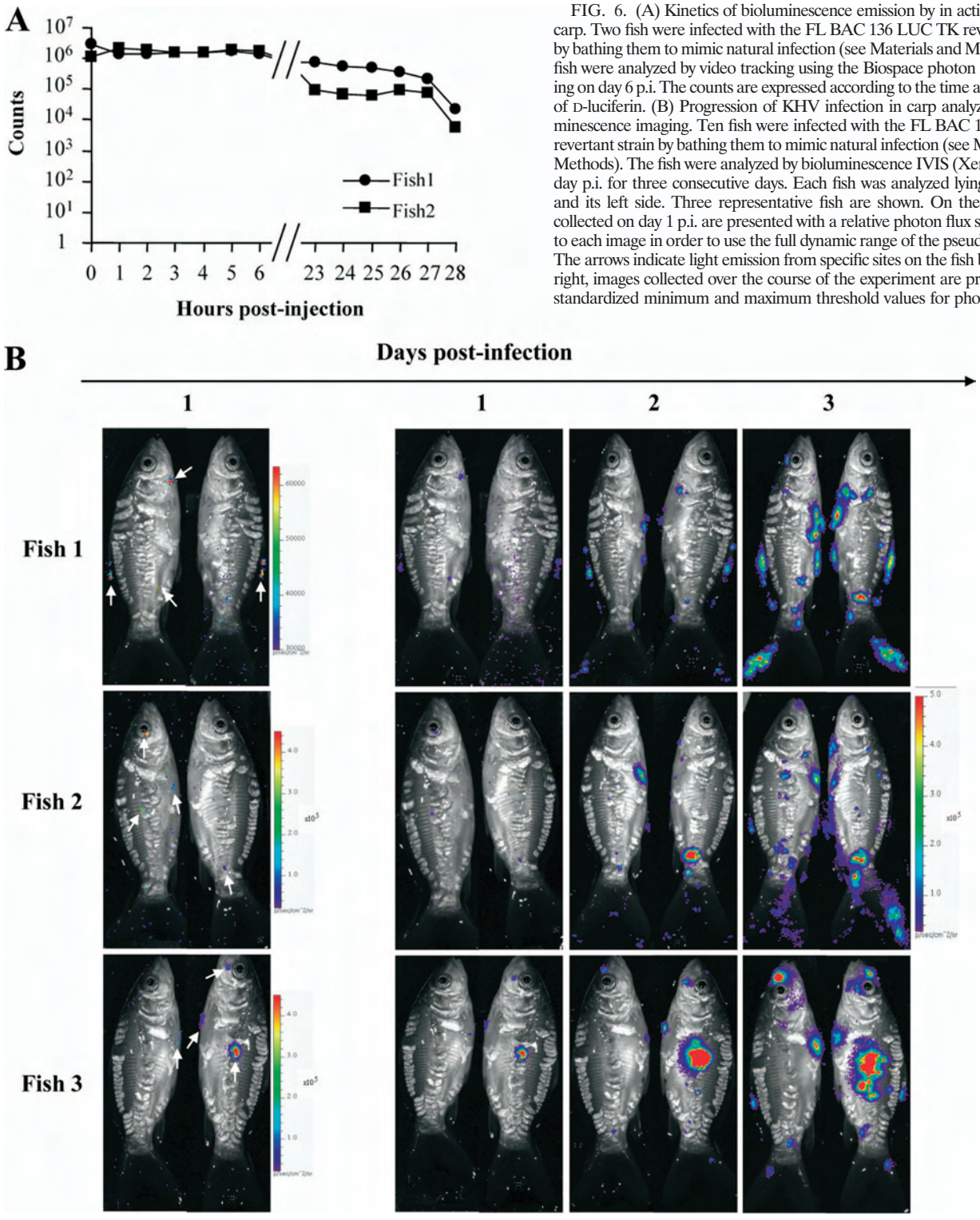

FIG. 6. (A) Kinetics of bioluminescence emission by in actio imaging of carp. Two fish were infected with the FL BAC 136 LUC TK revertant strain by bathing them to mimic natural infection (see Materials and Methods). The fish were analyzed by video tracking using the Biospace photon imager starting on day 6 p.i. The counts are expressed according to the time after injection of D-luciferin. (B) Progression of KHV infection in carp analyzed by bioluminescence imaging. Ten fish were infected with the FL BAC 136 LUC TK revertant strain by bathing them to mimic natural infection (see Materials and Methods). The fish were analyzed by bioluminescence IVIS (Xenogen) every day p.i. for three consecutive days. Each fish was analyzed lying on its right and its left side. Three representative fish are shown. On the left, images collected on day 1 p.i. are presented with a relative photon flux scale adapted to each image in order to use the full dynamic range of the pseudocolor scale. The arrows indicate light emission from specific sites on the fish body. On the right, images collected over the course of the experiment are presented with standardized minimum and maximum threshold values for photon flux.

fins is the portal of entry of the rhabdovirus *Infectious hematopoietic necrosis virus* into salmonids (10). It has also been suggested that *Viral hemorrhagic septicemia virus*, another important rhabdovirus of salmonids, enters fish through the skin, but this hypothesis has not been tested (3, 23). While the present study demonstrates that intact and healthy skin is the portal of entry for KHV in carp, further experiments are in progress to determine if ectoparasite infestations and/or rough handling of fish enhances the entry of KHV through the skin.

Viral particles were detected by electron microscopy exam-

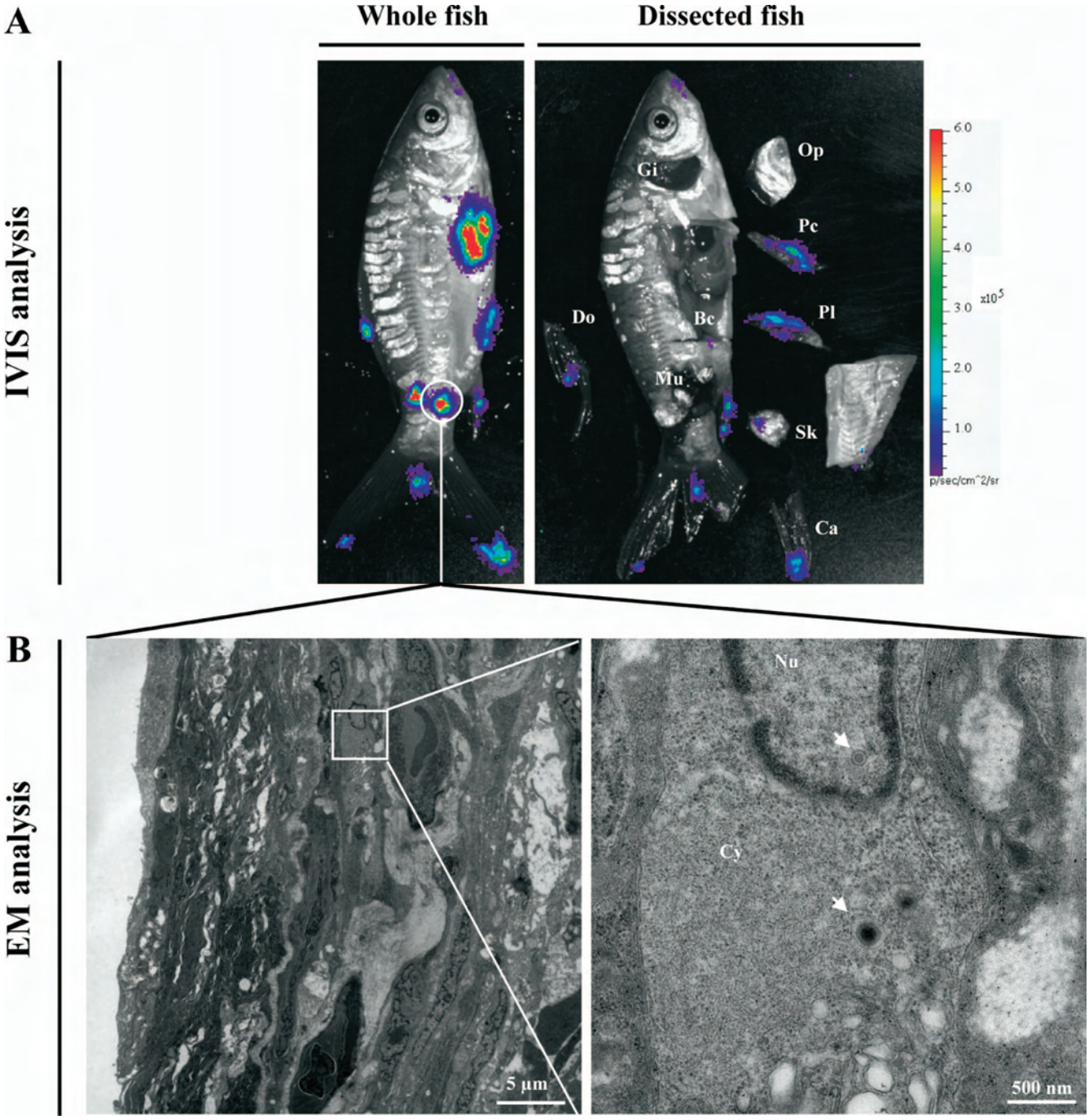

FIG. 7. In situ localization of luciferase activity and detection of viral replication in the skin. At the end of the experiment presented in Fig. 6, the fish were dissected immediately after bioluminescence imaging performed on day 3 p.i. The dissected fish and isolated organs were analyzed for ex vivo bioluminescence. (A) The analysis of one representative fish is presented. The left and right images represent bioluminescence imaging performed before and after dissection, respectively. Op, operculum; Pc, pectoral fin; Pl, pelvic fin; Sk, skin; Do, dorsal fin; Ca, caudal fin; Gi, gills; Mu, muscle; Bc, body cavity. (B) A skin fragment emitting bioluminescence was analyzed by electron microscopy (EM). The left image shows low magnification of the skin epithelium. The right image shows one representative infected epithelial cell at higher magnification. The arrows indicate viral particles present in the nucleus (Nu) and the cytosol (Cy).

ination of LUC-expressing skin fragments as early as 3 days p.i. (Fig. 7B). This early replication of the virus at the portal of entry should contribute not only to the spread of virus in the infected fish, but also to the spread of the virus in the fish population. Indeed, as early as 2 to 3 days p.i., infected fish rubbed themselves against each other or against objects. This behavior could contribute to a "skin-to-skin" mode of transmission. Later during infection, this mode of transmission could also occur when uninfected fish peck macroscopic skin herpetic lesions developed by infected fish (15).

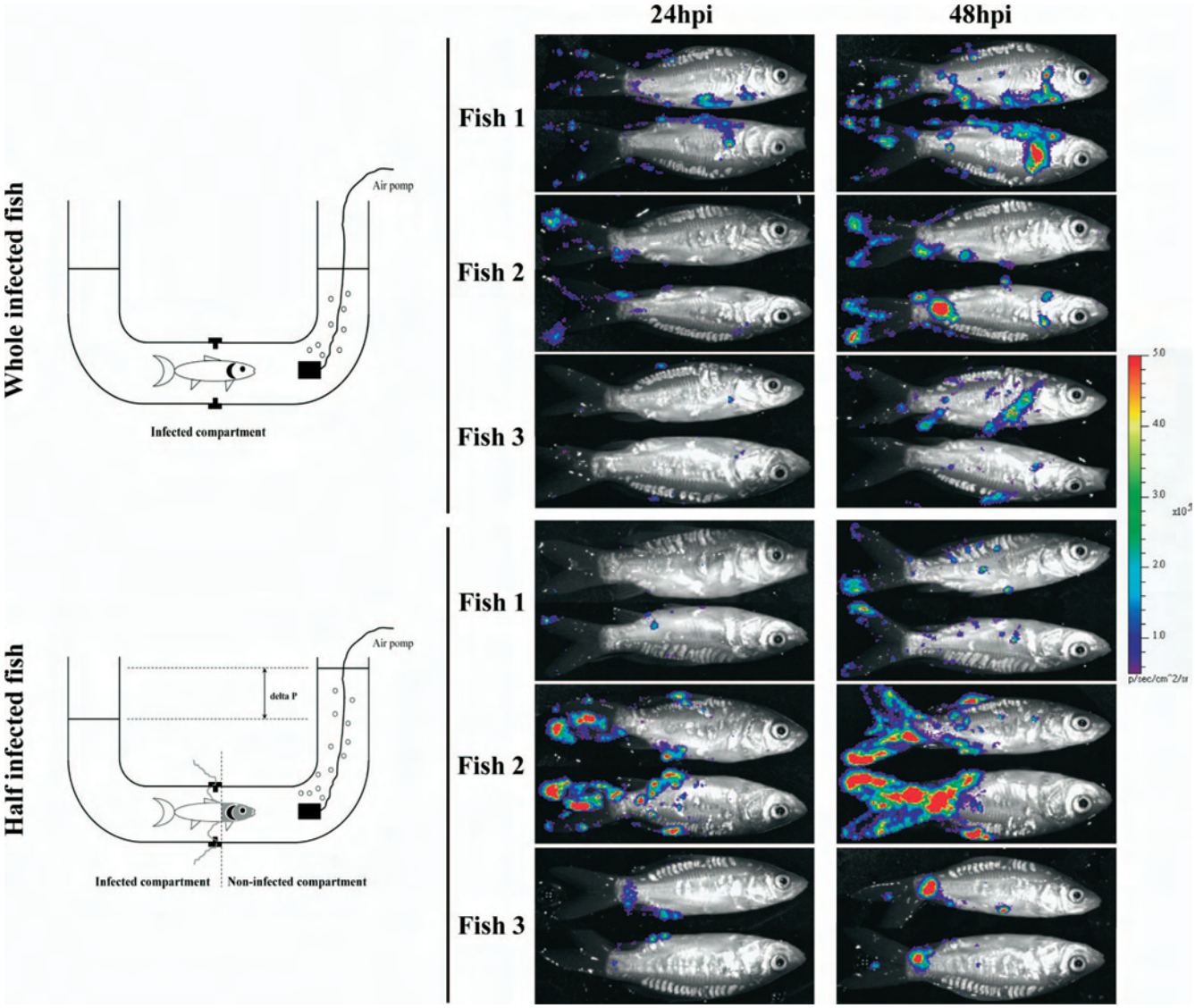

FIG. 8. Viral inoculation restricted to the fish skin. A schematic representation of the system used for this experiment is shown on the left and is explained in detail in Materials and Methods. The lower drawing presents the conditions under which fish ($n = 6$) were inoculated by restricted contact 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 in water containing 2×10^3 PFU/ml of the FL BAC 136 LUC TK revertant strain for 24 h. All fish were analyzed 24 h p.i. by bioluminescence imaging. After an additional incubation period of 24 h in individual tanks containing fresh water, they were reanalyzed by bioluminescence imaging (48 h p.i.). Three representative fish are shown. The images are presented with standardized minimum and maximum threshold values for photon flux.

In the present study, we observed that during the first 2 days p.i., the expression of the LUC reporter protein was restricted to the skin. On day 3 p.i., only 2 out of 10 dissected fish had positive gills, and only 1 of them also had some signal associated with the gut (Fig. 7A). These data contrast with earlier reports based on PCR analysis, which described an early and fast systemic spread of the virus in infected fish (7, 19, 21, 22). Different hypotheses could explain the discrepancy or the apparent paradox between these data. First, the discrepancy could be explained by a higher sensitivity of PCR assays compared to bioluminescence imaging. Even though we cannot completely exclude this hypothesis, the results presented in Fig. 4 demonstrate the high sensitivity of detection of the bioluminescence reporter gene, at least in vitro. A second and preferred hypothesis to explain the apparent paradox between the data reported above could be that the rapid (day 2 p.i.) and systemic dissemination observed by PCR reflects the secondary infection of blood cells (22), which could not be detected by bioluminescence imaging. Further experiments are required to understand the pathogenesis of KHV and to unravel how the virus spreads from the portal of entry to a secondary site of replication and a site of latency and eventually reactivates. While bioluminescence imaging will certainly contribute to addressing these questions, the data reported in this study

demonstrate that internal signals cannot be detected without dissection of the fish.

In conclusion, the present study has demonstrated for the first time that the portal of entry for KHV in carp is the skin. Together with an earlier study addressing the portal of entry of the rhabdovirus *Infectious hematopoietic necrosis virus* in salmonids (10), the present study suggests that the skin of teleost fish represents an efficient portal of entry for viruses.

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REFERENCES

1. **Aoki, T., I. Hirono, K. Kurokawa, H. Fukuda, R. Nahary, A. Eldar, A. J. Davison, T. B. Waltzek, H. Bercovier, and R. P. Hedrick.** 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.
2. **Bretzinger, A., T. Fischer-Scherl, R. Oumouma, R. Hoffmann, and U. Truyen.** 1999. Mass mortalities in koi, *Cyprinus carpio*, associated with gill and skin disease. Bull. Eur. Assoc. Fish Pathol. **19:**182–185.
3. **Brudeseth, B. E., H. F. Skall, and O. Evensen.** 2008. Differences in virulence of marine and freshwater isolates of viral hemorrhagic septicemia virus in vivo correlate with in vitro ability to infect gill epithelial cells and macrophages of rainbow trout (*Oncorhynchus mykiss*). J. Virol. **82:**10359–10365.
4. **Costes, B., G. Fournier, B. Michel, C. Delforge, V. S. Raj, B. Dewals, L. Gillet, P. Drion, A. Body, F. Schynts, F. Lieffrig, and A. Vanderplasschen.** 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.
5. **Costes, B., M. Thirion, B. Dewals, J. Mast, M. Ackermann, N. Markine-Goriaynoff, L. Gillet, and A. Vanderplasschen.** 2006. Felid herpesvirus 1 glycoprotein G is a structural protein that mediates the binding of chemokines on the viral envelope. Microbes Infect. **8:**2657–2667.
6. **Dishon, A., A. Perelberg, J. Bishara-Shieban, M. Ilouze, M. Davidovich, S. Werker, and M. Kotler.** 2005. Detection of carp interstitial nephritis and gill necrosis virus in fish droppings. Appl. Environ. Microbiol. **71:**7285–7291.
7. **Gilad, O., S. Yun, F. J. Zagmutt-Vergara, C. M. Leutenegger, H. Bercovier, and R. P. Hedrick.** 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.
8. **Gillet, L., V. Daix, G. Donofrio, M. Wagner, U. H. Koszinowski, B. China, M. Ackermann, N. Markine-Goriaynoff, and A. Vanderplasschen.** 2005. Development of bovine herpesvirus 4 as an expression vector using bacterial artificial chromosome cloning. J. Gen. Virol. **86:**907–917.
9. **Haenen, O. L. M., K. Way, S. M. Bergmann, and E. Ariel.** 2004. The emergence of koi herpesvirus and its significance to European aquaculture. Bull. Eur. Assoc. Fish Pathol. **24:**293–307.
10. **Harmache, A., M. LeBerre, S. Droineau, M. Giovannini, and M. Bremont.** 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.
11. **Hedrick, R. P.** 1996. Movement of pathogens with the international trade of live fish: problems and solutions. Rev. Sci. Technol. **15:**523–531.
12. **Hedrick, R. P., O. Gilad, S. Yun, J. Spangenberg, R. Marty, M. Nordhausen, M. Kebus, H. Bercovier, and A. Eldar.** 2000. A herpesvirus associated with mass mortality of juvenile and adult koi, a strain of common carp. J. Aquat. Anim. Health. **12:**44–55.
13. **Hedrick, R. P., R. Marty, M. Nordhausen, M. Kebus, H. Bercovier, and A. Eldar.** 1999. An herpesvirus associated with mass mortality of juvenile and adult koi *Cyprinus carpio*. Fish Health Newsl. **27:**7.
14. **Hutoran, M., A. Ronen, A. Perelberg, M. Ilouze, A. Dishon, I. Bejerano, N. Chen, and M. Kotler.** 2005. Description of an as yet unclassified DNA virus from diseased *Cyprinus carpio* species. J. Virol. **79:**1983–1991.
15. **Ilouze, M., A. Dishon, T. Kahan, and M. Kotler.** 2006. Cyprinid herpes virus-3 (CyHV-3) bears genes of genetically distant large DNA viruses. FEBS Lett. **580:**4473–4478.
16. **Karsi, A., S. Menanteau-Ledouble, and M. L. Lawrence.** 2006. Development of bioluminescent *Edwardsiella ictaluri* for noninvasive disease monitoring. FEMS Microbiol. Lett. **260:**216–223.
17. **Mast, J., C. Nanbru, T. van den Berg, and G. Meulemans.** 2005. Ultrastructural changes of the tracheal epithelium after vaccination of day-old chickens with the La Sota strain of Newcastle disease virus. Vet. Pathol. **42:**559–565.
18. **McGeoch, D. J., F. J. Rixon, and A. J. Davison.** 2006. Topics in herpesvirus genomics and evolution. Virus Res. **117:**90–104.
19. **Miyazaki, T., Y. Kuzuya, S. Yasumoto, M. Yasuda, and T. Kobayashi.** 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.
20. **Neukirch, M., K. Böttcher, and S. Bunnajrakul.** 1999. Isolation of a virus from koi with altered gills. Bull. Eur. Assoc. Fish Pathol. **19:**221–224.
21. **Perelberg, A., M. Smirnov, M. Hutoran, A. Diamant, Y. Bejerano, and M. Kotler.** 2003. Epidemiological description of a new viral disease afflicting cultured *Cyprinus carpio* in Israel. Isr. J. Aquaculture **55:**5–12.
22. **Pikarsky, E., A. Ronen, J. Abramowitz, B. Levavi-Sivan, M. Hutoran, Y. Shapira, M. Steinitz, A. Perelberg, D. Soffer, and M. Kotler.** 2004. Pathogenesis of acute viral disease induced in fish by carp interstitial nephritis and gill necrosis virus. J. Virol. **78:**9544–9551.
23. **Quillet, E., M. Dorson, G. Aubard, and C. Torhy.** 2001. In vitro viral haemorrhagic septicaemia virus replication in excised fins of rainbow trout: correlation with resistance to waterborne challenge and genetic variation. Dis. Aquat. Organ. **45:**171–182.
24. **Roberts, R. J., and A. E. Ellis.** 2001. The anatomy and physiology of teleosts, p. 12–54. *In* R. J. Roberts (ed.), Fish pathology, 3rd ed. W. B. Saunders, London, United Kingdom.
25. **Roberts, R. J. (ed.).** 2001. Fish pathology, 3rd ed. W. B. Saunders, London, United Kingdom.
26. **Roberts, R. J.** 2001. The parasitology of teleosts, p. 254–297. *In* R. J. Roberts (ed.), Fish pathology, 3rd ed. W. B. Saunders, London, United Kingdom.
27. **Ronen, A., A. Perelberg, J. Abramowitz, M. Hutoran, S. Tinman, I. Bejerano, M. Steinitz, and M. Kotler.** 2003. Efficient vaccine against the virus causing a lethal disease in cultured *Cyprinus carpio*. Vaccine **21:**4677–4684.
28. **Smail, D. A., and A. L. S. Munro.** 2001. The virology of teleosts, p. 169–254. *In* R. J. Roberts (ed.), Fish pathology, 3rd ed. W. B. Saunders, London, United Kingdom.
29. **Vanderplasschen, A., and G. L. Smith.** 1997. A novel virus binding assay using confocal microscopy: demonstration that the intracellular and extracellular vaccinia virions bind to different cellular receptors. J. Virol. **71:**4032–4041.
30. **Waltzek, T. B., G. O. Kelley, D. M. Stone, K. Way, L. Hanson, H. Fukuda, I. Hirono, T. Aoki, A. J. Davison, and R. P. Hedrick.** 2005. Koi herpesvirus represents a third cyprinid herpesvirus (CyHV-3) in the family Herpesviridae. J. Gen. Virol. **86:**1659–1667.
31. **Warming, S., N. Costantino, D. L. Court, N. A. Jenkins, and N. G. Copeland.** 2005. Simple and highly efficient BAC recombineering using *galK* selection. Nucleic Acids Res. **33:**e36.
32. **Zinn, K. R., T. R. Chaudhuri, A. A. Szafran, D. O'Quinn, C. Weaver, K. Dugger, D. Lamar, R. A. Kesterson, X. Wang, and S. J. Frank.** 2008. Noninvasive bioluminescence imaging in small animals. ILAR J. **49:**103–115.

The results of the previous chapter demonstrate that the major portal of entry for CyHV-3 in carp after immersion in infectious water is the skin covering the fins and the body. This study together with an earlier report addressing the portal of entry of the rhabdovirus Infectious hematopoietic necrosis virus in salmonids suggest that the skin of teleost fish represents an efficient portal of entry for some viruses.

Fish skin is a complex limiting structure providing mechanical, chemical and immune protection against injury and pathogenic microorganisms. 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 neutralize microorganisms. 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. However, despite the abundance of studies on fish skin immunity and skin bacterial infection, there are little 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.

In the present study, we used the luciferase CyHV-3 recombinant strain described above and bioluminescence imaging to investigate the roles of epidermal mucus as an innate immune barrier against CyHV-3 entry. Our results demonstrate that the mucus of the skin inhibits CyHV-3 binding to epidermal cells and contains soluble molecules able to neutralize CyHV-3 infectivity.

Experimental section

3rd chapter:

Skin mucus of *Cyprinus carpio* inhibits Cyprinid herpesvirus 3 binding to epidermal cells

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V. Stalin Raj*, G. Fournier*, K. Rakus, M. Ronsmans, P.Ouyang, B. Michel, C. Delforges, B. Costes, F.Farnir, B. Leroy, R. Wattiez, C. Melard, J. Mast, F. Lieffrig, A. Vanderplasscen

**These authors contributed equally.*

Experimental section: Preamble 3

VETERINARY RESEARCH

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Skin mucus of *Cyprinus carpio* inhibits cyprinid herpesvirus 3 binding to epidermal cells

Victor Stalin Raj[1†], Guillaume Fournier[1†], Krzysztof Rakus[1], Maygane Ronsmans[1], Ping Ouyang[1], Benjamin Michel[1], Cédric Delforges[1], Bérénice Costes[1], Frédéric Farnir[2], Baptiste Leroy[3], Ruddy Wattiez[3], Charles Melard[4], Jan Mast[5], François Lieffrig[6] and Alain Vanderplasschen[1*]

Abstract

Cyprinid herpesvirus 3 (CyHV-3) is the aetiological agent of a mortal and highly contagious disease in common and koi carp. The skin is the major portal of entry of CyHV-3 in carp after immersion in water containing the virus. In the present study, we used in vivo bioluminescence imaging to investigate the effect of skin mucus removal and skin epidermis lesion on CyHV-3 entry. 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. 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. The present study demonstrates that skin mucus removal and epidermal lesions enhance CyHV-3 entry in carp. It highlights the role of fish skin mucus as an innate immune protection against viral epidermal entry.

Introduction

The koi herpesvirus (KHV), also known as cyprinid herpesvirus 3 (CyHV-3; species *Cyprinid herpesvirus 3*, genus *Cyprinivirus*, family *Alloherpesviridae*, order *Herpesvirales*), is the aetiological agent of a lethal disease in common (*Cyprinus carpio carpio*) and koi (*Cyprinus carpio koi*) carp [1-5]. Since its emergence, in the late 1990s, this highly contagious disease has caused severe economic losses in both common and koi carp culture industries worldwide [6,7].

Recently, we demonstrated using a CyHV-3 recombinant strain expressing luciferase (LUC) and in vivo bioluminescence imaging that the major portal of entry for CyHV-3 in carp after immersion in infectious water is the skin covering the fins and the body [8]. This study together with an earlier report addressing the portal of entry of the rhabdovirus *Infectious hematopoietic necrosis virus* in salmonids [9] suggest that the skin of teleost fish represents an efficient portal of entry for some viruses.

The skin of teleost fish is made up of five structures (Figure 1b, left panel). The mucus layer or cuticle covers the epidermis [10]. The latter is a stratified squamous epithelium composed of three cell layers: (i) the superficial layer, composed of flattened squamous cells, (ii) the intermediate layer, "*stratum germinativum*", encompassing squamous and cuboidal cells and (iii) the basal layer "*stratum basale*" composed of columnar epithelial cells covering the basement membrane. Importantly, unlike its mammalian counterpart, fish epidermis is living and capable of mitotic division at all levels, even at the outermost squamous layer. The predominant cell type in the epidermis is the Malphigian cells. However, glandular cells such as goblet cells secreting mucus and club cells secreting potent alarm substances are also present. The epidermis and the dermis are separated by a relatively thick basement membrane containing pigment

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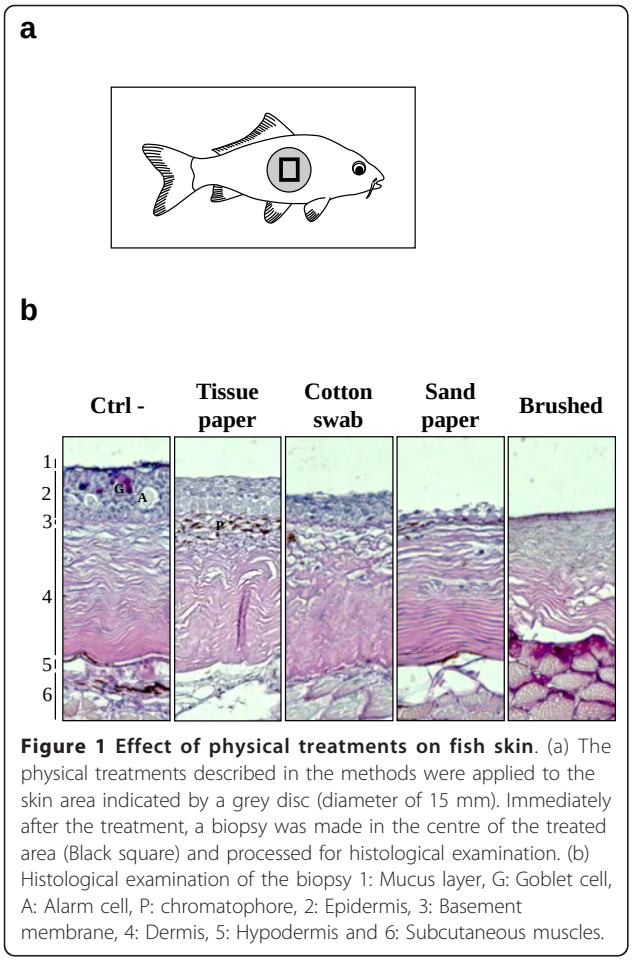

Figure 1 Effect of physical treatments on fish skin. (a) The physical treatments described in the methods were applied to the skin area indicated by a grey disc (diameter of 15 mm). Immediately after the treatment, a biopsy was made in the centre of the treated area (Black square) and processed for histological examination. (b) Histological examination of the biopsy 1: Mucus layer, G: Goblet cell, A: Alarm cell, P: chromatophore, 2: Epidermis, 3: Basement membrane, 4: Dermis, 5: Hypodermis and 6: Subcutaneous muscles.

cells. The scales are dermis structures and consequently are covered by the epidermis.

Fish skin is a complex limiting structure providing mechanical, chemical and immune protection against injury and pathogenic microorganisms [11]. 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 [11-15]. 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 [10]. However, despite the abundance of studies on fish skin immunity and skin bacterial infection, there are few 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 [16-20].

In the present study, we investigated the roles of epidermal mucus as an innate immune barrier against CyHV-3 entry. Our results demonstrate that the mucus of the skin inhibits CyHV-3 binding to epidermal cells and is able to neutralise CyHV-3 infectivity.

Materials and methods

Cells and virus

Cyprinus carpio brain cells (CCB) [21] were cultured in minimum essential medium (MEM) (Invitrogen, Merelbeke, Belgium) containing 4.5 g/L glucose (D-glucose monohydrate, Merck, Darmstadt, Germany) and 10% fetal calf serum [21]. Cells were cultured at 25°C in a humid atmosphere containing 5% CO_2 [22]. The KHV FL BAC 136 LUC TK revertant strain of CyHV-3 was described previously [8]. This recombinant strain encodes a firefly luciferase (LUC) expression cassette inserted in the intergenic region between open reading frame (ORF) 136 and ORF137. The KHV FL BAC recovered strain of CyHV-3 was described previously [22]. This recombinant strain encodes an enhanced green fluorescent protein (EGFP) expression cassette inserted at the end of ORF55.

Fish

Koi carp *(Cyprinus carpio koi)* (Hazorea Aquatics, Kibbutz Hazorea, Israel) and common carp *(Cyprinus carpio carpio)* (CEFRA, University of Liège, Belgium), with an average weight of 16 g, were kept in 60-liter tanks at 24°C. Microbiological, parasitical and clinical examinations of the fish just before the experiments demonstrated that these fish were fully healthy.

Physical treatments of the skin

Four physical treatments were applied on a defined area of the carp epidermis (disc shape, diameter of 15 mm): rubbing with a soft tissue paper (TORK premium, Goteborg, Sweden), rubbing with a cotton swab (Swube Applicator, Becton Dickinson Microbiology system, Maryland, USA), brushing with a rotary electric tooth brush (Philips Sensiflex HX 1513, Anderlecht, Belgium) for 2 s or rubbing with sandpaper (average particle diameter of 265 μm, Medium p60, LUX Wermelskirchen, Germany).

Histochemistery and microscopy analysis

Fish skin explants were fixed by immersion in Carnoy solution (ethanol 6: acetic acid 1: chloroform 3, v/v/v) for 2 h at 4°C. After dehydration with ethanol, samples were embedded in paraffin [23]. Five μm thick sections were stained by a combined Alcian Blue (AB) and Periodic acid-Schiff (PAS) staining [24]. Mounted samples

were observed using a Nikon Eclipse TE 2000-S microscope equipped with a DC 300F charge-coupled device (CCD) camera (Leica, Heerbrugg, Switzerland).

Culture of tail fin explants

Fish were euthanized using benzocaine (100 mg/L of water) (Sigma-Aldrich, Saint Louis, Missouri). The ventral lobe of the tail fin was clipped with forceps before section. Fin fragments maintained in forceps were immerged in a vertical position in minimum essential medium (GIBCO, Invitrogen, Paisley, UK) containing 4.5 g/liter glucose (D-glucose monohydrate; Merck, Damstadt, Germany) and 10% fetal calf serum (FCS) (Greiner Bio One, Frickenhausen, Germany). Tail fin explants were cultured at 25°C in a humid atmosphere containing 5% CO_2.

CyHV-3 inoculation of carp

For viral inoculation mimicking natural infection, fish were kept for 2 h in water containing 10^3 plaque forming unit (PFU)/mL of the KHV FL BAC 136 LUC TK revertant strain. At the end of the incubation period, fish were returned to larger tanks. To avoid removal of skin mucus, fish were caught using a container rather than a fish net, and they were manipulated with great care wearing humidified latex gloves. The animal study was accredited by the local ethics committee of the University of Liège, Belgium (Laboratory accreditation N° 1610008, protocol N°810).

Bioluminescence imaging

Imaging of firefly (*Photinus pyralis*) LUC was performed using an "in vivo imaging system" (IVIS) (IVIS®spectrum, Xenogen, Caliper LifeSciences, Hopkinton, Massachusetts, USA) as described previously [8]. For in vivo analysis, fish were anesthetized with benzocaine (50 mg/L of water). Ten minutes before bioluminescence analysis, D-luciferin (150 mg/kg body weight) (Xenogen, Caliper LifeSciences, Hopkinton, Massachusetts, USA) was administrated by intraperitoneal injection. Each fish was analyzed lying on its left and right side. For analysis of tail fin explants cultured ex vivo, culture medium was replaced by fresh medium containing D-luciferin (150 μg/mL) ten minutes before bioluminescence analysis. All the images presented in this study were acquired using a field view of 15 cm, a 1 min exposure time, a binning factor of 4 and a f/stop of 1. Relative intensities of transmitted light from bioluminescence were represented as a pseudocolor image ranging from violet (least intense) to red (most intense). Corresponding grey-scale photographs and color luciferase images were superimposed using the LivingImage analysis software (Xenogen, Caliper LifeSciences, Hopkinton, Massachusetts, USA).

Transmission electron microscopy

Samples were fixed in 0.1% glutaraldehyde (Sigma-Aldrich, Saint Louis, Missouri, USA). Epon blocks and sections were prepared as described elsewhere [25]. Sections were analyzed using a Tecnai Spirit transmission electron microscope (FEI, Eindhoven, The Netherlands), and electron micrographs were taken using a bottom-mounted 4-by-4 K Eagle camera (FEI).

Collection of carp epidermal mucus and production of clarified mucus extract

Epidermal mucus was collected from common carp (average weight of 5 kg) kept at 22°C (CEFRA, University of Liège, Belgium). Immediately after euthanasia, epidermal mucus was collected by gentle scraping of fish flanks using a soft rubber spatula. Mucus samples were pooled and stored on ice. Clarified mucus extract (CME) was then prepared as follows. Mucus was first clarified by centrifugation (2000 *g* for 10 min at 4°C). Clarified mucus was diluted five times in MEM on ice. To enhance mucus solubilisation, β2-mercaptoethanol (Sigma-Aldrich) was added at the final concentration of 5 mM. The sample was then processed five times through a 7 mL Dounce homogenizer (tight pestle, VWR, Chicago, USA). After an incubation of 30 min on ice, the sample was ultracentrifuged at 100 000 *g* for 30 min at 4°C. The supernatant was collected and sterilized by filtration through a 0.45 μm filter (0.45 μm filter PES, VWR). Finally, the sample was concentrated five times by centrifugation through an Amicon Ultra 3K column (Millipore). The resulting product, hereafter called CME, was stored at -80°C until use. The CME used in the present study had an estimated protein concentration of 0.95 mg/mL as determined with the non-interfering protein assay (GBiosciences, St Louis, USA).

CyHV-3 neutralisation assay by CME

The KHV FL BAC recovered strain of CyHV-3 was diluted in MEM to reach a concentration of 5.10^4 plaque forming unit (pfu)/mL. The effect of CME on CyHV-3 infectivity was tested under two conditions hereafter called pre-incubation and post-incubation addition of CME. For pre-incubation addition of CME, the virus suspension was mixed with adequate volumes of CME and MEM supplemented with 5 mM β2-mercaptoethanol to reach CME final concentrations (vol/vol) of 1/2, 1/4, 1/8, 1/16 and 1/32. Samples were then incubated at 25°C for 2 h. For post-incubation addition of CME, the samples were processed as described above with the exception that the CME volumes were added after the 2 h incubation period. A negative control (NC) sample consisted of incubating the viral suspension with an equal volume of MEM supplemented with 5 mM β2-mercaptoethanol before the 2 h incubation period. All samples were then diluted 200 times in MEM and

CyHV-3 infectivity was titrated on CCB monolayers grown in 24 well plates (BD, Erembodegen, Belgium) as described elsewhere [8]. Viral plaques were counted 3 days post-infection (dpi) using an epifluorescent microscope (Eclipse TE2000-S, Nikon). Statistical analyses of the results were performed by post hoc tests on least squares means for pair wise group comparisons. These analyses were done using SAS version 9.1.

Results

Physical treatments applied to carp epidermis

The goal of the present study was to investigate the effects of epidermal mucus removal and progressive epidermal abrasion on CyHV-3 entry in carp. To reach that goal, four different physical treatments were applied on the defined area of carp skin as depicted in Figure 1a. To avoid an effect on untreated areas, fish were handled with care by the head and the superior lobe of the tail fin. Immediately after treatment, the centre of the treated area was submitted to histological examination (Figure 1b). Gentle rubbing of the epidermis with a soft tissue paper induced removal of the mucus without apparent damage of the epithelial cells. In contrast, the use of a cotton swab induced removal of the mucus and the upper most layers of epidermal cells. After rubbing with sand paper, only a few epidermal columnar cells were left on the basement membrane; while all cells were removed after brushing with an electric tooth brush.

Effect of carp epidermis lesion on CyHV-3 entry in carp

The results presented above demonstrated that the different physical treatments applied locally on carp skin resulted in progressive damaging of the epidermis. These treatments were used to investigate the effect of epidermal mucus removal and progressive epidermal abrasion on CyHV-3 entry in carp. Carp skin was treated on a defined area (Figure 2) just before inoculation with the CyHV-3 KHV FL BAC 136 LUC TK revertant strain expressing LUC as a reporter gene. Sites of CyHV-3 entry in carp were revealed by IVIS examination of carp at different times post-inoculation (Figure 3).

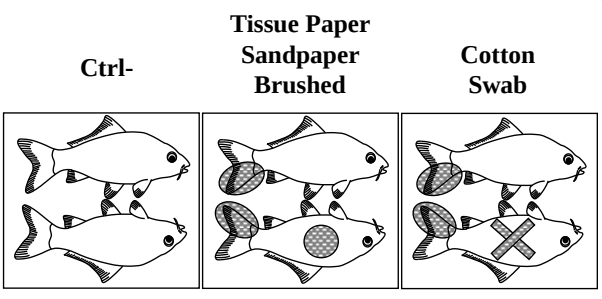

Figure 2 Localised skin physical treatments applied to the fish skin just before inoculation. Schematic diagram representing the area (in grey) of the fish skin to wish the indicated physical treatments were applied just before viral inoculation of the fish analysed in figure 3. Each panel represents the same fish lying on its left and right side.

Mucus removal and superficial abrasion of carp epidermis induced by rubbing with soft tissue paper and cotton swab enhanced CyHV-3 entry in carp. As early as 12 h post-inoculation, a strong LUC signal correlated with the area of the skin treated (Figure 3). Similarly to fish of the control group, the treated fish exhibited small foci of LUC emission distributed randomly revealing entry of the virus through unaffected skin as described earlier. According to post-inoculation time, the spread of the infection on the skin was observed as well as an increase of light emission for a determined site of infection.

Deep abrasion of skin epidermis induced on the flank of fish correlated at 12 h post-inoculation with no LUC signal at the centre of the lesion while the edge of the lesion expressed LUC activity (Figure 3, Sandpaper, Brushed). The absence of LUC activity at the centre of the lesion can be explained by the removal of sensitive cells induced by the treatment; while the presence of a signal at the edge most probably resulted from mucus removal and superficial epidermis abrasion induced at the periphery of the treated area. Interestingly, starting at 24 h post-inoculation a LUC signal appeared at the centre of the treated area while it was negative 12 h earlier. This result can only be explained by an extremely fast regeneration of the epidermis throughout the centre of the lesion providing sensitive cells for viral infection. To address this hypothesis, the kinetics of epidermis healing was investigated after epidermis excoriation on a 15 mm diameter disc (Figure 4). Histological examination performed immediately after lesion induction confirmed the excoriation of the epidermis leaving the basement membrane exposed to water (Figure 4, time 0). Surprisingly, as early as 2 h post-lesion, cell migration was observed from the edge of the lesion toward its centre. The cell migration front consisted of a cell monolayer, while the number of cell layer increased progressively moving away from the centre of the lesion. At 6 h post-lesion, the migration front was nearly closing the wound. At 12 h post-lesion, the epidermis was entirely covering the basement membrane and was uniformly composed of 5-7 layers of epidermal cells with no obvious polarization of the epithelium. At 24 h post-lesion; the polarization of the epidermis was back to normal with the exception of the number of cell layers which was still inferior to normal. At 48 h post-lesion, the epidermis of the treated area could not be differentiated from the control undamaged epidermis.

Independently of the treatment applied locally to the skin, treated fish had more LUC emission foci located

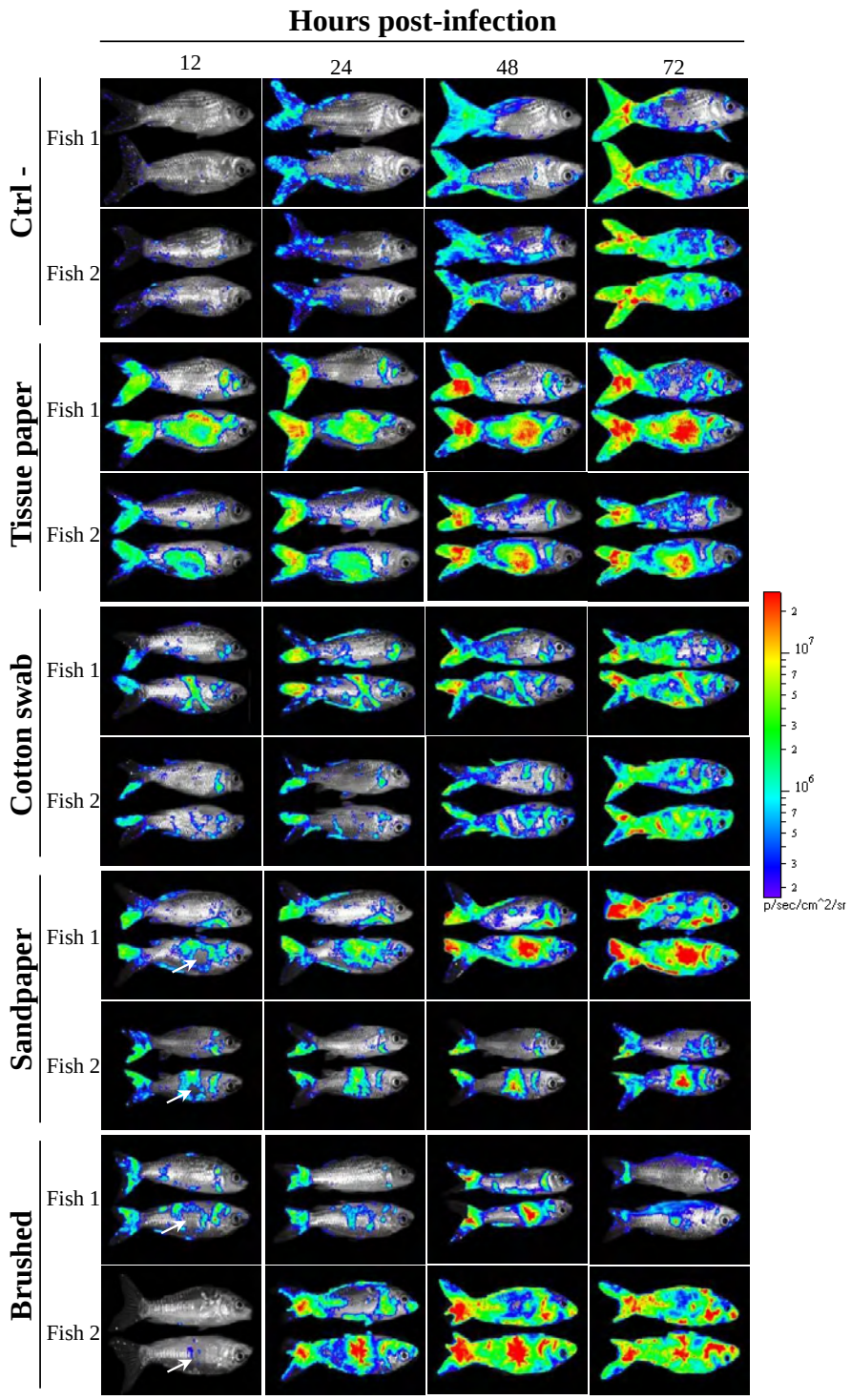

Figure 3 Effect of skin physical treatments on CyHV-3 entry in carp analyzed by bioluminescence imaging. Each physical treatment depicted in Figure 2 was applied to a group of 7 fish. Immediately after skin treatment, fish were inoculated by immersion in water containing the FL BAC 136 LUC TK revertant strain (10^3 PFU/mL of water for 2 h) to mimic natural infection. 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. White arrows indicate the centre of epidermis lesions which was associated with no bioluminescent signal at 12 h post-inoculation but an intense signal later during infection. The images collected over the course of the experiment are presented with standardized minimum and maximum threshold values for photon flux.

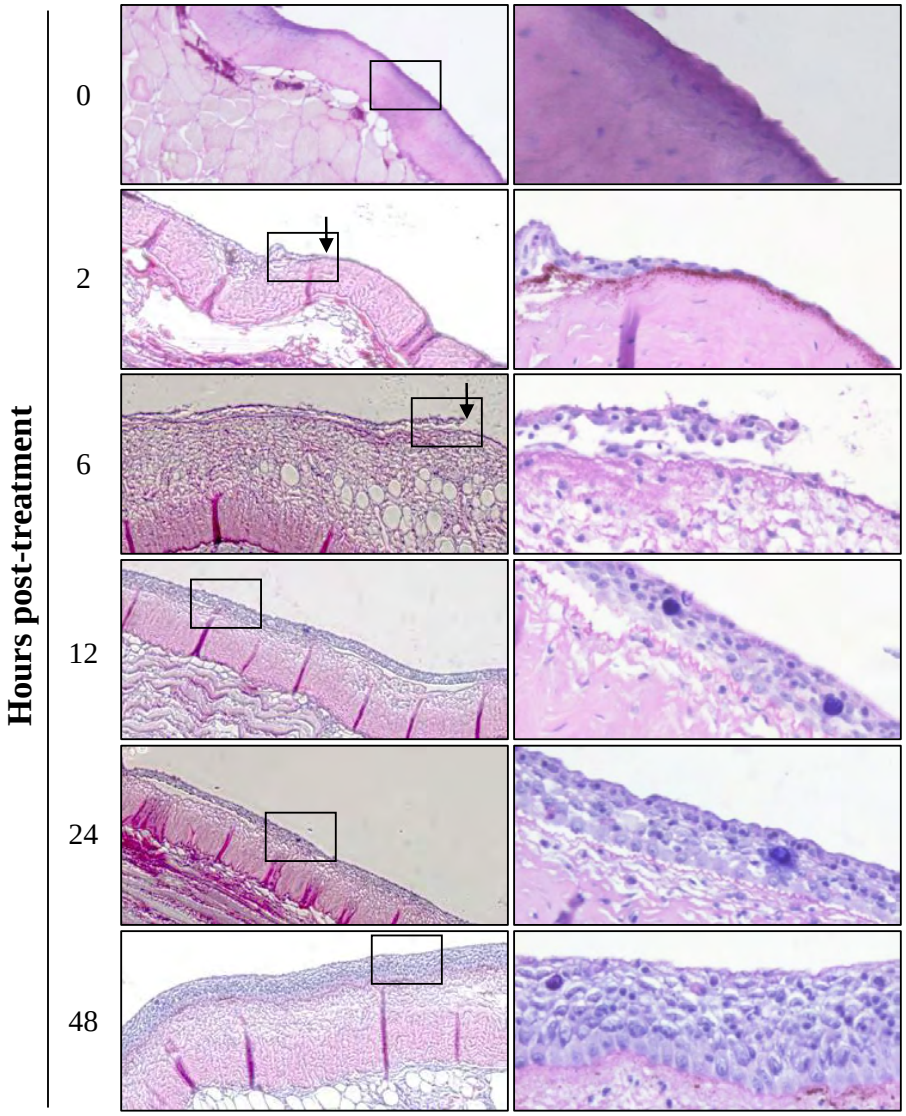

Figure 4 Kinetic of epidermis healing in carp. At time 0, the mucus and the epidermis of the skin were removed by brushing with a rotary electric tooth brush on a 15 mm circular area located on the side of the fish body. At the indicated time post-lesion, a biopsy was performed at the centre of the treated area, and processed for histological examination. Right panels represent higher magnification of the area marked in the left panels. The arrows indicate migration of epidermal cells towards the wound centre.

on the head than fish from the control group (Figure 3). This observation is likely to be the consequence of mucus removal on the head when handling the fish. Light emission was not detected from mock-infected carp used as negative controls (data not shown).

Removal of epidermal mucus enhances CyHV-3 binding to epidermal cells

The results presented above demonstrated that removal of epidermal mucus enhances the entry of CyHV-3 in carp. This observation led to the hypothesis that epidermal mucus could act as an innate immune protection reducing CyHV-3 binding to epidermal cells. To test this hypothesis, tail fin explants with or without mucus were inoculated ex vivo with CyHV-3 (Figure 5). After an incubation of 2 h, viral binding to epidermal cells was investigated by electron microscopy examination. While no viral particles could be detected on fin explants with an intact mucus layer, numerous viral particles were observed on the surface of the fin infected after removal of mucus. Virus

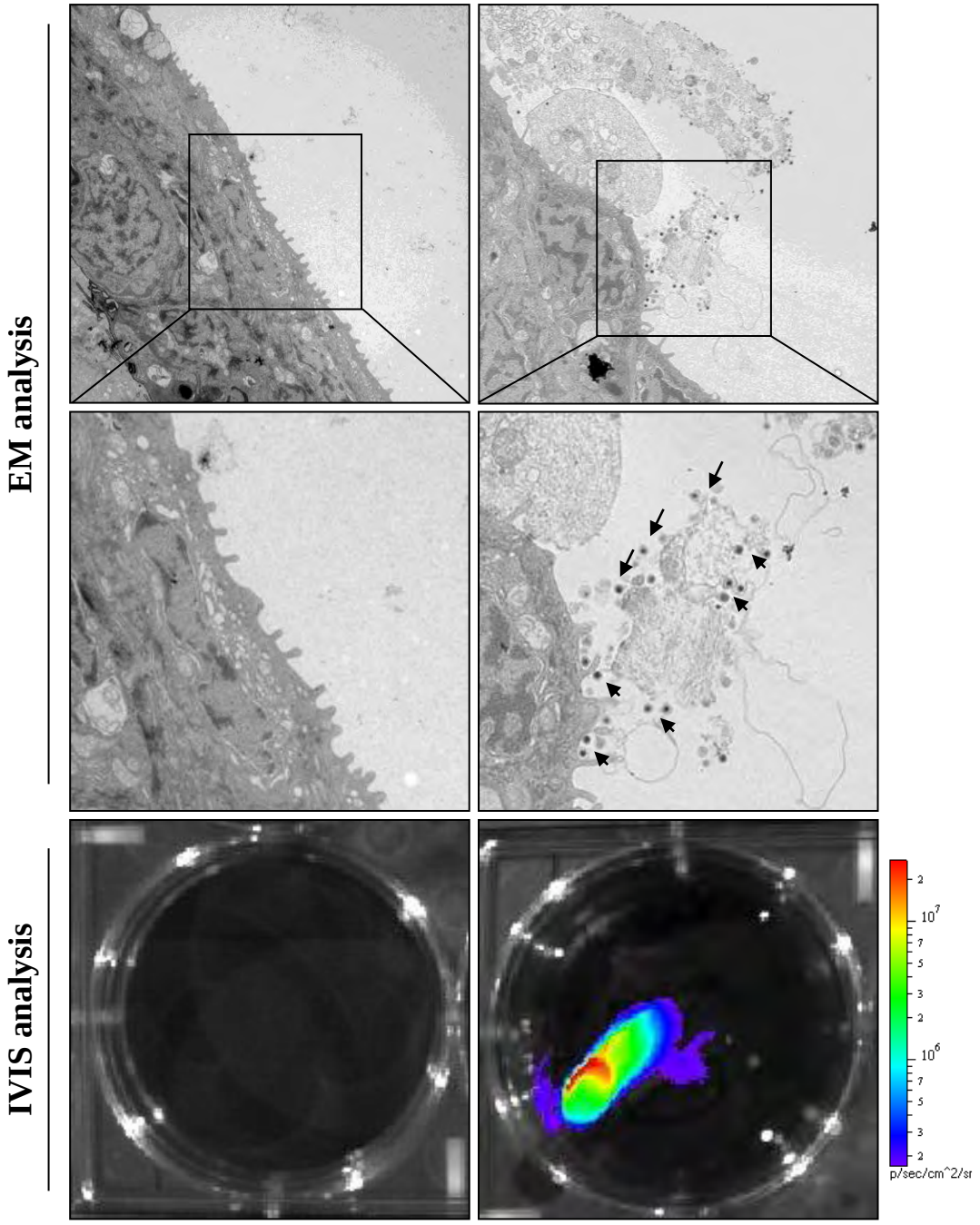

Figure 5 Effect of skin mucus removal on CyHV-3 binding to carp epidermal cells. Tail fin ventral lobes of carp were mock-treated or treated by rubbing with a soft tissue paper to removal epidermal mucus (see methods). Immediately after skin treatments, tail fin explants were harvested and inoculated ex vivo with the FL BAC 136 LUC TK revertant strain (10^6 PFU/mL of culture medium for 2 h). At the end of the 2 h inoculation period, a fragment of the fin was collected and processed for electron microscopy examination (EM analysis). The arrows indicate CyHV-3 particles bound to cells or cell debris. Twenty-four hours post-inoculation, duplicate tail explant cultures were analyzed by bioluminescence imaging (lower panels).

particles were found attached to structurally normal cells but also to lysed cells and cell debris still attached to the epidermis by desmosomes. As damaged cells were not observed in the control untreated sample (without removal of the mucus), they were thought to be the consequence of the mucus removal procedure. IVIS analysis of duplicate fin explants 24 h after inoculation confirmed that CyHV-3 infection of carp skin was drastically enhanced by mucus removal just before inoculation (Figure 5, bottom panels).

Epidermal mucus neutralises CyHV-3 infectivity

In the last section of this study, we investigated whether epidermal mucus can neutralise CyHV-3 infectivity (Figure 6). CME was prepared from epidermal mucus and tested for its ability to neutralise CyHV-3 as described in the materials and methods. Incubation of CyHV-3 with CME at the concentration (vol/vol) of 1/2 down to 1/16 led to a statistically significant reduction of the number of viral plaques compared to the NC sample (Figure 6, pre-incubation addition of CME). In contrast, none of the concentrations tested led to a significant neutralisation effect when CME was added to the sample after the incubation period (Figure 6, post-incubation addition of CME). The latter results demonstrate that diluted CME present in both types of samples (pre- and post-addition of CME) during the final titration step did not influence CyHV-3 infectivity significantly.

Discussion

Mucus covering fish surfaces exposed to water acts as an innate and adaptive first line of defence against pathogen entry [13]. Only very few studies addressed in vivo the role of epidermal mucus as an innate immune protection against bacterial infections [16-20]; while no study has demonstrated so far its role in preventing viral entry in fish. Here, 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. The data of the present study demonstrated that epidermal mucus inhibits CyHV-3 binding to epidermal cells at least partially by neutralisation of viral infectivity, and that epidermal lesions enhance CyHV-3 entry in carp.

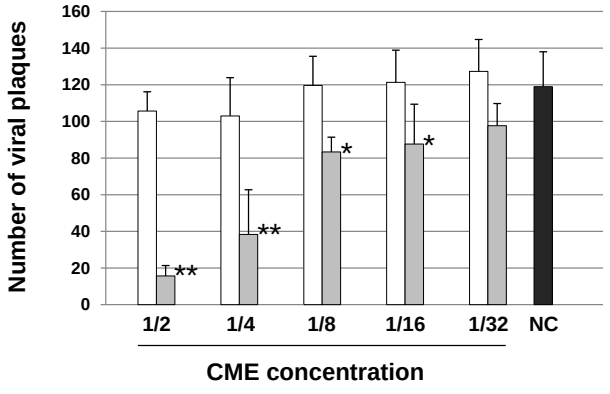

Figure 6 Effect of CME on CyHV-3 infectivity. CME was prepared from common carp epidermal mucus and tested for its ability to neutralise CyHV-3 as described in the materials and methods. Grey and white bars represent the results obtained when adding CME to the samples before and after the 2 h incubation period, respectively. NC represents the negative control sample in which no CME was added. The data presented are the means ± SE of triplicate measurements. The means that are significantly different from the mean of the NC group are marked (* ≤ 0.05, ** ≤ 0.0001).

Carp epidermal mucus inhibits CyHV-3 binding on epidermal cells (Figure 5). As mentioned in the introduction, epidermal mucus confers an innate immune protection against pathogen entry. This protection relies on mechanical reduction of pathogen access to epidermal cells and eventually on pathogen neutralisation by active molecules [13]. The results presented in Figure 6 demonstrated that epidermal mucus neutralises CyHV-3 in a dose dependent manner. Fish epidermal mucus contains a growing list of molecules that could contribute to virus neutralisation, such as for example complement factors, C-reactive protein, immunoglobulines, lectins and defensins [11-15,26,27]. Future studies are required to determine the mechanisms by which epidermal mucus neutralises CyHV-3.

Despite the ability of skin mucus to inhibit CyHV-3 binding to epidermal cells, immersion of carp in infectious water led to viral entry in carp through the skin (Figure 3, Ctrl-). Two hypotheses that are not mutually exclusive can conciliate these observations. Firstly, it is likely that the inhibition of virus binding to epidermal cells by mucus is partial rather than total. Secondly, the sites of primary skin infection could represent areas of the fish body that are uncovered by mucus [13] or covered by a thinner layer compared to the rest of the body. The heterogeneity of the thickness of the mucus layer over the surface of the fish could represent physiological differences or be the consequence of mucus removal caused by physical contact. Consistent with the latter hypothesis, we observed that the sites of primary infection are mainly located at the periphery of the fins (Figure 3).

Mucus removal and epidermal lesions enhance CyHV-3 entry in carp (Figure 3). The results of the present study suggest that skin lesions caused for example by ectoparasite infestations, rough handling or inappropriate environment (as for example a tank with abrasive walls) should enhance the entry of CyHV-3 through the skin and consequently the spread of the disease. At the early stage of the disease, CyHV-3 replicates at the portal of entry [8]. This early replication in the skin

probably explains why infected fish rubbed themselves against each other or against objects. This behaviour could represent an efficient "skin-to-skin" mode of transmission of CyHV-3 in the carp population by inducing physical contact between the skin of infected and naive carp with simultaneous removal of mucus. This hypothesis could at least partly explain the higher transmission dynamics of CyHV-3 in wildlife between adult carps during the host breeding season [28].

In conclusion, the present study demonstrates the role of fish epidermal mucus as an innate immune protection against a viral infection. This study further supports the role of epidermal mucus as an important component of fish innate immunity. It also provides a model to study the effect of immunostimulants on this component of fish innate immunity.

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Authors' contributions
VSR, GF and BC participated in the design of the study. VSR, GF, MR, PO, KR and BM performed the experiments and drafted the figures. BC coordinated some of the experiments. CM and FL controlled the sanitary statue of the carp and took care of zootechnique aspects. CD elaborated ex vivo culture of carp fins. JM performed electron microscopy analyses. FF performed statistical analyses. BL and RW produced and characterized CME. AV conceived the study and drafted the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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References

1. Bretzinger A, Fischer-Scherl T, Oumouma R, Hoffmann R, Truyen U: **Mass mortalities in koi, Cyprinus carpio, associated with gill and skin disease.** *Bull Eur Assoc Fish Pathol* 1999, **19**:182-185.
2. Hedrick RP, Gilad O, Yun S, Spangenberg J, Marty R, Nordhausen M, Kebus M, Bercovier H, Eldar A: **A herpesvirus associated with mass mortality of juvenile and adult koi, a strain of common carp.** *J Aquat Anim Health* 2000, **12**:44-55.
3. Hedrick RP, Marty GD, Nordhausen RW, Kebus M, Bercovier H, Eldar A: **An herpesvirus associated with mass mortality of juvenile and adult koi Cyprinus carpio.** *Fish Health Newsletter, FHS, Am Fish Soc* 1999, **27**:7.
4. Walster C: **Clinical observations of severe mortalities in Koi carp, *Cyprinus carpio*, with gill disease.** *Fish Vet J* 1999, **3**:54-58.
5. Michel B, Fournier G, Lieffrig F, Costes B, Vanderplasschen A: **Cyprinid herpesvirus 3.** *Emerg Infect Dis* 2010, **16**:1835-1843.
6. Haenen O, Way K, Bergmann SM, Ariel E: **The emergence of koi herpesvirus and its significance to European aquaculture.** *Bull Eur Assoc Fish Pathol* 2004, **24**:293-307.
7. Hedrick RP: **Movement of pathogens with the international trade of live fish: problems and solutions.** *Rev Sci Tech* 1996, **15**:523-531.
8. Costes B, Raj VS, Michel B, Fournier G, Thirion M, Gillet L, Mast J, Lieffrig F, Brémont M, Vanderplasschen A: **The major portal of entry of koi herpesvirus in Cyprinus carpio is the skin.** *J Virol* 2009, **83**:2819-2830.
9. Harmache A, LeBerre M, Droineau S, Giovannini M, Brémont M: **Bioluminescence imaging of live infected salmonids reveals that the fin bases are the major portal of entry for Novirhabdovirus.** *J Virol* 2006, **80**:3655-3659.
10. Roberts RJ, Ellis AE: In *The anatomy and physiology of teleosts.* Edited by: Roberts RJ, Fp. WB Saunders, London, United Kingdom; 2001:12-54.
11. Fontenot DK, Neiffer DL: **Wound management in teleost fish: biology of the healing process, evaluation, and treatment.** *Vet Clin North Am Exot Anim Pract* 2004, **7**:57-86.
12. Ellis AE: **Innate host defense mechanisms of fish against viruses and bacteria.** *Dev Comp Immunol* 2001, **25**:827-839.
13. Shephard KL: **Functions for fish mucus.** *Rev Fish Biol Fisheries* 1994, **4**:401-429.
14. Subramanian S, Ross NW, MacKinnon SL: **Comparison of antimicrobial activity in the epidermal mucus extracts of fish.** *Comp Biochem Physiol B Biochem Mol Biol* 2008, **150**:85-92.
15. Palaksha KJ, Shin GW, Kim YR, Jung TS: **Evaluation of non-specific immune components from the skin mucus of olive flounder (Paralichthys olivaceus).** *Fish Shellfish Immunol* 2008, **24**:479-488.
16. Fouz B, Devasa S, Gravningen K, Barja JL, Toranzo AE: **Antibacterial action of the mucus of turbot.** *Bull Eur Assoc Fish Pathol* 1990, **10**:56-59.
17. Harrell LW, Etlinger HM, Hodgins HO: **Humoral-factors important in resistance of salmonid fish to bacterial disease. II. Anti-vibrio anguillarum activity in mucus and observations on complement.** *Aquaculture* 1976, **7**:363-370.
18. Hjelmeland K, Christie M, Raa J: **Skin mucus protease from rainbow-trout, salmo-gairdneri richardson, and its biological significance.** *J Fish Biol* 1983, **23**:13-22.
19. Kanno T, Nakai T, Muroga K: **Mode of transmission of vibriosis among ayu Plecoglossus altivelis.** *J Aquat Anim Health* 1989, **1**:2-6.
20. Takahashi Y, Itami T, Konegawa K: **Enzymatic-properties of bacteriolytic substances in skin mucus and intestine of carp.** *Fish Pathol* 1986, **21**:187-191.
21. Neukirch M, Böttcher K, Bunnajrakul S: **Isolation of a virus from koi with altered gills.** *Bull Eur Ass Fish Pathol* 1999, **19**:221-224.
22. Costes B, Fournier G, Michel B, Delforge C, Raj VS, Dewals B, Gillet L, Drion P, Body A, Schynts F, Lieffrig F, Vanderplasschen A: **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* 2008, **82**:4955-4964.
23. Prophet E, Mills B, Arrington JB, Sobin LH, eds: *Laboratory Methods in Histotechnology* Armed Forces Institute of Pathology, Washington DC; 1992, 25-59.
24. Mowry RW: **Alcian Blue technics for the histochemical study of acedic carbohydrates.** *J Histochem Cytochem* 1956, **4**:407.
25. Mast J, Nanbru C, van den Berg T, Meulemans G: **Ultrastructural changes of the tracheal epithelium after vaccination of day-old chickens with the La Sota strain of Newcastle disease virus.** *Vet Pathol* 2005, **42**:559-565.
26. Jin JY, Zhou L, Wang Y, Li Z, Zhao JG, Zhang QY, Gui JF: **Antibacterial and antiviral roles of a fish beta-defensin expressed both in pituitary and testis.** *PLoS One* 2010, **5**:e12883.
27. Suzuki Y, Tasumi S, Tsutsui S, Okamoto M, Suetake H: **Molecular diversity of skin mucus lectins in fish.** *Comp Biochem Physiol B Biochem Mol Biol* 2003, **136**:723-730.
28. Uchii K, Telschow A, Minamoto T, Yamanaka H, Honjo MN, Matsui K, Kawabata Z: **Transmission dynamics of an emerging infectious disease in wildlife through host reproductive cycles.** *ISME J* 2011, **5**:244-251.

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The data of the two previous chapters demonstrate that the skin is the major portal of entry after inoculation of carp by immersion in water containing CyHV-3. While this model of infection mimics some natural conditions in which infection takes place, other epidemiological conditions could favour entry of virus through the digestive tract. Firstly, droppings from infected carp have been shown to contain infectious virus. Ingestion of infectious droppings or food contaminated by droppings by naïve subjects could represent a source of oral inoculation. Secondly, carp express cannibalistic and necrophagous behaviour. By ingestion of infectious tissues of CyHV-3 infected carp, naïve subjects could infect themselves through the oral route. Finally, recent studies performed in habitats with CyHV-3 history suggested that aquatic invertebrates feeding by water filtration could accumulate and store CyHV-3. Ingestion of contaminated invertebrates could represent another possible source of CyHV-3 oral infection. Together with the observation that CyHV-3 replicates intensively in the intestine during the disease it causes, the possible sources of CyHV-3 oral contamination listed above stimulated the study of the role of carp digestive tract as a possible portal of entry for the virus.

In the present study, we investigated the role of the carp digestive tract as a viral portal of entry using bioluminescence imaging. We found that feeding carp with infectious materials induces CyHV-3 entry through infection of the pharyngeal periodontal mucosa.

Experimental section

4th chapter:

Feeding *Cyprinus carpio* with infectious materials mediates cyprinid herpesvirus 3 entry through infection of pharyngeal periodontal mucosa

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Feeding Cyprinus carpio with infectious materials mediates cyprinid herpesvirus 3 entry through infection of pharyngeal periodontal mucosa

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ABSTRACT

Cyprinid herpesvirus 3 (CyHV-3), also known as Koi herpesvirus, is the etiological agent of a mortal disease in common and koi carp. Recently, we investigated the entry of CyHV-3 in carp using bioluminescence imaging and a CyHV-3 recombinant strain expressing luciferase (LUC). We demonstrated that the skin is the major portal of entry after inoculation of carp by immersion in water containing CyHV-3. While this model of infection mimics some natural conditions in which infection takes place, other epidemiological conditions could favour entry of virus through the digestive tract. Here, we investigated whether ingestion of infectious materials mediates CyHV-3 entry through the digestive tract. Carp were fed with materials contaminated with the CyHV-3 LUC recombinant (oral contamination) or immersed in water containing the virus (contamination by immersion). Bioluminescence imaging analyses performed at different times post-infection led to the following observations: (i) The pharyngeal periodontal mucosa is the major portal of entry after oral contamination, while the skin is the major portal of entry after contamination by immersion. (ii) Both modes of inoculation led to the spreading of the infection to the various organs tested. However, the timing and the sequence in which the organs turned positive were different between the two modes of inoculation. Finally, we compared the disease induced by the two inoculation modes. They led to comparable clinical signs and mortality rate. The results of the present study suggest that, based on epidemiological conditions, CyHV-3 can enter carp either by skin or periodontal pharyngeal mucosal infection.

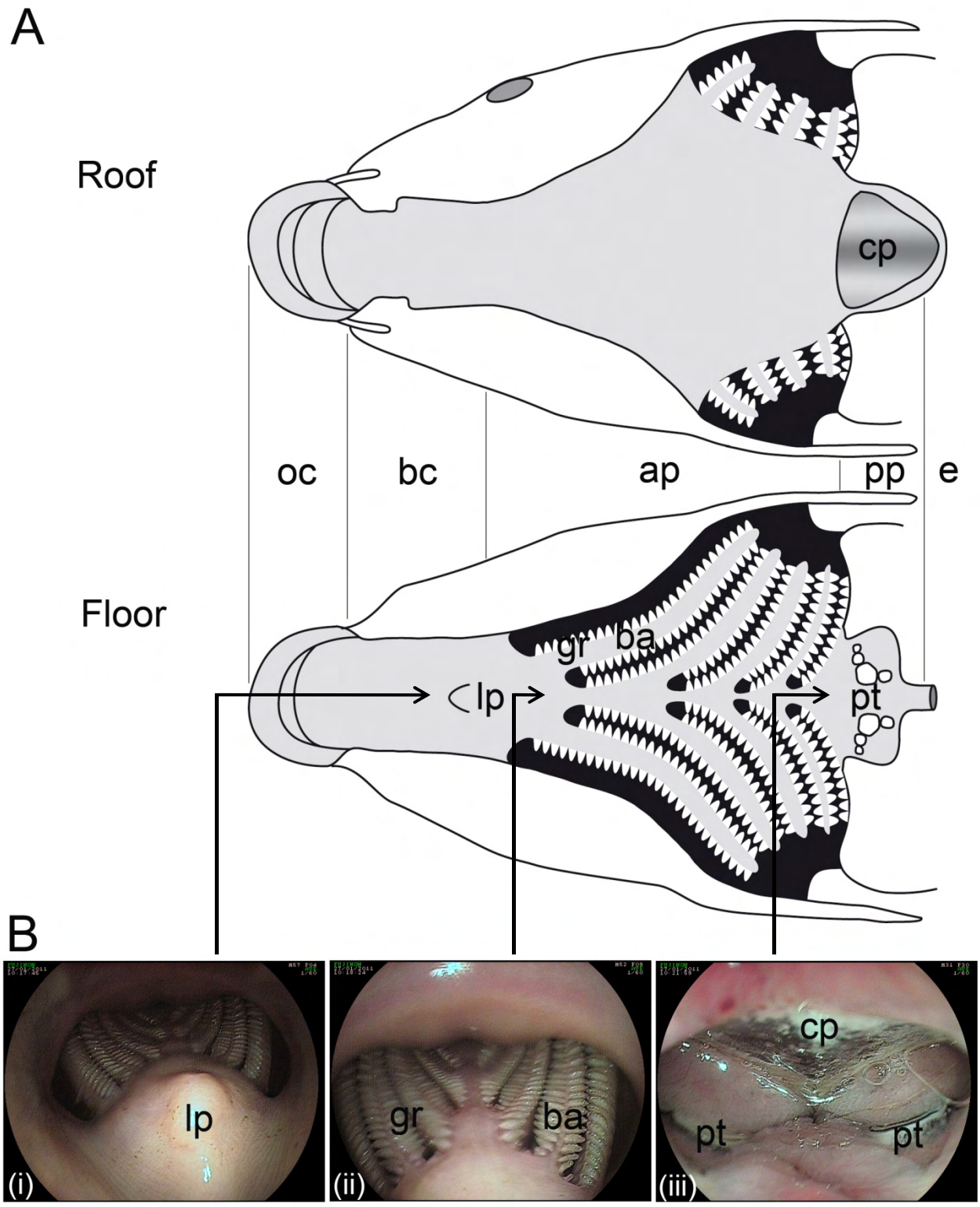

Figure 1: Oropharyngeal cavity of carp.
(A) Schematic representation of the roof and floor of the carp oropharyngeal cavity (adapted from Sibbing 1988). The cavity is subdivided into four sections: oral cavity (oc), buccal cavity (bc), anterior pharynx (ap), posterior pharynx (pp). esophagus (e); lp, lingual process; ba, branchial arch; gr, gill raker; pt, pharyngeal teeth; and cp, chewing pad. (B) Endoscopy views of carp oropharyngeal cavity. A 2 kg carp was anesthetized before exploration of its oropharyngeal cavity by endoscopy. Panels i–iii illustrate bc, ap, and pp, respectively.

INTRODUCTION

The Cyprinid herpesvirus 3 (CyHV-3; species *Cyprinid herpesvirus 3*, genus *Cyprinivirus*, family *Alloherpesviridae*, order *Herpesvirales*), also known as koi herpesvirus, is the aetiological agent of a contagious and mortal disease in common (*Cyprinus carpio carpio*) and koi (*Cyprinus carpio koi*) carp [1-5]. Since its emergence, in the late 1990s, CyHV-3 has caused severe economic losses in both common and koi carp culture industries worldwide [4, 6, 7].

The recent publication of the CyHV-3 sequence [8], together with the cloning of its genome as an infectious bacterial artificial chromosome (BAC) [9], allowed the production of CyHV-3 recombinant strains. Recently, we took advantage of these advances to construct a luciferase (LUC)-expressing recombinant strain by intergenic insertion of a LUC expression cassette [10]. Using this recombinant strain, bioluminescent imaging, and an original system to perform percutaneous infection restricted to the posterior part of the fish, we showed that the skin covering the fins and the body, and not the gills, is the major portal of entry after inoculation by immersion in water containing the virus [10]. This study, together with an earlier report addressing the portal of entry of a rhabdovirus (infectious hematopoietic necrosis virus) in salmonids [11], suggests that the skin of teleost fish is an efficient portal of entry for certain viruses.

The skin is the major portal of entry of CyHV-3 in carp after inoculation by immersion in water containing the virus [10]. While this model of infection certainly mimics some natural condition of infections, other conditions could favor entry of the virus through the digestive tract. Firstly, droppings from infected carp have been shown to contain infectious virus [12]. Ingestion of infectious droppings or food contaminated by droppings by naïve subjects could represent a source of oral inoculation. Secondly, carp express cannibalistic and necrophagous behavior. By ingestion of infectious tissues of CyHV-3 infected carp [10, 13], naïve subjects could infect themselves through the oral route. Finally, recent studies performed in habitats with CyHV-3 history suggested that aquatic invertebrates feeding by water filtration could accumulate and store CyHV-3 [14]. Ingestion of contaminated invertebrates could represent another possible source of CyHV-3 oral infection. Together with the observation that CyHV-3 replicates intensively in the intestine during the disease it causes [12], the possible sources of CyHV-3 oral contamination listed above stimulated the study of the role of carp digestive tract as a possible portal of entry for the virus.

The digestive tract of common carp is composed of the oropharyngeal cavity, the esophagus and the intestine [15-17]. The oropharyngeal cavity is subdivided into four sections: the oral cavity, the buccal cavity, the anterior pharynx (syn. branchial cavity) and the posterior pharynx (syn. chewing cavity) (Fig. 1). The three first sections are involved in respiration and food selection, while the posterior pharynx between pharyngeal teeth and chewing pad is involved in mastication. Common carp are stomach-less fish. The short esophagus connects the posterior pharynx to the anterior part of the intestine also called pseudogaster [18].

In the present study, we investigated the role of the carp digestive tract as a viral portal of entry using bioluminescence imaging. We found that feeding carp with infectious materials induces CyHV-3 entry through infection of the pharyngeal periodontal mucosa. The results of the present study suggest that, based on epidemiological conditions, CyHV-3 can enter carp either by skin (immersion in infectious water) or periodontal pharyngeal mucosal infection (ingestion of infectious materials).

MATERIALS AND METHODS

Virus

The KHV FL BAC 136 LUC TK revertant strain of CyHV-3, hereafter called LUC strain, was described previously [10]. This recombinant strain encodes a firefly luciferase (LUC) expression cassette inserted in the intergenic region between open reading frame (ORF) 136 and ORF137.

Fish

Common carp *(Cyprinus carpio carpio)* (CEFRA, University of Liège, Belgium) with an average weight of 10g and 100g were kept in 60-liter tanks at 24 °C. Microbiological, parasitical and clinical examinations of the fish just before the experiments demonstrated that these fish were fully healthy. Two common carp with a weight of 2 and 3 kg were collected from a private pond.

CyHV-3 inoculation of carp

Common carp were inoculated by one of two different inoculation modes. To mimic contamination through infectious water, 10g fish (5 fish/ L) were immersed for 2 h in water containing 300 PFU/ml of the CyHV-3 LUC strain. At the end of the contamination period, the fish were returned to the larger tank. To mimic contamination by the oral route, (10g and 100g) fish kept individually in 2 L of water were fed with three pellets of food (Ichi Food Summer mini 2-3 mm; Aquatic Science) contaminated with the CyHV-3 LUC strain. Food pellets were contaminated with CyHV-3 by immersion of ten food pellets per ml of CyHV-3 LUC strain (2.8×10^5 PFU/ml) for 10 min. Pellets were distributed to fish immediately after incubation. To determine the number of infectious particles contained in a pellet, 5 contaminated pellets (in triplicate) were disrupted by flushing through a 5 ml pipette in 5 ml of minimum essential medium (MEM) (Invitrogen, Merelbeke, Belgium). After centrifugation (6000 *g* for 20 min at 4 °C), the supernatant was collected and sterilized by filtration through a 0.45 µm filter (0.45 µm filter PES, VWR). Infectious particles were then titrated as described elsewhere [10]. Titration of CyHV-3 in the pellets revealed that they contained 754.5 ± 59.6 PFU/pellet (mean ± SE of triplicate measurements). Fish were regrouped in the larger tank after ingestion of the food. The animal studies presented in this manuscript have been accredited by the local ethics committee of the University of Liège, Belgium (N° LA1610008/810, 1059 and 1063).

Bioluminescence imaging

Imaging of firefly (*Photinus pyralis*) LUC was performed using an "in vivo imaging system" (IVIS) (IVIS®spectrum, Xenogen, Caliper LifeSciences, Hopkinton, Massachusetts, USA) as described previously [10]. Fish were anesthetized with benzocaine (50 mg/L of water). Fifteen minutes before bioluminescence analysis, D-luciferin (150 mg/kg body weight) (Xenogen, Caliper LifeSciences, Hopkinton, Massachusetts, USA) was administered by intraperitoneal injection. Each fish was analysed in vivo lying on its right and its left side and ex vivo after euthanasia and dissection. All the images presented in this study were acquired using a field view of 15 cm, an auto-exposure time with a maximum of 1 minute, a binning factor of 4 and a f/stop of 1. Relative intensities of transmitted light from bioluminescence were represented as a pseudocolor image ranging from violet (least intense) to red (most intense). Corresponding grey-scale photographs and color luciferase images were superimposed using the LivingImage analysis software (Xenogen, Caliper LifeSciences, Hopkinton, Massachusetts, USA). For quantitative comparisons, the Living Image software (Caliper Life Sciences) was used to obtain the total flux ($p.s^{-1}$) over each region of interest (ROI). All the ROI automatically identified by the IVIS software as positive (Fig. 4A) were standing out against background with a difference of at least 3 log.

Transmission electron microscopy

Samples were fixed in 0.1% glutaraldehyde (Sigma-Aldrich, Saint Louis, Missouri, USA). Epon blocks and sections were prepared as described elsewhere for histological and electron microscopic examination [19]. Sections were analysed using a Tecnai Spirit transmission electron microscope (FEI, Eindhoven, The Netherlands), and electron micrographs were taken using a bottom-mounted 4-by-4 K Eagle camera (FEI).

Statistical analyses

A possible difference in the dynamics of IVIS positive organs (Fig. 4B) or in the dynamics of mortality (Fig. 5) was tested using a permutation test as follows. Firstly, occurrences were recorded in the real dataset. Then, in successive repetitions (1000 or 10000) of the same procedure, these occurrences were randomly allocated to each of the 2 groups (immersion and oral inoculation), so mimicking the observed data but without introducing any systematic difference between the 2 groups. A measure of the global difference between the 2 curves – taken as the sum over the days of the absolute difference at any given day – was then obtained for these shuffled dataset and compared to the really observed one. The proportion of shuffled datasets with a measure greater or equal to the real difference was then taken as the p-value.

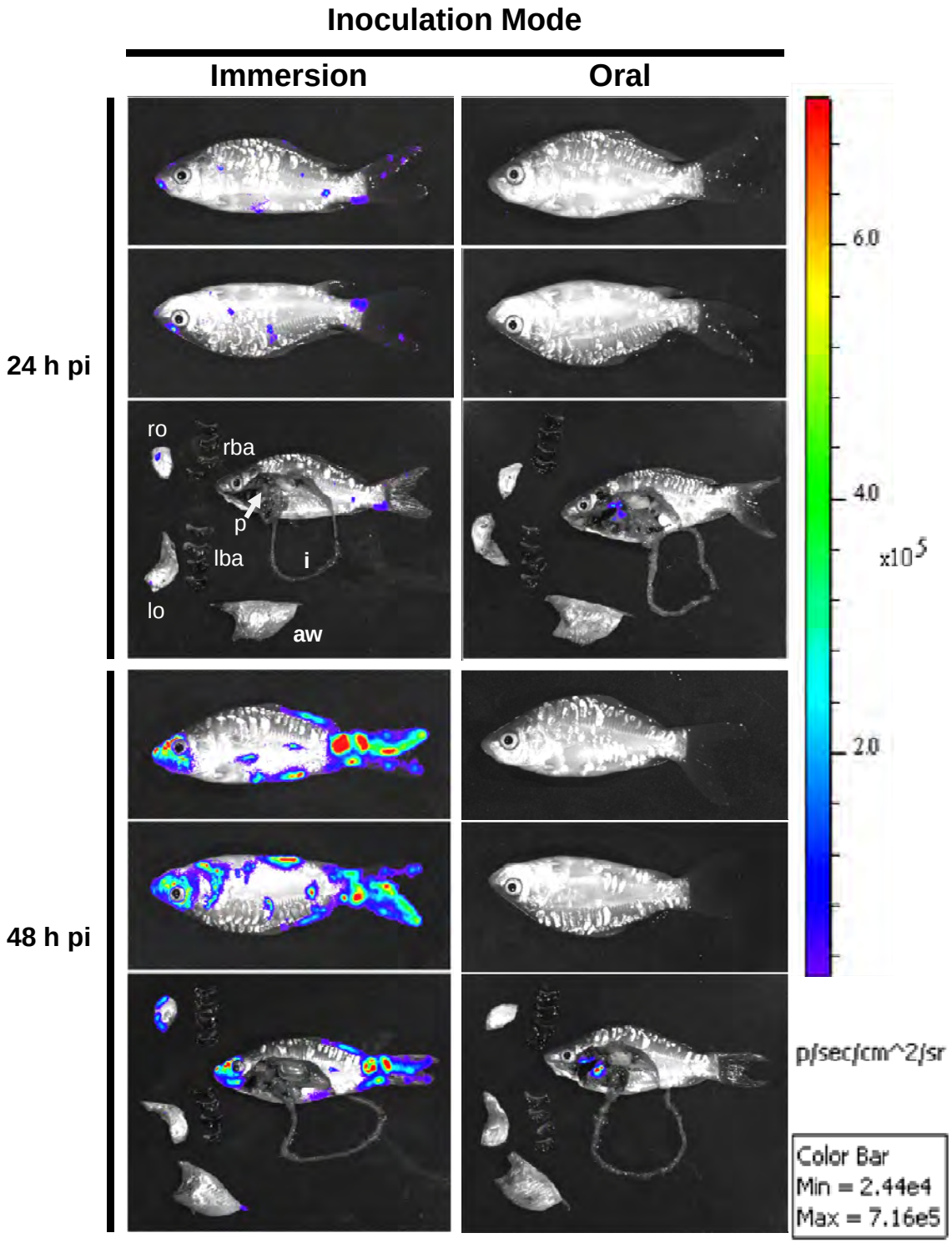

Figure 2: The portal of entry of CyHV-3 in carp analysed by bioluminescence imaging.
Two groups of fish (mean weight 10 g) were infected with the CyHV-3 LUC strain either by bathing them in water containing the virus (Immersion, left column) or by feeding them with food pellets contaminated with the virus (Oral, right column). At the indicated time pi, six fish per group were analysed by bioluminescence IVIS. Each fish was analysed lying on its right and its left side. To analyze internal signals, fish were euthanized and dissected immediately after in vivo bioluminescence imaging. Dissected fishes and isolated organs were analysed for ex vivo bioluminescence. The analysis of one fish is presented for each time point and inoculation mode. Pictures collected over the course of this experiment are presented with a standardized minimum and maximum threshold value for photon flux. rba, right branchial arches; lba, left branchial arches; ro, right operculum; lo, left operculum; p, pharynx; aw, abdominal wall; i, intestine.

RESULTS AND DISCUSSION

CyHV-3 portal of entry after inoculation by immersion in infectious water or by feeding with contaminated materials

In the present study, we investigated the role of carp digestive tract as a putative portal of entry for CyHV-3 using bioluminescence imaging. Carp were infected with the CyHV-3 LUC strain using two modes of inoculation: immersion in water containing the virus and feeding with contaminated materials (Fig. 2). Fish were analysed by IVIS 24 and 48 h post-infection. Because photon emission is drastically attenuated in fish tissues [10], each fish was analysed in vivo lying on its right and then left side, and ex vivo after euthanasia and dissection. The results can be summarized as follows: In fish inoculated by immersion, 5 out of 6 fish analysed 1 day post-infection (dpi) expressed at least one focal source of light on the body surface. The signals were detected from various anatomic sites of the fish body, but principally on the fins. Analyses performed 2 dpi revealed that all of the fish had LUC signals on their surface (n = 6). In comparison to day 1, the number and the intensity of light focal sources detected 2 dpi increased in number and intensity. None of the fish inoculated by immersion expressed internal LUC signals neither at 1 dpi nor at 2 dpi. These observations confirmed our former results [10] demonstrating that the skin is the major portal of entry of CyHV-3 after inoculation by immersion in infectious water. Analysis of fish inoculated by ingestion of infectious materials led to unexpected results (Fig. 2, oral inoculation mode). While none of the six fish analysed 1 dpi displayed LUC signals on the skin, one of them expressed LUC at the posterior part of the carp pharyngeal cavity. At 2 dpi, all of the analysed fish (n = 6) had intense light-emitting foci in the posterior part of the pharyngeal cavity. For 5 of the fish, no other LUC signal was detected elsewhere on or in the body (Fig. 2). In addition to a strong pharyngeal signal, one single fish expressed a focal source of light on one branchial arch (data not shown). Because of the small size of the common carp used for this experiment, it was difficult to identify precisely the site of light emission within the pharyngeal cavity. Consequently this part of the experiment was repeated with larger carp (100 g, n = 5) (Fig. 3).

The results obtained were consistent with those generated in smaller fish. All fish (n = 5) that we analysed 2 dpi expressed LUC at the posterior part of the pharyngeal cavity. Ex vivo bioluminescent analysis of dissected pharyngeal cavities revealed that luciferase expression was localized to the protruding periodontal pharyngeal mucosa between the pharyngeal teeth and the chewing pad (Fig. 3A). This mucosa forms protruding foliaceous papillae within the pharyngeal cavity (Fig. 1B, panel iii) [17]. The stratified oropharyngeal epithelium consists of common epithelial cells, as well as several specialized cells, such as mucous cells, club cells, chloride cells, and sensory cells [17]. Next, to investigate whether LUC expression detected on the pharyngeal mucosa was associated with viral replication, and if so to identify the cell type(s) supporting the infection, a biopsy specimen of positive mucosa was analysed by electron microscopy (Fig. 3C-D). A detailed examination of ultrathin sections revealed cells supporting viral replication in the mucosa epithelium. The infected

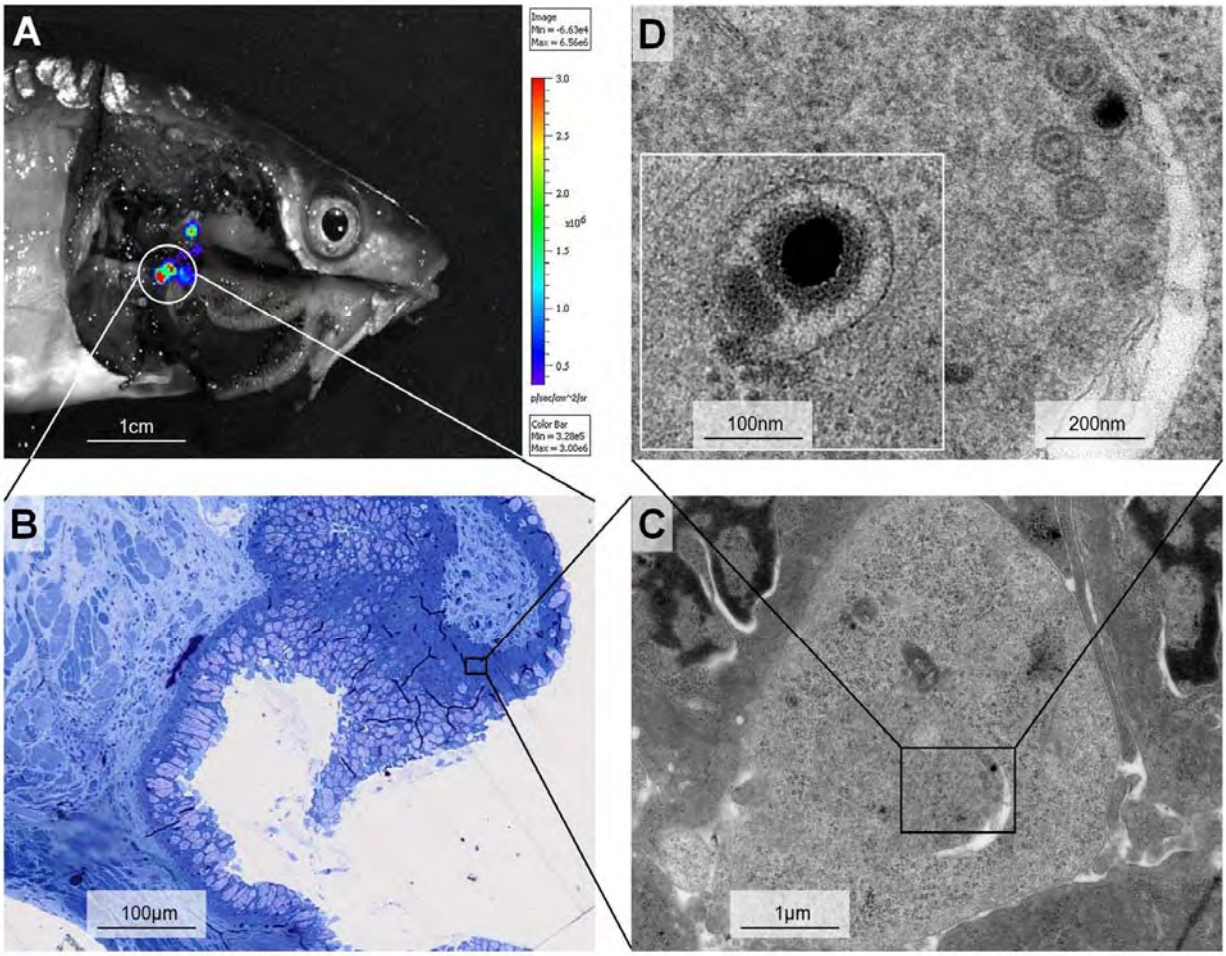

Figure 3: In situ localization of LUC activity and detection of viral replication in carp periodontal pharyngeal mucosa.

Carp weighing 100 g were fed with food pellets contaminated with the CyHV-3 LUC strain. At 2 dpi, carp were anesthetized, injected with luciferine, and euthanized immediately before dissection of the oropharyngeal cavity. Dissected fish were analysed for ex vivo bioluminescence (A). A fragment of periodontal pharyngeal mucosa emitting bioluminescence was harvested and processed for histological (B) and electron microscopy analysis (C and D). Panel C shows low magnification of the epithelium. Panel D shows one representative infected epithelial cell at higher magnification.

cells could be identified at low magnification based on their less-electron-dense cytoplasm and nucleus. Viral capsids and enveloped particles were observed in the nuclei and the cytosol of the infected cells, respectively. All of the infected cells that we detected were common epithelial cells.

While common epithelial cells are abundant throughout the oropharyngeal cavity, LUC signal was restricted to the pharyngeal periodontal mucosa (Fig. 2 and 3). There are two hypotheses that could explain this observation. First, the common epithelial cells in this area could express cell-surface molecules that make them highly sensitive to CyHV-3 infection. A second, more likely, hypothesis relies on a mechanical phenomenon: during mastication, mucus removal and/or microlesions could be induced in protruding foliaceous papillae by food and/or the pharyngeal teeth, creating an efficient portal of entry for CyHV-3. This hypothesis is consistent with our recent observation that skin mucus removal with or without associated epidermal lesions drastically enhance CyHV-3 entry [20]. To investigate the likelihood of this hypothesis, we recorded carp mastication movements by video endoscopy (Video 1, second section). The video showed that the protruding periodontal mucosa covers pharyngeal teeth and is likely to be affected by mastication.

CyHV-3 replicates intensively in the intestine and is excreted in droppings during the disease it causes [12] (see data of Fig. 4 below). However, the data of the present study suggest that the intestine does not act as a portal of entry for CyHV-3 after oral contamination. Several hypotheses could explain these observations. Firstly, it is possible that CyHV-3 is quickly inactivated in the lumen of the anterior part of the digestive tract. Intestinal mucus and/or secreted enzymes could inactivate CyHV-3 infectivity. This hypothesis is supported by the recent observation that epidermal soluble mucus extract is able to neutralise CyHV-3 infectivity [20]. Secondly, it is possible that enterocytes which are polarized cells express CyHV-3 receptor(s) for entry on their basolateral plasma membrane but not on their luminal apical membrane.

CyHV-3 pathogenesis after inoculation by immersion in infectious water or by feeding with contaminated materials

The results presented above suggest that according to epidemiological conditions, CyHV-3 enters carp through skin (immersion in infectious water) or periodontal pharyngeal infection (feeding on contaminated materials). In the second part of this study, we investigated whether the two modes of inoculation induce similar CyHV-3 disease. First, we investigated by bioluminescence imaging how the virus spreads from the portal of entry to secondary sites of replications. Two groups of fish were infected either by immersion in infectious water or by feeding with infectious materials (Fig. 4). At 1, 2, 4, 6 and 8 dpi, 6 fish per group were analysed by bioluminescence imaging and the emission of photons was quantified for selected tissues/organs (Fig. 4A). Figure 4B illustrates the dynamics of the infection within tested organs according to the two modes of inoculation. Statistical analyses of these data (permutation test, 10000 permutations) demonstrated that the dynamic of positive organs differed significantly between the two modes of inoculation for the skin ($p = 0.004$) and the pharynx ($p =$

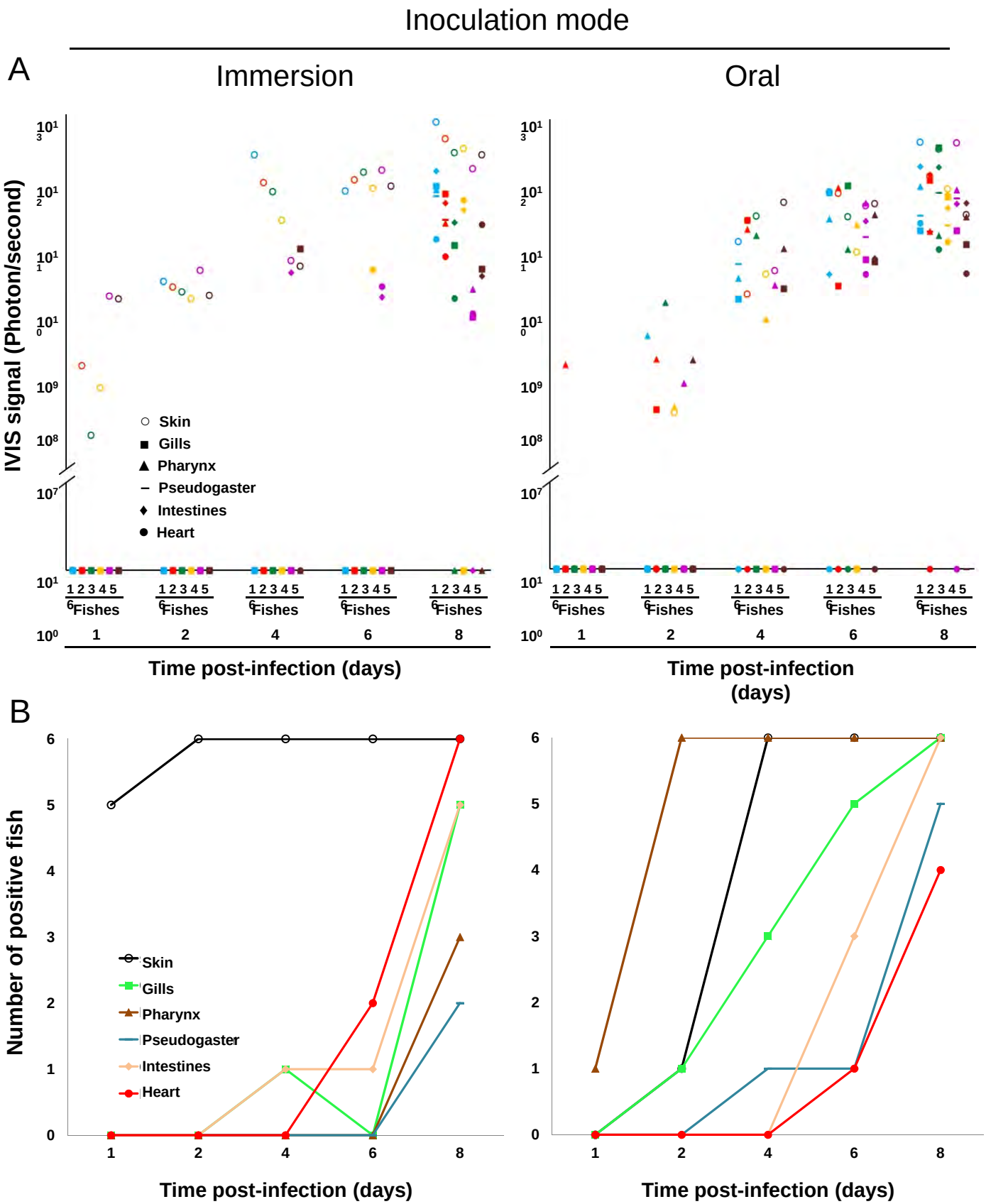

Figure 4: Progression of CyHV-3 infection in carp analysed by bioluminescence imaging.
Two groups of fish (mean weight of 10 g) were infected with the CyHV-3 LUC strain either by bathing them in water containing the virus (Immersion, left column) or by feeding them with food pellets contaminated with the virus (Oral, right column). At the indicated time post-infection, six fish per group were analysed by in vivo and ex vivo bioluminescence imaging. A/ For each fish, the IVIS signal (Photon/second) was determined for several organs (skin, gills, pharynx, pseudogaster, intestine, and heart) as described in the materials and methods. B/ For each analysed organ, the number of positive fish is presented according to time post-infection. This experiment is representative of two independent experiments.

0.0025), while gills (p = 0.059) almost reached significance. Pseudogaster (p = 0.237), guts (p = 0.832) and heart (p = 0.833) were found not to differ.

The analyses performed at 1 and 2 dpi confirmed that the skin and the periodontal pharyngeal mucosa are the major portal of entry after inoculation by immersion in infectious water and by feeding with infectious materials, respectively. In the latter case, soon after positivity of the pharynx, the skin became positive closely followed by the gills (Fig. 4B). Two hypotheses could explain that the skin is the second place the virus is seen after the pharynx following oral exposure. Firstly, it is possible that the skin signal detected 2 dpi represents a low level of infection that occurred at the time of feeding. Indeed, it is likely that contaminated food pellets released virions in the water before they were ingested by carp. Due to the low concentration of the virus in the water, the resulting skin infection was perhaps not be detected 1 dpi but rather at 2 dpi. Secondly, it is possible that the skin signal observed on day 2 pi represents spreading of the viral infection on the fish surface from the periodontal pharyngeal mucosa.

In the fish inoculated by immersion, the gills were not positive until day 6 when they were positive earlier in the oral route (Fig. 4). The most likely explanation of this observation is that the earlier infection of the gills observed in the oral route represents the spreading of the viral infection from the pharyngeal mucosa by continuity of tissue; while the infection of the gills observed after bath exposure could reflect the systemic spreading of the infection.

Next, we investigated whether the mode of inoculation (immersion versus oral) could affect the disease induced in term of clinical signs and mortality rate (Fig. 5). Three groups of fish each consisting of 17 carp were inoculated either by immersion in infectious water (1 group) or by feeding with contaminated materials (2 groups). Daily examination of carp did not reveal any significant difference between the two modes of infection. All groups of fish expressed the clinical signs associated with CyHV-3 disease, including apathy, folding of the dorsal fin, increased mucus secretion, suffocation, erratic swimming, and loss of equilibrium. The intensities of the clinical signs were comparable in the three groups. Comparison of the survival rates between the three groups led to the following conclusions. Survival curves for the two orally inoculated samples were compared using a binomial comparison of the survival rates 30 days after inoculation. No significant difference was found (p=0.08). Accordingly, the two samples were pooled and the survival rate after 30 days was compared between the pooled sample and the sample with inoculation by immersion. Again, a binomial test confirmed that no significant survival rate difference can be detected (p = 0.15). A possible difference in the mortality dynamics according to the mode of inoculation was then tested using a permutation test (1000 permutations). The obtained p-value of p = 0.331 showed that no significant difference exists between the two inoculation mode dynamics.

The results presented above suggest that CyHV-3 induces a comparable disease after entry through infection of the skin or periodontal pharyngeal mucosa (Fig. 4 and 5). Based on the IVIS data of the present study and earlier studies [12, 21], we propose a model for CyHV-3 pathogenesis.

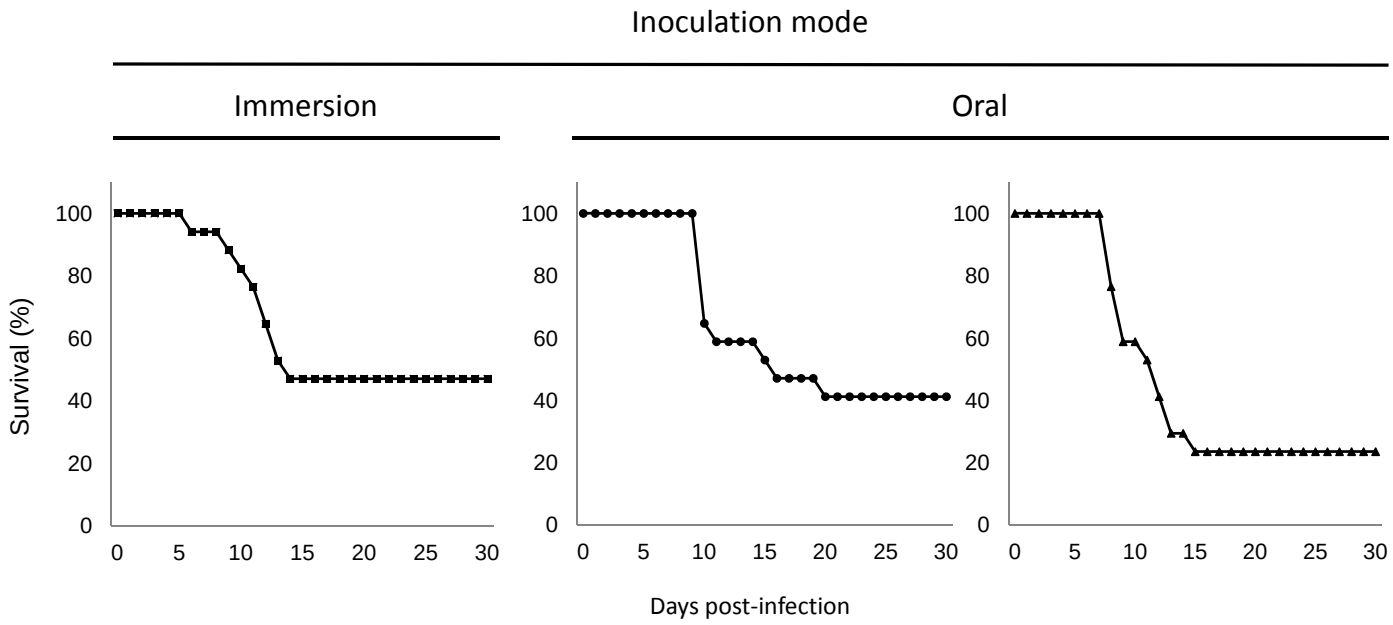

Figure 5: **Survival rates of carp infected with the CyHV-3 LUC strain**.
On day zero, three groups of fish, each consisting of 17 common carp (mean weight of 10g) kept in separate tanks, were infected either by bathing them in water containing the virus (left graph, immersion) or by feeding them with food pellets contaminated with the virus (middle and right graphs, Oral) as described in Materials and Methods. The fish were examined daily for clinical signs of CyHV-3 disease, and dead fish were removed. The percentage of survival is expressed according to time post-infection.

According to epidemiological conditions (immersion in water containing the virus or ingestion of infectious materials), CyHV-3 enters fish through skin or pharyngeal periodontal infection (Fig. 2) [10, 20]. Earlier reports based on PCR analysis described an early and fast systemic spread of the virus in infected fish [13, 22-24], while our IVIS data suggested that active replication within secondary sites occurs only 4-6 days after contamination. To explain these data we propose that the rapid (2 dpi) and systemic dissemination observed by PCR reflects the secondary infection of blood cells [24], which could not be detected by bioluminescence imaging. Infected blood cells supporting a replicative infection could act as Trojan horse for the virus leading to a systemic distribution of the virus within the infected host. Associated with this phase of systemic distribution, the virus could reach secondary sites of replication among which some will contribute to excretion of infectious particles in the environment (intestine and gills). A recent study on CyHV-3 pathogenesis support the role of infected blood cells described above both during clinical infection as well as during latency [21].

In conclusion, this study demonstrated that according to epidemiological conditions, CyHV-3 can enter carp either through infection of the skin (immersion in infectious water) or through infection of the pharyngeal periodontal mucosa (feeding on infectious materials). The existence of these two portal of entry adapted to different epidemiological conditions most probably contributes to the high contagious nature of the virus.

COMPETING INTEREST

The authors declare that they have no competing interest.

AUTHOR'S CONTRIBUTIONS

GF, MB and VSR contributed to the design of the study. GF, MB, VSR performed the experiments and drafted the figures. EP and PV provided expertise in the oropharyngeal cavity of cyprinids. DP performed endoscopy exploration of carp pharyngeal cavity. FL controlled the sanitary statue of the carp and took care of zootechnique aspects. JM performed electron microscopy analyses. FF performed statistical analyses. AV conceived the study and drafted the manuscript. All authors read and approved the final manuscript.

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REFERENCES

1. Bretzinger A, Fischer-Scherl, T., Oumouma, R., Hoffmann, R., Truyen, U.: **Mass mortalities in koi, Cyprinus carpio, associated with gill and skin disease.** *Bull Eur Assoc Fish Pathol* 1999, **19:**182-185.
2. Hedrick RP, Marty, R., Nordhausen, M., Kebus, M., Bercovier, H., Eldar, A.: **An herpesvirus associated with mass mortality of juvenile and adult koi *Cyprinus carpio*.** *Fish Health Newsletter, FHS, Am Fish Soc* 1999, **27:**7.
3. Hedrick RP, Gilad, O., Yun, S., Spangenberg, J., Marty, R., Nordhausen, M., Kebus, M., Bercovier, H., Eldar, A.: **A herpesvirus associated with mass mortality of juvenile and adult koi, a strain of common carp.** *J Aquat Anim Health* 2000, **12:**44-55.
4. Michel B, Fournier G, Lieffrig F, Costes B, Vanderplasschen A: **Cyprinid herpesvirus 3: an interesting virus for applied and fundamental research.** *Emerging Infectious Diseases* 2010, **16:**1835-1843.
5. Walster: **Clinical observations of severe mortalities in koi carp, Cyprinus carpio, with gill disease.** *Fish Veterinary Journal* 1999, **3:**54-58.
6. Haenen OL, Engelsma M: **Global distribution of KHV with particular reference to Europe.** 2004.
7. Hedrick RP: **Movement of pathogens with the international trade of live fish: problems and solutions.** *Rev Sci Tech* 1996, **15:**523-531.
8. Aoki T, Hirono I, Kurokawa K, Fukuda H, Nahary R, Eldar A, Davison AJ, Waltzek TB, Bercovier H, Hedrick RP: **Genome sequences of three koi herpesvirus isolates representing the expanding distribution of an emerging disease threatening koi and common carp worldwide.** *J Virol* 2007, **81:**5058-5065.
9. Costes B, Fournier G, Michel B, Delforge C, Raj VS, Dewals B, Gillet L, Drion P, Body A, Schynts F, et al: **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* 2008, **82:**4955-4964.
10. Costes B, Stalin Raj V, Michel B, Fournier G, Thirion M, Gillet L, Mast J, Lieffrig F, Bremont M, Vanderplasschen A: **The major portal of entry of koi herpesvirus in cyprinus carpio is the skin.** *J Virol* 2009, **83:**2819-2830.
11. Harmache A, LeBerre M, Droineau S, Giovannini M, Bremont M: **Bioluminescence imaging of live infected salmonids reveals that the fin bases are the major portal of entry for Novirhabdovirus.** *J Virol* 2006, **80:**3655-3659.
12. Dishon A, Perelberg A, Bishara-Shieban J, Ilouze M, Davidovich M, Werker S, Kotler M: **Detection of carp interstitial nephritis and gill necrosis virus in fish droppings.** *Appl Environ Microbiol* 2005, **71:**7285-7291.
13. Gilad O, Yun S, Zagmutt-Vergara FJ, Leutenegger CM, Bercovier H, Hedrick RP: **Concentrations of a Koi herpesvirus (KHV) in tissues of experimentally infected Cyprinus carpio koi as assessed by real-time TaqMan PCR.** *Dis Aquat Organ* 2004, **60:**179-187.
14. Kielpinski M, Kempter J, Panicz R, Sadowski J, Schütze H, Ohlemeyer S, Bergmann SM: **Detection of KHV in Freshwater Mussels and Crustaceans from Ponds with KHV History in Common Carp (Cyprinus carpio).** *The Israeli Journal of Aquaculture* 2010, **62:**28-37.
15. Atema J: **chemical senses, chemical signals and feeding behavior in fishes.** In *Fish Behavior and its use in the capture and culture of fishes. Volume* 5. Edited by Bardach JE, Magnuson JJ, May RC, Reinhart JM. Manila: ICLARM; 1980: 512

16. Lamb CF, Finger TE: **Gustatory control of feeding behavior in goldfish.** *Physiol Behav* 1995, **57:**483-488.
17. Sibbing F: **Specializations and limitations in the utilization of food resources by the carp, Cyprinus carpio : a study of oral food processing.** *Environmental Biology of Fishes* 1988, **22:**161-178.
18. Kalat T, Shabanipour N: **Internal Anatomy of Common Carp (Cyprinus carpio) as Revealed by Magnetic Resonance Imaging.** *Applied Magnetic Resonance* 2010, **38:**361-369.
19. Mast J, Nanbru C, van den Berg T, Meulemans G: **Ultrastructural changes of the tracheal epithelium after vaccination of day-old chickens with the La Sota strain of Newcastle disease virus.** *Vet Pathol* 2005, **42:**559-565.
20. Raj VS, Fournier G, Rakus K, Ronsmans M, Ouyang P, Michel B, Delforges C, Costes B, Farnir F, Leroy B, et al: **Skin mucus of Cyprinus carpio inhibits cyprinid herpesvirus 3 binding to epidermal cells.** *Vet Res* 2011, **42:**92.
21. Eide KE, Miller-Morgan T, Heidel JR, Kent ML, Bildfell RJ, Lapatra S, Watson G, Jin L: **Investigation of koi herpesvirus latency in koi.** *J Virol* 2011, **85:**4954-4962.
22. Miyazaki T, Kuzuya Y, Yasumoto S, Yasuda M, Kobayashi T: **Histopathological and ultrastructural features of Koi herpesvirus (KHV)-infected carp Cyprinus carpio, and the morphology and morphogenesis of KHV.** *Dis Aquat Organ* 2008, **80:**1-11.
23. Perelberg A, Smirnov, M., Hutoran, M., Diamant, A., Bejerano, Y., Kotler, M.: **Epidemiological description of a new viral disease afflicting cultured Cyprinus Carpio in Israel.** *The Israeli Journal of Aquaculture* 2003, **55:**5-12.
24. Pikarsky E, Ronen A, Abramowitz J, Levavi-Sivan B, Hutoran M, Shapira Y, Steinitz M, Perelberg A, Soffer D, Kotler M: **Pathogenesis of acute viral disease induced in fish by carp interstitial nephritis and gill necrosis virus.** *J Virol* 2004, **78:**9544-9551.

Discussion et perspectives

L'intensification récente de l'aquaculture a contribué à révéler l'existence de nombreux herpèsvirus de poisson. Depuis son émergence dans les années 1990, l'herpèsvirus cyprin 3 (CyHV-3), s'est montré particulièrement contagieux et mortel, entrainant des pertes économiques majeures dans l'industrie des carpes koi et commune. Le nombre croissant d'études menées sur ce pathogène révèle l'intérêt qu'il suscite sur le plan de la recherche fondamentale et appliquée. Le but de cette thèse était d'identifier les portes d'entrée du CyHV-3 chez son hôte naturel la carpe commue (*Cyprinus carpio*). La démarche scientifique appliquée au cours de cette thèse a mené à quatre études.

La première étude décrit le clonage du génome complet du CyHV-3 sous la forme d'un BAC clone stable et infectieux (Costes *et al.*, 2008). Evitant les écueils liés à la taille, aux répétitions et aux mutations génomiques accumulées en culture *in vitro*, le BAC CyHV-3 permet le maintien stable et la mutagenèse du génome viral en système procaryote. Plusieurs souches recombinantes ont ainsi été produites à partir du BAC. L'obtention de cet outil est une avancée précieuse permettant notamment l'étude des portes d'entrée du virus par la production d'une souche recombinante exprimant un gène rapporteur.

Plusieurs auteurs ont postulé que les branchies pourraient être la porte d'entrée du CyHV-3 chez la carpe (Dishon *et al.*, 2005; Gilad *et al.*, 2004; Ilouze *et al.*, 2006; Miyazaki *et al.*, 2008; Pikarsky *et al.*, 2004). Dans la deuxième étude, cette hypothèse a été testée par imagerie bioluminescente *in vivo* (IVIS). Grâce au BAC clone du CyHV-3, une souche virale recombinante exprimant la LUC en tant que rapporteur a été produite (Costes *et al.*, 2009). Au moyen de cette souche LUC et d'un astucieux système limitant l'infection cutanée à la partie postérieure du poisson, il a été démontré que l'entrée virale se faisait par la peau de la carpe (Costes *et al.*, 2009). Ces résultats, associés à ceux d'une étude antérieure explorant la porte d'entrée d'un rhabdovirus chez les salmonids (Harmache *et al.*, 2006), suggèrent que la peau des poissons téléostéens représente une porte d'entrée efficace pour certains virus.

Chez les poissons téléostéens, l'épiderme est un épithélium stratifié squameux qui, à la différence des mammifères, est constitué dans son intégralité de cellules vivantes. La peau des poissons est couverte d'un mucus servant de barrière mécanique et contenant un ensemble de molécules capables de neutraliser les infections microbiennes et les infestations parasitaires (Ellis, 2001; Fontenot & Neiffer, 2004; Palaksha *et al.*, 2008; Shephard, 1994; Subramanian *et al.*, 2008). Dans la troisième étude, grâce au CyHV-3 LUC, nous avons étudié l'effet du retrait du mucus et de lésions progressives de l'épiderme sur l'entrée cutanée du virus chez la carpe. Il a été démontré que le mucus épidermique inhibe efficacement l'accès du CyHV-3 aux cellules épidermiques superficielles de la peau. Par ailleurs, l'activité neutralisante anti-CyHV-3 d'extraits solubles de mucus a été démontrée *in vitro*. L'ensemble de ces résultats démontre le rôle du mucus cutané des poissons comme protection immune innée contre certaines infections virales.

Les deux précédentes études ont envisagé l'exposition de la peau et des branchies au virus par immersion. D'autres conditions épidémiologiques pourraient faciliter l'entrée du virus par le tractus

digestif. Cette hypothèse a été testée dans la quatrième étude. L'ingestion d'un aliment contaminé avec du virus a révélé la pénétration du CyHV-3 dans la carpe par infection de la muqueuse pharyngienne péri-odontale.

La première partie de cette thèse a été consacrée à l'obtention d'un outil essentiel à la mutagenèse, le KHV BAC. Depuis une dizaine d'années, la manipulation des herpèsvirus a été facilitée par le clonage de leur génome sous la forme d'un BAC (Borst *et al.*, 1999; Wagner *et al.*, 2002). Ces vecteurs permettent le maintien stable du génome viral et sa mutagenèse en *Escherichia coli*, ainsi que la reconstitution des particules virales infectieuses par transfection du plasmide BAC en cellules permissives. Le CyHV-3 procure un modèle extrême pour l'étude de la mutagenèse des herpèsvirus à plus d'un titre. Premièrement, avec 295 kpb (Aoki *et al.*, 2007), le CyHV-3 possède le plus grand génome au sein des *Herpesvirales*. Deuxièmement, sa séquence révèle de nombreuses régions répétées (Aoki *et al.*, 2007). Enfin, la multiplication prolongée en culture cellulaire de ce virus entraine une dérive génomique (Ronen *et al.*, 2003) qui rend difficile la mutagenèse par recombinaison homologue classique en système eucaryote.

L'obtention du BAC CyHV-3 démontre la possibilité de manipuler un génome d'herpèsvirus supérieur aux 235 kpb du Cytomégalovirus humain (HCMV) (Borst *et al.*, 1999). A l'origine développé pour le séquençage du génome humain (Shizuya *et al.*, 1992), le BAC conserve durablement la séquence clonée en bactéries (Etude 1, Figure 3), ce qui permet d'amplifier et de purifier une grande quantité d'ADN viral cloné, facilitant le séquençage du génome du CyHV-3. On note que lors du clonage, une seule répétition terminale (TR) a été conservée. Cette caractéristique résout les problèmes d'auto-recombinaisons et facilitera la mutagenèse des neuf gènes qu'elle comporte. Mais l'intérêt majeur du BAC clone réside dans les possibilités de mutagenèse qu'il offre. En effet, grâce à la recombinaison en système procaryote, il est possible de modifier (déléter, remplacer, tronquer, supprimer ou insérer) n'importe quelle partie du génome viral (gènes essentiels ou non, promoteurs, micro-satellites,…) avec n'importe quelle séquence ADN endogène ou exogène. Afin d'illustrer l'une de ces nombreuses possibilités, l'ORF 16 codant potentiellement pour une GPCR a été délété et le phénotype de la souche virale recombinante caractérisé *in vitro* et *in vivo* (Costes *et al.*, 2008). L'insertion inter-génique d'un gène rapporteur (LUC) (Costes *et al.*, 2009) et son utilisation ont également été démontrées au cours des études 2, 3 et 4.

Deux grandes contraintes lors de la mutagenèse existent cependant. Une première contrainte, commune aux BAC-clones d'herpèsvirus, réside dans le choix du gène rapporteur, du promoteur (endogène ou exogène, précoce immédiat, précoce, tardif, de latence) et leur site d'insertion. En effet, ceux-ci peuvent affecter certaines propriétés biologies du virus et/ou de la cellule hôte (Baens *et al.*, 2006) et par conséquent la réponse de l'hôte face à l'infection. Les études présentent dans cette thèse, basées sur l'utilisation d'une souche CyHV-3 LUC, n'échappent pas à cette critique. En effet, il est possible que certaines cellules infectées n'aient pas été détectées par défaut d'expression de la LUC.

Le virus recombinant LUC produit est un outil précieux pour l'étude future de la latence virale. Cela dit, il y aura lieu de vérifier que l'expression constitutive de la LUC par le recombinant ne représente pas une cible pour l'immunité adaptative du sujet infecté.

La deuxième contrainte est plus spécifique au CyHV-3. Lors du clonage, la cassette BAC a été insérée au sein de l'ORF 55, une des rares séquences connues du CyHV-3 au début de ce projet (Bercovier *et al.*, 2005) et décrite comme étant non-essentielle *in vitro* chez les poxvirus et les herpèsvirus (Coen *et al.*, 1989; Panicali & Paoletti, 1982). L'ORF55 est donc interrompu par la cassette BAC. Plusieurs solutions à ce problème ont été adoptées. Lors de la reconstitution de particules virales infectieuses, il est possible de laisser la cassette BAC en place, générant ainsi un virus exprimant le marqueur EGFP mais dont l'ORF 55 est tronqué. Il est également possible d'exciser la cassette BAC (virus tronqué pour l'ORF 55) ou de restaurer l'ORF55 (Costes *et al.*, 2008). Enfin, une dernière solution, qui n'a pas été développée dans cette thèse, a consisté en la génération d'un nouveau BAC clone du CyHV-3 (G. Fournier, résultats non montrés). En effet, après la publication de la séquence virale (Aoki *et al.*, 2007), il était en effet possible de cibler une région intergénique pour l'insertion de la cassette BAC.

Malgré ces contraintes, l'intérêt du BAC clone dans l'étude fondamentale du CyHV-3 est évident : par l'altération de n'importe quel gène viral, il est possible d'étudier d'une part le caractère essentiel ou non d'un gène *in vitro* et *in vivo*, d'autre part les conséquences de l'absence ou de l'altération de ce gène ou groupe de gènes dans la biologie de l'infection. Cette approche, couplée à l'étude de ces mêmes gènes exprimés en système isolé, à l'étude du protéome, ou encore à la réponse immune de l'hôte, devrait profondément enrichir notre compréhension du virus et de la maladie qu'il occasionne chez la carpe. Parmi les 156 ORFs du CyHV-3, la plupart sont inconnus, mais certains sont des homologues d'autres gènes d'herpèsvirus (Aoki *et al.*, 2007). Ainsi au cours de notre première étude nous avons montré : (i) que les ORF16 et 55 n'étaient pas essentiels *in vitro* ou *in vivo*, (ii) que l'ORF16 ne semblait pas jouer un rôle majeur dans la biologie du virus et (iii) que la troncation de l'ORF55 réduisait la virulence du CyHV-3. Des expériences complémentaires menées en collaboration avec le Prof. Balzarini du Rega Institute de Louvain ont permis de caractériser d'un point de vue fonctionnel le produit d'expression de l'ORF55 comme étant une Thymidine Kinase (TK) de type II, et celui de l'ORF140 une Thymidilate Kinase (TmpK), deux gènes impliqués dans la virulence du CyHV-3 (G. Fournier, résultats non montrés). Certaines protéines des *Herpesviridae* sont impliquées dans l'évasion de la réponse immune de l'hôte. Par exemple, la glycoprotéine G (gG) des alphaherpèsvirus peut séquestrer des chimiokines activant le système immunitaire (Costes *et al.*, 2006), ou encore le gène K3 des gammaherpèsvirus qui limite la présentation antigénique par le complexe majeur d'histocompatibilité (MHC) de classe I des cellules infectées (Stevenson *et al.*, 2009). Le CyHV-3 possède également une série de gènes potentiellement impliqués dans l'évasion immune. Les ORFs 4 et 12, en tant qu'homologues de TNFR et l'ORF 134, homologue d'une IL-10 sont actuellement étudiés dans notre laboratoire. Le CyHV-3 représente dès lors un passionnant sujet

d'étude grâce à ces différentes séquences homologues dont il est possible d'étudier les fonctions prédites, mais également grâce à ces nombreux gènes inconnus qui sont autant de nouvelles protéines ou fonctions à découvrir.

Face aux pertes économiques engendrées par le virus en aquaculture, plusieurs laboratoires se sont consacrés au CyHV-3 sous l'angle de la recherche appliquée et notamment au développement d'un vaccin. Ainsi, une première approche décrite dans la littérature consiste à protéger les carpes 5 jours après l'infection en les mettant à 30°C, température à la fois non-permissive pour le virus et favorable au développement d'une réponse immune (Gilad *et al.*, 2003). Si cette méthode peut limiter les mortalités dans une situation d'épidémie, elle ne protège pas complètement les poissons et contribue à la dissémination de la maladie dans la population de carpes. Une deuxième approche met à profit l'accumulation aléatoire de mutations chez le CyHV-3 en culture cellulaire pour générer une souche atténuée (Ronen *et al.*, 2003). Cependant, la souche ainsi obtenue ne procure pas une innocuité satisfaisante et ne peut se prémunir d'une possible réversion vers la virulence. La technologie du BAC se révèle là encore très intéressante pour la recherche appliquée. En effet, l'accumulation d'une ou plusieurs délétions devrait aboutir au développement d'un vaccin atténué. Différents gènes ont été testés et les premiers résultats révèlent une atténuation complète associée à une protection immune. Ces travaux ont mené au dépôt d'un brevet et n'ont donc pu être présentés dans ce manuscrit. La première qualité attendue d'un vaccin réside dans l'innocuité, la sécurité pour l'hôte, le consommateur et l'environnement. L'utilisation de la technologie BAC permet la délétion complète et spécifique de gènes impliqués dans la virulence, dans l'évasion immune ou codants pour des glycoprotéines non-essentielles. Cette approche permet de générer un virus apathogène et sans risque de retour à la virulence. La deuxième qualité primordiale d'un vaccin doit être de fournir une protection efficace. En cela, le vaccin atténué est plus efficient que les formes sous-unitaires, vectorielles ou ADN, car il stimule l'ensemble des acteurs du système immunitaire inné et surtout adaptatif. Il est donc crucial de déterminer les gènes essentiels et les antigènes majeurs à ne pas toucher. Enfin, grâce à la mutagenèse, la création d'un vaccin DIVA (Differentiation of Infected and Vaccinated Animals) peut-être envisagée. Classiquement, ce type de vaccin présente une délétion pour une protéine non essentielle mineure (d'un point de vue antigénique). Ainsi, les poissons vaccinés développent une réponse sérologique différente de celle des poissons infectés par la souche sauvage qui auront des anticorps dirigés contre la protéine délétée. La différenciation entre individus vaccinés et infectés est très importante pour l'exportation de poissons vers une exploitation indemne. Les glycoprotéines de la famille de l'ORF25, étudiées au laboratoire (Michel, 2010), pourraient être de bons candidats pour le développement d'un vaccin DIVA à condition d'être présentes chez toutes les souches sauvages et d'être suffisamment immunogènes. Une dernière qualité essentielle au vaccin réside dans sa facilité d'administration. La vaccination de groupe par une souche atténuée présente, là encore, un énorme avantage par rapport aux vaccins inactivés ou sous-unitaires pour lesquelles chaque individu doit subir une injection du vaccin. Toutefois, ces derniers pourraient être préférés par certains éleveurs de carpes

koi réticents à introduire une souche de CyHV-3 même vaccinale au sein de leur élevage. La vaccination ADN devient alors une alternative de choix pour autant que la protéine produite par ce vaccin soit suffisamment immunogénique. Il a été démontré que chez les poissons, ce type de vaccination peut se révéler particulièrement efficace (Lorenzen & LaPatra, 2005). Il est fascinant de voir à travers ces quelques exemples que la frontière entre recherche fondamentale et appliquée n'est pas toujours évidente et que les avancées de la seconde prennent inexorablement racine dans les résultats de la première.

Les études 2 et 4 ont été consacrées à l'identification des portes d'entrée du CyHV-3 chez *Cyprinus carpio* au moyen d'une souche virale recombinante exprimant la LUC. L'étude 2 s'est intéressée à l'entrée du virus lors d'une infection par immersion dans de l'eau contaminée. Suite à l'observation de comportements cannibales et à la détection de CyHV-3 dans des proies potentielles de la carpe (Kielpinski *et al.*, 2010), l'étude 4 a envisagé l'infection de la carpe par ingestion de matériel contenant des particules infectieuses. Ces deux études sont complémentaires et couvrent les différentes conditions épidémiologiques possibles. En effet, si l'on envisage une carpe et par extension un poisson téléostéen dans son entièreté, on peut identifier les tissus en contact avec l'environnement extérieur tels que la peau, les yeux, mais aussi les branchies et le tube digestif. La peau des poissons téléostéens est constituée d'écailles élasmoïdes, structure dermique recouverte d'un épiderme contenant des cellules en division dans toute l'épaisseur (Sire & Akimenko, 2004). L'ensemble de ces tissus est couvert d'une couche de mucus. L'apparition d'un signal lumineux chez un poisson infecté avec un virus LUC nécessite le contact physique, l'attachement et la pénétration du virus dans une cellule sensible. Au cours des différentes expériences d'infection immersive menées, nous avons remarqué que le signal apparaissait surtout sur les bords des nageoires et de l'opercule, où le revêtement de mucus est le plus fin. Cette tendance a été confirmée dans la troisième étude, où le retrait de mucus a facilité l'entrée virale par la peau. La quatrième étude a révélé que l'ingestion d'aliments contaminés induit la pénétration du virus par infection des cellules épithéliales communes de la muqueuse pharyngienne périodontale. L'absence de signal LUC dans le reste du tube digestif aux premiers jours de l'infection contraste avec l'apparition plus tardive d'un signal et l'excrétion virale dans les fèces. Cette apparente contradiction peut s'expliquer par une barrière de mucus efficace dans la lumière digestive, des cellules polarisées et/ou un phénomène de transcytose virale (Faulkner *et al.*, 2000). Il est probable que l'effet remarqué dans la troisième étude soit généralisable à l'ensemble du corps des poissons téléostéens. Ainsi, le retrait de mucus par frottement, parasitisme, blessure ou mastication, doit certainement faciliter l'infection des cellules sensibles en les rendant physiquement plus accessibles par le virus. Afin de tester cette hypothèse, il serait intéressant d'appliquer un traitement de retrait systématique du mucus sur tous les tissus de surface exposés : les yeux, les branchies, le pseudogaster ou encore l'intestin.

D'un point de vue évolutif, on peut se demander l'intérêt d'une peau, exposant des cellules sensibles au virus, protégée par un simple revêtement de mucus inconstant. Chez les mammifères les infections virales se font par les muqueuses, la peau étant kératinisée. Sur le plan évolutif, différentes voies ont été explorées pour protéger l'organisme des agressions physiques et biologiques. On distingue notamment les plaques, différents types d'écailles (placoïdes, cosmoïdes, élasmoïdes), les plumes ou encore la kératinisation et les poils. Même si quelques espèces de poisson, pour certains organes, ont révélé la présence de kératine (Mittal & Whitear, 1979), le phénomène de kératinisation systématique de la peau n'est apparu qu'au Permien chez les cotylosauriens (Alibardi, 2001), amniotes cherchant à s'affranchir du milieu aquatique.

La peau des poissons offre tout de même une protection mécanique, chimique et immune contre les blessures et les pathogènes (Fontenot & Neiffer, 2004). Cependant, c'est le mucus qui joue le rôle de barrière défensive et non la kératine. Sa viscosité laisse deviner ce rôle mécanique. Grâce aux mucines, glycoprotéines qui s'organisent en réseau piégeant l'eau (Shephard, 1994), le mucus cutané des poissons englue les microorganismes pathogènes. Un phénomène de flux, généré par la production continue de mucus et la nage, permet l'élimination de ces agents par dispersion du mucus dans l'eau au niveau de la nageoire caudale. Ce mécanisme rappelle le tapis muco-ciliaire des voies respiratoires des pulmonés supérieures. Dans la troisième étude, nous avons démontré que le mucus inhibait l'attachement viral aux cellules épithéliales de la nageoire caudale *ex vivo*. Il serait intéressant de comparer l'efficacité neutralisante du mucus de poisson à celle d'un mucus de remplacement.

Parallèlement à cette protection mécanique, le mucus des poissons contient un ensemble de molécules et de cellules capable de neutraliser les infections microbiennes et les infestations parasitaires. (Ellis, 2001; Fontenot & Neiffer, 2004; Palaksha *et al.*, 2008; Shephard, 1994; Subramanian *et al.*, 2008). On relève notamment la présence d'interféron (INF) de type-I, de cellules tueuses naturelles (NK), de facteurs du complément, mais également d'Immunoglobulines (Ig), de protéases, de peptides, de lysozyme, de phagocytes, d'agglutinines ou encore de lectines. Au cours de la troisième étude, il a été démontré que le retrait de mucus épidermique *in vivo* facilitait l'entrée virale au niveau de la peau de la carpe et que ce rôle antiviral a pu en partie être attribué *in vitro* à des éléments solubles contenus dans l'extrait de mucus clarifié (Raj *et al.*, 2011). Cette étude, ainsi qu'une étude précédente chez la truite (*Oncorhynchus mykiss*), démontre le rôle protecteur du mucus chez le poisson téléostéen, tant comme barrière physique qu'immunologique (Cain *et al.*, 1996). Cependant, la description des composants et des mécanismes qui sous-tendent cette activité antivirale reste encore à élucider. C'est pourquoi, le laboratoire d'accueil envisage une approche protéomique pour déterminer les molécules présentes spécifiquement dans le mucus épidermique de la carpe et leurs activités antivirales. Dans un premier temps, un traitement protéase devrait confirmer ou infirmer la nature protéique des molécules impliquées. Dans un deuxième temps, Le fractionnement de l'extrait de mucus par chromatographie, l'analyse du pouvoir neutralisant des différentes fractions et l'identification des protéines présentes par une analyse spectrométrique de masse devraient permettre

d'identifier la ou les protéines responsables de la neutralisation observée dans l'extrait de mucus clarifié.

Au travers des trois études exploitant la souche CyHV-3 LUC, nous avons déterminé les voies d'entrée possibles du virus. Un autre aspect de la pathogénie mis en lumière par l'étude 4 est la séquence chronologique des organes infectés par le virus une fois entré dans le poisson. Il est intéressant de noter que la propagation du virus dans le poisson dépend de la voie d'entrée. Ainsi la carpe infectée par la peau présente une réplication virale cutanée les premiers jours qui se généralise après 6 à 8 jours, tandis que lors d'une infection orale, après une réplication dans la muqueuse pharyngienne, le virus va rapidement se répliquer dans les branchies et la peau, probablement par continuité de tissu, avant de se généraliser au reste du corps également entre 6 et 8 jours (Fournier *et al.*, 2012). Les organes identifiés à l'IVIS avaient déjà été décrits précédemment comme site de multiplication virale (Gilad *et al.*, 2004; Pikarsky *et al.*, 2004), mais la chronologie est différente puisque certains organes ont souvent été décrits comme positifs tels que le rein ou le foie bien avant le sixième jour. Ceci peut s'expliquer par un signal bioluminescent labile dans certains organes (Costes *et al.*, 2009), une plus grande sensibilité de détection des PCR ou encore la présence du virus dans le sang qui reste sous la limite de détection de l'IVIS en début de virémie, mais se manifeste dans les organes très vascularisés lors de la généralisation de l'infection : cœur, foie, reins. Enfin, les intestins deviennent positifs dans une phase tardive. Cette donnée, avec la présence de virus dans les fèces (Dishon *et al.*, 2005), supporte l'idée que les intestins sont une voie d'excrétion du virus. Une étude réalisée dans le laboratoire d'accueil montre également la présence de bioluminescence dans les bandelettes olfactives et le cerveau de la carpe, plaidant pour une latence dans des ganglions nerveux sensoriels olfactifs, mais là encore, le signal est transitoire et rend difficile l'étude de la latence via la bioluminescence *in vivo*. Afin de pallier à cette difficulté, la création d'une souche recombinante virale exprimant la β-galactosidase est actuellement en cours et devrait permettre d'accroitre la sensibilité de détection du virus *ex vivo*. Toutefois, une étude récente (Eide *et al.*, 2011) montre que des poissons cliniquement sains ayant survécu à un lointain épisode de KHV présentent de l'ADN viral uniquement dans les leucocytes circulants avant stress, mais que l'ADN viral est à nouveau détectable dans de nombreux organes, un mois après l'induction d'un stress thermique. Cette étude supporte l'existence du phénomène de latence au niveau des leucocytes. L'effet de l'âge sur la sensibilité des carpes au CyHV-3 est un autre facteur important pour la dissémination du virus. Il semble que les jeunes poissons de quelques grammes sont plus sensibles que les sujets plus grands (Perelberg, 2003), et que les larves ne soient pas sensibles au virus (Ito *et al.*, 2007). Cependant, les premiers résultats d'une étude menée au laboratoire d'accueil montrent, à l'aide de la souche LUC, que les larves sont sensibles dès l'éclosion. L'analyse des mécanismes de latence et de sensibilité est importante pour comprendre et contrôler la propagation du virus et ouvre la réflexion sur une considération plus générale, celle des conditions de transmission du CyHV-3.

En effet, la transmission du virus chez *Cyprinus carpio* peut s'envisager de différentes manières, soit par contact direct des carpes, soit par le biais d'un vecteur. Dans la transmission directe, la voie la plus évidente semble le contact peau à peau entre poissons lorsque le virus s'y multiplie. Un deuxième scénario fait référence au cannibalisme décrit chez la carpe (van Damme *et al.,* 1989) dans des populations de taille hétérogène et de forte densité, mais aussi au cannibalisme « opportuniste » observé au cours des expériences menées au laboratoire. En effet, les premiers poissons malades, présentant des lésions herpétiques de la peau, se font attaquer par les poissons encore sains.

Une étude récente a démontré la présence d'ADN viral dans des organismes invertébrés (Kielpinski *et al.,* 2010). Par ailleurs, les matières fécales de poissons malades contiennent des particules virales infectieuses. Il est donc envisageable que des poissons s'infectent par voie orale tel que décrit dans l'étude 4. L'eau elle-même est un bon vecteur, puisque l'on peut infecter des poissons par immersion dans de l'eau contenant du virus (Costes 2009). De plus, la présence d'ADN viral a été notée dans le plancton (Minamoto *et al.,* 2011) et les sédiments d'un lac infecté (Honjo *et al.,* 2011). Le caractère infectieux de l'environnement est donc corrélé à la concentration en particules virales encore infectieuses. Certains auteurs ce sont intéressé au temps durant lequel, une fois libéré dans l'eau, le virus reste infectieux. Leurs conclusions sont assez variables, allant de quelques heures à quelques jours selon l'environnement (Perelberg, 2003; Shimizu *et al.,* 2006), peut-être plus dans les sédiments ou les invertébrés filtrants. Afin de tester la transmission indirecte du virus par l'eau, nous avons entrepris une expérience avec des aquariums supérieurs, où cohabitent des poissons naïfs et infectés, déversant leur eau par gravitation dans des aquariums inférieurs contenant des poissons naïfs. L'eau remonte des aquariums inférieurs vers les aquariums supérieurs grâce à des pompes filtrantes contenant une flore bactérienne. Par contre, trois conditions différentes sont appliquées au circuit descendant: (i) l'eau circulant de l'aquarium en amont vers celui en aval passe par une cuve de décantation simple laissant passer les macro-particules, (ii) un filtre est ajouté au système, ne laissant passer que les micro-particules, (iii) un système à Ultra-Violet est ajouté. Les premiers résultats suggèrent une gradation dans la vitesse de propagation du virus selon les conditions expérimentales. Ainsi, la transmission du virus dans la cohabitation directe est plus rapide que celle observée dans le partage de l'eau et des macro-particules. Celle-ci est elle-même plus rapide que dans le partage de l'eau et des microparticules, tandis que le virus ne résiste pas à l'ajout d'un traitement UV. Enfin, les autres espèces de poissons qui cohabitent avec la carpe en milieu naturel ou artificiel sont également des vecteurs potentiels. Par exemple, le poisson rouge (*Carassius auratus*) qui partage fréquemment les bassins des carpes koi a été décrit comme porteur sain (El-Matbouli *et al.,* 2007; Haenen & Hedrick, 2006), voire comme partiellement sensible (Bergmann *et al.,* 2010; Sadler *et al.,* 2008) et le carassin (*Carassius carassius*) semble également être porteur. Afin de déterminer le spectre d'hôte potentiel du CyHV-3, une étude préliminaire, menée dans le cadre de ma thèse, a été réalisée *in vitro* sur diverses cultures cellulaires de poissons, d'insectes et de mammifères à l'aide des virus EGFP (Costes *et al.,* 2008) et LUC (Costes *et al.,* 2009). Cependant, seules les cellules de carpes se sont

révélées permissives, même si certaines cellules de mammifères ont présenté une sensibilité réduite. Ces résultats n'ont pas permis d'identifier les autres espèces de poissons sensibles, mais permettent d'envisager, dans une toute autre perspective, l'utilisation du CyHV-3 comme vecteur vaccinal chez les espèces de mammifères concernées. Enfin, une étude sera prochainement réalisée, au moyen de la souche LUC, sur différentes espèces de poissons cohabitant avec la carpe dans son environnement naturel, afin de déterminer les réservoirs potentiels, peut-être même l'espèce hôte originelle du virus. Cette étude pourrait avoir deux répercussions importantes. D'une part, la détermination des espèces sensibles avec la possibilité de développer des vaccins vectoriels pour celles-ci. D'autre part, l'identification de l'espèce hôte « naturelle », « originale » du virus, puisqu'un certain nombre d'éléments laissent à penser qu'il y a eu un récent saut d'espèce.

En effet, durant des millions d'années, certains virus ont co-évolué avec leur hôte. Lors de ce processus, le système immunitaire de l'hôte infecté a sélectionné parmi la population virale les particules les mieux adaptées. Charles Darwin a redoutablement bien résumé ce mécanisme en une phrase : « *les espèces qui survivent ne sont pas les espèces les plus fortes, ni les plus intelligentes, mais celles qui s'adaptent le mieux aux changements* ». Ce processus a engendré des situations surprenantes ou des virus confèrent un avantage sélectif à l'hôte infecté. Ainsi, l'herpèsvirus alcélaphin 1 (AlHV-1) infecte asymptomatiquement son hôte naturel, le gnou (*Connochaetes taurinus*) (Plowright, 1990) et lui facilite l'accès aux plaines herbeuses en induisant une maladie mortelle chez les autres bovins sensibles. Cependant chez la carpe, le CyHV-3 produit une maladie mortelle qui ne reflète aucunement l'adaptation d'un virus à son hôte. Cette observation laisse penser que le génome du CyHV-3 a récemment subit des mutations donnant la capacité au virus de sauter de son espèce hôte naturelle à la carpe. De plus, aucun épisode de mortalité de masse associé à des signes cliniques typiques du CyHV-3 n'a été décrit avant la fin des années 1990, alors que l'élevage de la carpe remonte à l'époque de l'Empire Romain (Balon, 1995). On est donc confronté à une apparition brusque de la maladie. Enfin, des études récentes montrent une forte similarité des séquences génomiques de différentes souches du virus, notamment dans des régions où la dérive génétique devrait être forte comme les pseudogènes (Michel, 2010) ou encore les VNTR (Variable number of tandem repeat) (Avarre *et al.*, 2011). Tous ces arguments suggèrent que les souches actuelles de CyHV-3 dérivent toutes d'une même souche. Deux hypothèses sont possibles quant à l'origine de cette souche originelle. Tout d'abord, il se pourrait que le CyHV-3 soit une espèce virale asymptomatique présente depuis longtemps chez la carpe. Les souches de CyHV-3 actuelles seraient issues d'une souche mutante apparue au sein de la population. Cette première hypothèse est peu probable, puisque les carpes auraient dû présenter une immunité protectrice vis-à-vis de ce pathogène endémique. La seconde hypothèse beaucoup plus probable postule que le CyHV-3 serait le produit d'un « saut d'espèce » d'un virus d'une espèce hôte non identifiée chez la carpe. Cette hypothèse est corroborée par la description d'infections subliniques au CyHV-3 chez plusieurs espèces proches de *Cyprinus carpio*, comme par exemple le poisson rouge (*Carassius auratus*) (Bergmann *et al.*, 2010;

Sadler *et al.*, 2008). Des recherches complémentaires virologiques et épidémiologiques sont nécessaires pour déterminer l'origine du CyHV-3.

A cours de cette thèse, nous avons mis la technologie BAC à profit pour étudier le CyHV-3. Nous avons ainsi identifié les portes d'entrée du virus chez la carpe ainsi que les moyens de défense de cette dernière. Mais cette thèse a surtout permis l'acquisition d'outils essentiels à l'exploration du virus et au développement d'un vaccin. Le CyHV-3 s'est révélé être un sujet d'étude passionnant, abordant de nombreux domaines différents : de la recherche fondamentale à la recherche appliquée, de la biologie moléculaire à l'expérimentation animale, de l'étude de gènes à l'épidémiologie. Cette expérience m'a permis d'acquérir un bagage scientifique précieux grâce aux scientifiques qui m'ont guidé, m'ont appris à avoir un regard critique sur mes recherches, à formuler des hypothèses de travail originales et à mettre en valeur les résultats obtenus. Cette thèse fut également la source d'un essor intellectuel et philosophique. En effet, au cours de ces quatre années, j'ai rencontré des personnes de confiance qui ont su m'insuffler leur passion pour la science et la découverte. Un jour Anton Tchekhov a écrit « Quand nous avons soif, il nous semble que nous pourrions boire tout un océan : c'est la foi. Et quand nous nous mettons à boire, nous buvons un verre ou deux : c'est la science. » . Evidemment, la métaphore de l'eau se prête à mon sujet de thèse. Il est vrai que dans la recherche, il faut avoir la foi et qu'en débutant une thèse, on espère s'attaquer à l'océan et révolutionner le monde. Cependant, il arrive aussi au cours de cette aventure que l'on boive la tasse. C'est dans ces moments que l'on apprécie le soutien de toute une équipe de collègues et amis. Au bilan, on réalise que la thèse se termine trop vite, qu'il y a encore beaucoup de questions en suspens. Cette thèse n'est évidemment qu'un verre d'eau ou deux dans l'océan de la connaissance, mais je suis fier de cette contribution. J'espère encore longtemps goûter à la science car ma soif n'est nullement étanchée ! Comme l'a si joliment dit Edgar Allan Poe « Ce n'est pas dans la science qu'est le bonheur, mais dans l'acquisition de la science. »

Summary - Résumé

Summary

The common carp is one of the most important freshwater species in aquaculture and its colourful subspecies koi is grown for personal pleasure and exhibitions. Both subspecies are economically important. In the 1990s, a highly contagious and lethal pathogen called koi herpesvirus (KHV) or cyprinid herpesvirus 3 (CyHV-3) began to cause severe financial losses in these two carp industries worldwide. Because of its economic importance and its numerous original biological properties, CyHV-3 became rapidly an attractive subject for applied and fundamental research. The goal of this thesis was to identify the portals of entry of CyHV-3 in carp. This information is essential to understand the pathogenesis and the epidemiology of the infection, but also to develop efficacious vaccines. Prolonged CyHV-3 cultivation *in vitro* leads to the spontaneous attenuation of the virus. To circumvent this problem, the entire viral genome was cloned as a bacterial artificial chromosome (BAC). Then to test the usefulness of the BAC clone, several recombinants strains were generated as described in the first chapter. In the second chapter, we took profit of the CyHV-3 BAC clone to produce a recombinant strain encoding a firefly luciferase (LUC) expression cassette. Infection of carp by immersion in water containing the CyHV-3 LUC strain demonstrated, using bioluminescent *in vivo* imaging system (IVIS), that the skin, and not the gills, is the major portal of entry for CyHV-3. Fish skin provides mechanical, chemical and immune protection against injury and pathogenic microorganisms. Its mucus layer confers an innate immune protection against pathogen entry. However, there is little *in vivo* evidence on the role of skin mucus as a first line of innate immune protection against bacterial and viral infections. In the third chapter, we used the CyHV-3 LUC strain and IVIS to investigate the roles of epidermal mucus as an innate immune barrier against CyHV-3 entry. Our results demonstrate that the mucus of the skin inhibits CyHV-3 binding to epidermal cells and contains soluble molecules able to neutralize CyHV-3 infectivity.

The skin is the major portal of entry after inoculation by immersion in water containing CyHV-3. While this model of infection mimics some natural conditions in which infection takes place, other epidemiological conditions could favor entry of virus through the digestive tract. Consequently, in the fourth and last chapter, we investigated the role of the carp digestive tract as a viral portal of entry using bioluminescence imaging. We found that feeding carp with infectious materials induces CyHV-3 entry through infection of the pharyngeal periodontal mucosa. In conclusion, this study demonstrated that according to epidemiological conditions, CyHV-3 can enter carp either through infection of the skin (immersion in infectious water) or through infection of the pharyngeal periodontal mucosa (feeding on infectious materials). The existence of these two portal of entry adapted to different epidemiological conditions most probably contributes to the high contagious nature of the virus.

Résumé

Les carpes commune et koi sont importantes sur le plan économique. La première est une des espèces d'eau douce les plus importantes en aquaculture tandis que la seconde est très prisée des collectionneurs. Dans les années 1990, un pathogène mortel et très contagieux, appelé herpèsvirus de la carpe koi (KHV) ou herpèsvirus cyprin 3 (CyHV-3), a commencé à causer de lourdes pertes financières dans ces deux industries. En raison de ces pertes économiques et des propriétés biologiques originales du CyHV-3, il est rapidement devenu un sujet intéressant pour la recherche appliquée et fondamentale. Le but de cette thèse était d'identifier les portes d'entrée du CyHV-3 chez la carpe. Cette étape est essentielle pour comprendre la pathogénie et l'épidémiologie de l'infection, mais également pour le développement de vaccins efficaces. La culture prolongée du CyHV-3 *in vitro* mène à une atténuation spontanée. Pour contourner cette difficulté, l'entièreté de son génome a été clonée en tant que chromosome artificiel bactérien (BAC) comme décrit dans le premier chapitre de cette thèse. Ensuite, plusieurs souches virales recombinantes ont été produites, illustrant l'intérêt de cet outil moléculaire. Dans le deuxième chapitre, nous avons tiré profit du CyHV-3 BAC pour produire une souche recombinante exprimant le gène de la luciférase (LUC) de la luciole. L'infection de carpes, par immersion dans de l'eau contenant du CyHV-3 LUC, a permis de démontrer, grâce à la bioluminescence *in vivo* (IVIS), que la peau est la porte d'entrée principale du CyHV-3 et non les branchies. La peau des poissons fournit une protection mécanique, chimique et immunologique contre les blessures et les micro-organismes pathogènes. Le mucus cutané confère également une protection immune innée contre les pathogènes. Peu d'études existent sur le rôle *in vivo* du mucus comme protection immune innée contre les bactéries ou les virus. Le troisième chapitre de cette thèse décrit l'utilisation du CyHV-3 LUC et de l'IVIS pour investiguer le rôle du mucus cutané en tant que barrière immune innée contre l'entrée du virus. Les résultats démontrent que le mucus inhibe l'attachement du CyHV-3 aux cellules épidermiques et qu'il contient des molécules solubles capables de neutraliser l'infection virale. Les études précédentes démontrent que la peau est une porte d'entrée majeure lors d'une infection par immersion dans de l'eau contenant du virus. Tandis que ce modèle mime certaines conditions naturelles d'infection, d'autres conditions épidémiologiques privilégient une entrée du virus par le tractus digestif. Aussi, dans le quatrième et dernier chapitre, nous avons étudié par IVIS le tractus digestif de la carpe en tant que porte d'entrée virale. Cette étude démontre que nourrir des carpes avec du matériel infecté induit une entrée du CyHV-3 au niveau de la muqueuse périodontale pharyngienne.

En conclusion, cette thèse démontre qu'en fonction des conditions épidémiologiques, le CyHV-3 peut infecter la carpe soit par la peau, soit par le pharynx. L'existence de ces deux portes d'entrée adaptées aux différentes conditions épidémiologiques contribue très probablement à la nature hautement contagieuse du virus.

References

A

Ackermann, M. (2004). Herpesviruses: a brief overview. *Methods Mol Biol* **256**, 199-219.

Adkison, M. A., Gilad, O., and Hedrick, R. P. (2005). An enzyme linked immunosorbent assay (ELISA) for detection of antibodies to the koi herpesvirus (KHV) in the serum of koi Cyprinus carpio. *Fish Pathol* **40**, 53-62.

Alibardi, L. (2001). Keratinization in the epidermis of amphibians and the lungfish: comparison with amniote keratinization. *Tissue Cell* **33**, 439-449.

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.

Ariav, R., Tinman, S., Paperna, I., Bejerano, I. (1999). First report of newly emerging viral disease of Cyprinus carpio species in Israel. In *the EAFP 9th International Conference*. Rhodes, Greece.

Atema, J. (1980). chemical senses, chemical signals and feeding behavior in fishes. In *Fish Behavior and its use in the capture and culture of fishes*, p. 512. Edited by J. E. Bardach, J. J. Magnuson, R. C. May & J. M. Reinhart. Manila: ICLARM.

Avarre, J. C., Madeira, J. P., Santika, A., Zainun, Z., Baud, M., Cabon, J., Caruso, D., Castric, J., Bigarre, L., Engelsma, M. & Maskur, M. (2011). Investigation of Cyprinid herpesvirus-3 genetic diversity by a multi-locus variable number of tandem repeats analysis. *J Virol Methods* **173**, 320-327.

B

Baens, M., Noels, H., Broeckx, V., Hagens, S., Fevery, S., Billiau, A. D., Vankelecom, H. & Marynen, P. (2006). The dark side of EGFP: defective polyubiquitination. *PLoS One* **1**, e54.

Ballestas, M. E. & Kaye, K. M. (2001). Kaposi's sarcoma-associated herpesvirus latency-associated nuclear antigen 1 mediates episome persistence through cis-acting terminal repeat (TR) sequence and specifically binds TR DNA. *J Virol* **75**, 3250-3258.

Balon, E. K. (1995). Origin and domestication of the wild carp, *Cyprinus carpio*: from Roman gourmets to the swimming flowers. *Aquaculture* **129**, 3-48.

Bercovier, H., Fishman, Y., Nahary, R., Sinai, S., Zlotkin, A., Eyngor, M., Gilad, O., Eldar, A. & Hedrick, R. P. (2005). Cloning of the koi herpesvirus (KHV) gene encoding thymidine kinase and its use for a highly sensitive PCR based diagnosis. *BMC Microbiol* **5**, 13.

Bergmann, S. M., Riechardt, M., Fichtner, D., Lee, P. & Kempter, J. (2010a). Investigation on the diagnostic sensitivity of molecular tools used for detection of koi herpesvirus. *Journal of Virological Methods* **163**, 229-233.

Bergmann, S. M., Sadowski, J., Kiepiski, M., Bartomiejczyk, M., Fichtner, D., Riebe, R., Lenk, M. & Kempter, J. (2010b). Susceptibility of koi x crucian carp and koi x goldfish hybrids to koi herpesvirus (KHV) and the development of KHV disease (KHVD). *Journal of Fish Diseases* **33**, 267-272.

Bergmann, S. M., Stumpf, P., Schütze, H., Fichtner, D., Sadowski, J. & Kempter, J. (2007). Similarities and heterogenicity of koi herpes virus (KHV) genome detected in ornamental fish without clinical signs. *Aquaculture* **272**, S245-S245.

Black, M. E. & Hruby, D. E. (1990). Quaternary structure of vaccinia virus thymidine kinase. *Biochem Biophys Res Commun* **169**, 1080-1086.

Bly, J. E. & Clem, W. (1992). Temperature and teleost immune functions. *Fish & Shellfish Immunology* **2**, 159-171.

Body, A., Lieffrig, F., Charlier, G. & Collard, A. (2000). Isolation of Virus-like particles from Koi (Cyprinus carpio) suffering gill necrosis. *Bull Eur Assoc Fish Pathol* **20**, 87-88.

Bondad-Reantaso, M. G., Sunarto, A. & Subasinghe, R. P. (2007). Managing the koi herpesvirus disease outbreak in Indonesia and the lessons learned. *Dev Biol (Basel)* **129**, 21-28.

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.

Bretzinger, A., Fischer-Scherl, T., Oumouma, R., Hoffmann, R., Truyen, U. (1999). Mass mortalities in koi, Cyprinus carpio, associated with gill and skin disease. *Bull Eur Assoc Fish Pathol* **19**, 182-185.

Browne, H., Bell, S., Minson, T. & Wilson, D. W. (1996). An endoplasmic reticulum-retained herpes simplex virus glycoprotein H is absent from secreted virions: evidence for reenvelopment during egress. *J Virol* **70**, 4311-4316.

Brudeseth, B. E., Skall, H. F. & Evensen, O. (2008). Differences in virulence of marine and freshwater isolates of viral hemorrhagic septicemia virus in vivo correlate with in vitro ability to infect gill epithelial cells and macrophages of rainbow trout (Oncorhynchus mykiss). *J Virol* **82**, 10359-10365.

Burnside, J., Bernberg, E., Anderson, A., Lu, C., Meyers, B. C., Green, P. J., Jain, N., Isaacs, G. & Morgan, R. W. (2006). Marek's disease virus encodes MicroRNAs that map to meq and the latency-associated transcript. *J Virol* **80**, 8778-8786.

C

Cai, X., Lu, S., Zhang, Z., Gonzalez, C. M., Damania, B. & Cullen, B. R. (2005). Kaposi's sarcoma-associated herpesvirus expresses an array of viral microRNAs in latently infected cells. *Proc Natl Acad Sci U S A* **102**, 5570-5575.

Cain, K. D., Lapatra, S. E., Baldwin, T. J., Shewmaker, B., Jones, J. & Ristow, S. S. (1996). *Characterization of mucosal immunity in rainbow trout Oncorhynchus mykiss challenged with infectious hematopoietic necrosis virus : identification of antiviral activity*. Oldendorf, ALLEMAGNE: Inter-Research.

Cardin, R. D., Schaefer, G. C., Allen, J. R., Davis-Poynter, N. J. & Farrell, H. E. (2009). The M33 chemokine receptor homolog of murine cytomegalovirus exhibits a differential tissue-specific role during in vivo replication and latency. *J Virol* **83**, 7590-7601.

Chen, S. H., Cook, W. J., Grove, K. L. & Coen, D. M. (1998). Human thymidine kinase can functionally replace herpes simplex virus type 1 thymidine kinase for viral replication in mouse sensory ganglia and reactivation from latency upon explant. *J Virol* **72**, 6710-6715.

Cheng, L., Chen, C. Y., Tsai, M. A., Wang, P. C., Hsu, J. P., Chern, R. S. & Chen, S. C. (2011). Koi herpesvirus epizootic in cultured carp and koi, Cyprinus carpio L., in Taiwan. *J Fish Dis* **34**, 547-554.

Choi, D. L., Sohn, S. G., Bang, J. D., Do, J. W. & Park, M. S. (2004). Ultrastructural identification of a herpes-like virus infection in common carp Cyprinus carpio in Korea. *Dis Aquat Organ* **61**, 165-168.

Coen, D. M., Kosz-Vnenchak, M., Jacobson, J. G., Leib, D. A., Bogard, C. L., Schaffer, P. A., Tyler, K. L. & Knipe, D. M. (1989). Thymidine kinase-negative herpes simplex virus mutants establish latency in mouse trigeminal ganglia but do not reactivate. *Proc Natl Acad Sci U S A* **86**, 4736-4740.

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., Stalin Raj, V., 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.

Costes, B., Thirion, M., Dewals, B., Mast, J., Ackermann, M., Markine-Goriaynoff, N., Gillet, L. & Vanderplasschen, A. (2006). Felid herpesvirus 1 glycoprotein G is a structural protein that mediates the binding of chemokines on the viral envelope. *Microbes Infect* **8**, 2657-2667.

Coupar, B. E. H., Goldie, S. G., Hyatt, A. D. & Pallister, J. A. (2005). Identification of a Bohle iridovirus thymidine kinase gene and demonstration of activity using vaccinia virus. *Archives of Virology* **150**, 1797-1812.

D

Darlington, R. W. & Moss, L. H., 3rd (1968). Herpesvirus envelopment. *J Virol* **2**, 48-55.

Davidovich, M., Dishon, A., Ilouze, M. & Kotler, M. (2007). Susceptibility of cyprinid cultured cells to cyprinid herpesvirus 3. *Arch Virol* **152**, 1541-1546.

Davison, A. J. (1984). Structure of the genome termini of varicella-zoster virus. *J Gen Virol* **65 (Pt 11)**, 1969-1977.

Davison, A. J. (1992). Channel catfish virus: a new type of herpesvirus. *Virology* **186**, 9-14.

Davison, A. J. (2002). Evolution of the herpesviruses. *Vet Microbiol* **86**, 69-88.

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.

Davison, A. J. & Stow, N. D. (2005). New genes from old: redeployment of dUTPase by herpesviruses. *J Virol* **79**, 12880-12892.

Davison, A. J., Trus, B. L., Cheng, N., Steven, A. C., Watson, M. S., Cunningham, C., Le Deuff, R. M. & Renault, T. (2005). A novel class of herpesvirus with bivalve hosts. *J Gen Virol* **86**, 41-53.

Dewals, B., Boudry, C., Gillet, L., Markine-Goriaynoff, N., de Leval, L., Haig, D. M. & Vanderplasschen, A. (2006). Cloning of the genome of Alcelaphine herpesvirus 1 as an infectious and pathogenic bacterial artificial chromosome. *J Gen Virol* **87**, 509-517.

Dimmock, N. J., Easton, A. J. & Leppard, K. N. (2007). Towards a definition of a virus. In *Introduction to modern virology*, 6 edn, pp. 3 - 17. Edited by N. J. Dimmock, A. J. Easton & K. N. Leppard. Oxford: Wiley-Blackwel.

Dishon, A., Davidovich, M., Ilouze, M. & Kotler, M. (2007). Persistence of cyprinid herpesvirus 3 in infected cultured carp cells. *J Virol* **81**, 4828-4836.

Dishon, A., Perelberg, A., Bishara-Shieban, J., Ilouze, M., Davidovich, M., Werker, S. & Kotler, M. (2005). Detection of carp interstitial nephritis and gill necrosis virus in fish droppings. *Appl Environ Microbiol* **71**, 7285-7291.

Dong, C., Weng, S., Li, W., Li, X., Yi, Y., Liang, Q. & He, J. (2011). Characterization of a new cell line from caudal fin of koi, Cyprinus carpio koi, and first isolation of cyprinid herpesvirus 3 in China. *Virus Research* **161**, 140-149.

Doszpoly, A., Somogyi, V., LaPatra, S. E. & Benko, M. (2011). Partial genome characterization of acipenserid herpesvirus 2: taxonomical proposal for the demarcation of three subfamilies in Alloherpesviridae. *Arch Virol* **156**, 2291-2296.

E

Eide, K. E., Miller-Morgan, T., Heidel, J. R., Kent, M. L., Bildfell, R. J., Lapatra, S., Watson, G. & Jin, L. (2011). Investigation of koi herpesvirus latency in koi. *J Virol* **85**, 4954-4962.

El-Matbouli, M., Rucker, U. & Soliman, H. (2007a). Detection of Cyprinid herpesvirus-3 (CyHV-3) DNA in infected fish tissues by nested polymerase chain reaction, pp. 23-28.

El-Matbouli, M., Saleh, M. & Soliman, H. (2007b). Detection of cyprinid herpesvirus type 3 in goldfish cohabiting with CyHV-3-infected koi carp (Cyprinus carpio koi). *Vet Rec* **161**, 792-793.

Ellis, A. E. (2001). Innate host defense mechanisms of fish against viruses and bacteria. *Dev Comp Immunol* **25**, 827-839.

F

Faulkner, G. C., Krajewski, A. S. & Crawford, D. H. (2000). The ins and outs of EBV infection. *Trends Microbiol* **8**, 185-189.

Flint, S., Racaniello, V. R., Enquist, L. W., Skalka, A. M. & Krug, R. M. (2000). *Virology : molecular biology, pathogenesis, and control*. Washington, D. C: ASM press.

Fontenot, D. K. & Neiffer, D. L. (2004). Wound management in teleost fish: biology of the healing process, evaluation, and treatment. *The veterinary clinics of North America* **7**, 57-86.

Fournier, G., Boutier, M., Stalin Raj, V., Mast, J., Parmentier, E., Vandewalle, P., Peeters, D., Lieffrig, F., Farnir, F., Gillet, L. & Vanderplasschen, A. (2012). Feeding Cyprinus carpio with infectious materials mediates cyprinid herpesvirus 3 entry through infection of pharyngeal periodontal mucosa. *Vet res* **43**.

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.

G

Garber, D. A., Beverley, S. M. & Coen, D. M. (1993). Demonstration of circularization of herpes simplex virus DNA following infection using pulsed field gel electrophoresis. *Virology* **197**, 459-462.

Gilad, O., Yun, S., Adkison, M. A., Way, K., Willits, N. H., Bercovier, H. & Hedrick, R. P. (2003). Molecular comparison of isolates of an emerging fish pathogen, koi herpesvirus, and the effect of water temperature on mortality of experimentally infected koi. *J Gen Virol* **84**, 2661-2667.

Gilad, O., Yun, S., Andree, K. B., Adkison, M. A., Zlotkin, A., Bercovier, H., Eldar, A. & Hedrick, R. P. (2002). Initial characteristics of koi herpesvirus and development of a polymerase chain reaction assay to detect the virus in koi, Cyprinus carpio koi. *Dis Aquat Organ* **48**, 101-108.

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.

Gillet, L., Daix, V., Donofrio, G., Wagner, M., Koszinowski, U. H., China, B., Ackermann, M., Markine-Goriaynoff, N. & Vanderplasschen, A. (2005). Development of bovine herpesvirus 4 as an expression vector using bacterial artificial chromosome cloning. *J Gen Virol* **86**, 907-917.

Gomez, D. K., Joh, S. J., Jang, H., Shin, S. P., Choresca Jr, C. H., Han, J. E., Kim, J. H., Jun, J. W. & Park, S. C. (2011). Detection of koi herpesvirus (KHV) from koi (Cyprinus carpio koi) broodstock in South Korea. *Aquaculture* **311**, 42-47.

Granzow, H., Klupp, B. G., Fuchs, W., Veits, J., Osterrieder, N. & Mettenleiter, T. C. (2001). Egress of alphaherpesviruses: comparative ultrastructural study. *J Virol* **75**, 3675-3684.

Gray, W. L., Mullis, L., LaPatra, S. E., Groff, J. M., and Goodwin, A. (2002). Detection of koi herpesvirus DNA in tissues of infected fish. *J Fish Dis* **25**, 171-178.

Gunimaladevi, I., Kono, T., Venugopal, M. N. & Sakai, M. (2004). Detection of koi herpesvirus in common carp, Cyprinus carpio L., by loop-mediated isothermal amplification. *J Fish Dis* **27**, 583-589.

H

Haenen, O. L. & Engelsma, M. (2004). Global distribution of KHV with particular reference to Europe.

Haenen, O. L. & Hedrick, R. P. (2006). Koi herpesvirus workshop *Bull Eur Assoc Fish Pathol* **26**.

Haenen, O. L. M., Way, K., Bergmann, S.M., and Ariel, E. (2004). The emergence of koi herpesvirus and its significance to European aquaculture. *Bull Eur Assoc Fish Pathol* **24**, 293-307.

Hanson, L., Dishon, A. & Kotler, M. (2011). Herpesviruses that infect fish. *Viruses* **3**, 2160-2191.

Haramoto, E., Kitajima, M., Katayama, H. & Ohgaki, S. (2007). Detection of koi herpesvirus DNA in river water in Japan. *J Fish Dis* **30**, 59-61.

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.

Harrell, L. W., Etlinger, H. M. & Hodgins, H. O. (1976). Humoral-factors important in resistance of salmonid fish to bacterial disease. 2. Anti-vibrio anguillarum activity in mucus and observations on complement

Aquaculture **7**, 363-370.

Hedrick, R. P. (1996). Movement of pathogens with the international trade of live fish: problems and solutions. *Rev Sci Tech* **15**, 523-531.

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.

Hedrick, R. P., Marty, R., Nordhausen, M., Kebus, M., Bercovier, H., Eldar, A. (1999). An herpesvirus associated with mass mortality of juvenile and adult koi *Cyprinus carpio*. *Fish Health Newsletter, FHS, Am Fish Soc* **27**, 7.

Hjelmeland, K., Christie, M. & Raa, J. (1983). Skin mucus protease from rainbow trout, Salmo gairdneri Richardson, and its biological significance. *Journal of Fish Biology* **23**, 13-22.

Honess, R. W. & Roizman, B. (1974). Regulation of herpesvirus macromolecular synthesis. I. Cascade regulation of the synthesis of three groups of viral proteins. *J Virol* **14**, 8-19.

Honess, R. W. & Roizman, B. (1975). Regulation of herpesvirus macromolecular synthesis: sequential transition of polypeptide synthesis requires functional viral polypeptides. *Proc Natl Acad Sci U S A* **72**, 1276-1280.

Honjo, M. N., Minamoto, T. & Kawabata, Z. (2011). Reservoirs of Cyprinid herpesvirus 3 (CyHV-3) DNA in sediments of natural lakes and ponds. *Vet Microbiol*.

Honjo, M. N., Minamoto, T., Matsui, K., Uchii, K., Yamanaka, H., Suzuki, A. A., Kohmatsu, Y., Iida, T. & Kawabata, Z. (2010). Quantification of cyprinid herpesvirus 3 in environmental water by using an external standard virus. *Appl Environ Microbiol* **76**, 161-168.

Hsu, T. Y., Liu, M. W., Chang, Y. R., Pai, C. Y., Liu, M. Y., Yang, C. S. & Chen, J. Y. (1996). Functional analysis of C-terminal deletion mutants of Epstein-Barr virus thymidine kinase. *J Gen Virol* **77**, 1893-1899.

Hutoran, M., Ronen, A., Perelberg, A., Ilouze, M., Dishon, A., Bejerano, I., Chen, N. & Kotler, M. (2005). Description of an as yet unclassified DNA virus from diseased Cyprinus carpio species. *J Virol* **79**, 1983-1991.

I

Ilouze, M., Dishon, A., Kahan, T. & Kotler, M. (2006a). Cyprinid herpes virus-3 (CyHV-3) bears genes of genetically distant large DNA viruses. **580**, 4473-4478.

Ilouze, M., Dishon, A. & Kotler, M. (2006b). Characterization of a novel virus causing a lethal disease in carp and koi. *Microbiol Mol Biol Rev* **70**, 147-156.

Ishioka, T., Yoshizumi, M., Izumi, S., Suzuki, K., Suzuki, H., Kozawa, K., Arai, M., Nobusawa, K., Morita, Y., Kato, M., Hoshino, T., Iida, T., Kosuge, K. & Kimura, H. (2005). Detection and sequence analysis of DNA polymerase and major envelope protein genes in koi herpesviruses derived from Cyprinus carpio in Gunma prefecture, Japan. *Vet Microbiol* **110**, 27-33.

Ito, T., Sano, M., Kurita, J., Yuasa, K. & Iida, T. (2007). Carp larvae are not susceptible to Koi Herpesvirus. *Fish Pathol* **42**, 107-109.

J

Jacob, R. J., Morse, L. S. & Roizman, B. (1979). Anatomy of herpes simplex virus DNA. XII. Accumulation of head-to-tail concatemers in nuclei of infected cells and their role in the generation of the four isomeric arrangements of viral DNA. *J Virol* **29**, 448-457.

Jin, J. Y., Zhou, L., Wang, Y., Li, Z., Zhao, J. G., Zhang, Q. Y. & Gui, J. F. (2010). Antibacterial and antiviral roles of a fish beta-defensin expressed both in pituitary and testis. *PLoS One* **5**, e12883.

Johnson, D. C. & Spear, P. G. (1982). Monensin inhibits the processing of herpes simplex virus glycoproteins, their transport to the cell surface, and the egress of virions from infected cells. *J Virol* **43**, 1102-1112.

Jones, C. (2003). Herpes simplex virus type 1 and bovine herpesvirus 1 latency. *Clin Microbiol Rev* **16**, 79-95.

Jones, P. C. & Roizman, B. (1979). Regulation of herpesvirus macromolecular synthesis. VIII. The transcription program consists of three phases during which both extent of transcription and accumulation of RNA in the cytoplasm are regulated. *J Virol* **31**, 299-314.

K

Kalat, T. & Shabanipour, N. (2010). Internal Anatomy of Common Carp (Cyprinus carpio) as Revealed by Magnetic Resonance Imaging. *Applied Magnetic Resonance* **38**, 361-369.

Kanno, T., Nakai, T. & Muroga, K. (1989). Mode of transmission of vibriosis among ayu Plecoglossus altivelis. *Journal of Aquatic Animal Health* **1**, 2-6.

Karsi, A., Menanteau-Ledouble, S. & Lawrence, M. L. (2006). Development of bioluminescent Edwardsiella ictaluri for noninvasive disease monitoring. *FEMS Microbiol Lett* **260**, 216-223.

Kasai, H., Muto, Y. & Yoshimizu, M. (2005a). Virucidal effects of ultraviolet, heat treatment and disinfectants against Koi Herpesvirus (KHV). *Fish Pathol* **40**, 137-138.

Kasai, H., Muto, Y. & Yoshimizu, M. (2005b). Virucidal effects of ultraviolet, heat treatment and disinfectants against koi herpesvirus (KHV). *Fish Pathology* **40**, 137-138.

Kielpinski, M., Kempter, J., Panicz, R., Sadowski, J., Schütze, H., Ohlemeyer, S. & Bergmann, S. M. (2010). Detection of KHV in Freshwater Mussels and Crustaceans from Ponds with KHV History in Common Carp (Cyprinus carpio). *The Israeli Journal of Aquaculture* **62**, 28-37.

King, A., Lefkowitz, E., Adams, M. & Carstens, E. (2012). Order of presentation of virus taxonomic descriptions. In *Virus Taxonomy : Ninth Report of the International Committee on Taxonomy of Viruses*, pp. 23-36. Edited by A. King, E. Lefkowitz, M. Adams & E. Carstens. London: Elsevier.

Kongchum, P., Sandel, E., Lutzky, S., Hallerman, E. M., Hulata, G., David, L. & Palti, Y. (2011). Association between IL-10a single nucleotide polymorphisms and

resistance to cyprinid herpesvirus-3 infection in common carp (Cyprinus carpio). *Aquaculture* **315**, 417-421.

Kurita, J., Yuasa, K., Ito, T., Sano, M., Hedrick, R. P., Engelsma, M. Y., Haenen, O. L. M., Sunarto, A., Kholidin, E. B., Chou, H.-Y., Tung, M.-C., Pe, ntilde, a, L. d. l., Lio-Po, G., Tu, C., Way, K. & Iida, T. (2009). Molecular Epidemiology of Koi Herpesvirus. *Fish Pathology* **44**, 59-66.

L

Lamb, C. F. & Finger, T. E. (1995). Gustatory control of feeding behavior in goldfish. *Physiol Behav* **57**, 483-488.

Lee, M. A., Diamond, M. E. & Yates, J. L. (1999). Genetic evidence that EBNA-1 is needed for efficient, stable latent infection by Epstein-Barr virus. *J Virol* **73**, 2974-2982.

Liu, H., Shi, X., Gao, L. & Jiang, Y. (2002). Study on the aetiology of koi epizootic disease using the method of nested-polymerase chain reaction assay (nested-PCR). *J Huazhong Agric Univ* **21**, 414-418.

Lorenzen, N. & LaPatra, S. E. (2005). DNA vaccines for aquacultured fish. *Rev Sci Tech* **24**, 201-213.

Lu, F., Weidmer, A., Liu, C. G., Volinia, S., Croce, C. M. & Lieberman, P. M. (2008). Epstein-Barr virus-induced miR-155 attenuates NF-kappaB signaling and stabilizes latent virus persistence. *J Virol* **82**, 10436-10443.

M

Marek, A., Schachner, O., Bilic, I. & Hess, M. (2010). Characterization of Austrian koi herpesvirus samples based on the ORF40 region. *Dis Aquat Organ* **88**, 267-270.

Markine-Goriaynoff, N., Gillet, L., Karlsen, O. A., Haarr, L., Minner, F., Pastoret, P. P., Fukuda, M. & Vanderplasschen, A. (2004). The core 2 beta-1,6-N-acetylglucosaminyltransferase-M encoded by bovine herpesvirus 4 is not essential for virus replication despite contributing to post-translational modifications of structural proteins. *J Gen Virol* **85**, 355-367.

Masse, M. J., Jons, A., Dijkstra, J. M., Mettenleiter, T. C. & Flamand, A. (1999). Glycoproteins gM and gN of pseudorabies virus are dispensable for viral penetration and propagation in the nervous systems of adult mice. *J Virol* **73**, 10503-10507.

Mast, J., Nanbru, C., van den Berg, T. & Meulemans, G. (2005). Ultrastructural changes of the tracheal epithelium after vaccination of day-old chickens with the La Sota strain of Newcastle disease virus. *Vet Pathol* **42**, 559-565.

Matsui, K., Honjo, M., Yukihiro, K., Uchii, K., Yonekura, R. & Kawabata, Z. (2008). Detection and significance of koi herpesvirus (KHV) in freshwater environments. *Freshwat Biol* **53**, 1262-1272.

McGeoch, D. J., Davison, A. J., Dolan, A., Gatherer, D. & Sevilla-Reyes, E. E. (2008). Molecular evolution of the Herpesvirales. In *Origin and evolution of viruses*, 2 edn, pp. 447-476. Edited by E. Domingo, C. R. Parrish & J. J. Holland. Amsterdam: Elsevier.

McGeoch, D. J., Rixon, F. J. & Davison, A. J. (2006). Topics in herpesvirus genomics and evolution. *Virus Research* **117**, 90-104.

Mettenleiter, T. C. (2004). Budding events in herpesvirus morphogenesis. *Virus Res* **106**, 167-180.

Mettenleiter, T. C., Klupp, B. G. & Granzow, H. (2009). Herpesvirus assembly: an update. *Virus Res* **143**, 222-234.

Michel, B. (2010). Structural proteome of Cyprinid herpesvirus 3. In *Infectious and parasitic disease*. Liege: University of Liege.

Michel, B., Fournier, G., Lieffrig, F., Costes, B. & Vanderplasschen, A. (2010a). Cyprinid herpesvirus 3: an interesting virus for applied and fundamental research. *Emerging Infectious Diseases* **16**, 1835-1843.

Michel, B., Leroy, B., Stalin Raj, V., Lieffrig, F., Mast, J., Wattiez, R., Vanderplasschen, A. F. & Costes, B. (2010b). The genome of cyprinid herpesvirus 3 encodes 40 proteins incorporated in mature virions. *J Gen Virol* **91**, 452-462.

Minamoto, T., Honjo, M. N., Uchii, K., Yamanaka, H., Suzuki, A. A., Kohmatsu, Y., Iida, T. & Kawabata, Z. (2009). Detection of cyprinid herpesvirus 3 DNA in river water during and after an outbreak. *Vet Microbiol* **135**, 261-266.

Minamoto, T., Honjo, M. N., Yamanaka, H., Tanaka, N., Itayama, T. & Kawabata, Z. (2011). Detection of cyprinid herpesvirus-3 DNA in lake plankton. *Res Vet Sci* **90**, 530-532.

Mittal, A. K. & Whitear, M. (1979). Keratinization of fish skin with special reference to the catfish Bagarius bagarius. *Cell Tissue Res* **202**, 213-230.

Miwa, S., Ito, T. & Sano, M. (2007). Morphogenesis of koi herpesvirus observed by electron microscopy. *J Fish Dis* **30**, 715-722.

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.

Morgan, R. W., Cantello, J. L. & McDermott, C. H. (1990). Transfection of chicken embryo fibroblasts with Marek's disease virus DNA. *Avian Dis* **34**, 345-351.

Mowry, R. W. (1956). Alcian Blue technics for the histochemical study of acidic carbohydrates. *J Histochem Cytochem* **4**, 407.

N

Neukirch, M., Böttcher, K. & Bunnajrakul, S. (1999). Isolation of a virus from koi with altered gills. *Bull Eur Assoc Fish Pathol* **19**, 221-224.

Neukirch, M. & Kunz, U. (2001). Isolation and preliminary characterization of several viruses from koi (Cyprinus carpio) suffering gill necrosis and mortality. *Bull Eur Assoc Fish Pathol* **21**, 125-135.

O

Ødegård, J., Olesena, I., Dixonb, P., Jeneyc, Z., Nielsena, H.-M., Wayb, K., Joinerb, C., Jeneyc, G., Ardóc, L., Rónyaic, A. & Gjerdea, B. (2010). Genetic analysis of common carp (Cyprinus carpio) strains. II: Resistance to koi herpesvirus and Aeromonas hydrophila and their relationship with pond survival *Aquaculture* **304**, 7-13.

Oh, M. J., Jung, S.J., Choi, T.J., Kim, H.R., Rajendran, K.V., Kim, Y.J., Park, M.A., Chun, S.K. (2001). A viral disease occurring in cultured carp Cyprinus carpio in Korea. *Fish pathology* **36**, 147-151.

P

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.

Panicali, D. & Paoletti, E. (1982). Construction of poxviruses as cloning vectors: insertion of the thymidine kinase gene from herpes simplex virus into the DNA of infectious vaccinia virus. *Proc Natl Acad Sci U S A* **79**, 4927-4931.

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.

Perelberg, A., Ronen, A., Hutoran, M., Smith, Y. & Kotler, M. (2005). Protection of cultured Cyprinus carpio against a lethal viral disease by an attenuated virus vaccine. *Vaccine* **23**, 3396-3403.

Perelberg, A., Smirnov, M., Hutoran, M., Diamant, A., Bejerano, Y., Kotler, M. (2003). Epidemiological description of a new viral disease afflicting cultured Cyprinus Carpio in Israel. *The Israeli Journal of Aquaculture* **55**, 5-12.

Pfeffer, S., Sewer, A., Lagos-Quintana, M., Sheridan, R., Sander, C., Grasser, F. A., van Dyk, L. F., Ho, C. K., Shuman, S., Chien, M., Russo, J. J., Ju, J., Randall, G., Lindenbach, B. D., Rice, C. M., Simon, V., Ho, D. D., Zavolan, M. & Tuschl, T. (2005). Identification of microRNAs of the herpesvirus family. *Nat Methods* **2**, 269-276.

Pikarsky, E., Ronen, A., Abramowitz, J., Levavi-Sivan, B., Hutoran, M., Shapira, Y., Steinitz, M., Perelberg, A., Soffer, D. & Kotler, M. (2004). Pathogenesis of acute viral disease induced in fish by carp interstitial nephritis and gill necrosis virus. *J Virol* **78**, 9544-9551.

Plowright, W. (1990). Malignant catarrhal fever virus. In *Virus infections of ruminants*, pp. 123-150. Edited by Z. Dinter & B. Morein. Amsterdam: Elsevier Sci Pub.

Pokorova, D., Vesely, T., Piackova, V., Reschova, S., Hulova, J. (2005). Current knowledge on koi herpesvirus (KHV): a review. *Vet Med-Czech* **5**, 139-147.

Prophet, E. B. (1992). Tissue processing: dehydratation, clearing and infiltration. In *Laboratory Methods in Histotechnology* pp. 25-59. Edited by E. B. Prophet, B. Mills, J. B. Arrington & L. H. Sobin. Washington DC: Armed Forces Institute of Pathology.

Q

Quillet, E., Dorson, M., Aubard, G. & Torhy, C. (2001). In vitro viral haemorrhagic septicaemia virus replication in excised fins of rainbow trout: correlation with resistance to waterborne challenge and genetic variation. *Dis Aquat Organ* **45**, 171-182.

R

Raj, V. S., 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.

Rakus, K. L., Wiegertjes, G. F., Jurecka, P., Walker, P. D., Pilarczyk, A. & Irnazarow, I. (2009). Major histocompatibility (MH) class II B gene polymorphism influences disease resistance of common carp (Cyprinus carpio L.). *Aquaculture* **288**, 44-50.

Roberts, R. J. (2001a). *Fish pathology*. London: W. B. Saunders.

Roberts, R. J. (2001b). The parasitology of teleosts,. In *Fish pathology*, 3rd ed. edn, pp. 254–297. Edited by R. J. Roberts. London: W. B. Saunders.

Roberts, R. J. & Ellis, A. E. (2001). The anatomy and physiology of teleosts. In *Fish pathology*, 3rd ed edn, pp. 12–54. Edited by R. J. Roberts. London, United Kingdom: W. B. Saunders.

Roizman, B. & Pellet, P. E. (2007). The Family: Herpesviridae a brief introduction. In *Fields virology*, 5 edn, pp. 2479-2499. Edited by D. M. Knipe & P. M. Howley. Philadelphia: Lippincott Williams and Wilkins.

Roizmann, B., Desrosiers, R. C., Fleckenstein, B., Lopez, C., Minson, A. C. & Studdert, M. J. (1992). The family Herpesviridae: an update. The Herpesvirus Study Group of the International Committee on Taxonomy of Viruses. *Arch Virol* **123**, 425-449.

Ronen, A., Perelberg, A., Abramowitz, J., Hutoran, M., Tinman, S., Bejerano, I., Steinitz, M. & Kotler, M. (2003). Efficient vaccine against the virus causing a lethal disease in cultured Cyprinus carpio. *Vaccine* **21**, 4677-4684.

Rosenkranz, D., Klupp, B. G., Teifke, J. P., Granzow, H., Fichtner, D., Mettenleiter, T. C. & Fuchs, W. (2008). Identification of envelope protein pORF81 of koi herpesvirus. *J Gen Virol* **89**, 896-900.

S

Sadler, J., Marecaux, E. & Goodwin, A. E. (2008). Detection of koi herpes virus (CyHV-3) in goldfish, Carassius auratus (L.), exposed to infected koi, pp. 71-72.

Sano, M., Ito, T., Kurita,J., Yanai, T., Watanabe, N., Satoshi, M., and Iida, T. (2004). First detection of koi herpes virus in cultured common carp Cyprinus carpio in Japan. *Fish Pathol* **39**, 165-168.

Savin, K. W., Cocks, B. G., Wong, F., Sawbridge, T., Cogan, N., Savage, D. & Warner, S. (2010). A neurotropic herpesvirus infecting the gastropod, abalone, shares ancestry with oyster herpesvirus and a herpesvirus associated with the amphioxus genome. *Virol J* **7**, 308.

Shapira, Y., Magen, Y., Zak, T., Kotler, M., Hulata, G. & Levavi-Sivan, B. (2005). Differential resistance to koi herpes virus (KHV)/carp interstitial nephritis and gill necrosis virus (CNGV) among common carp (Cyprinus carpio L.) strains and crossbreds. *Aquaculture* **245**, 1-11.

Shephard, K. L. (1994). Functions for fish mucus. *Reviews in Fish Biology and Fisheries* **4**, 401-429.

Shimizu, T., Yoshida, N., Kasai, H. & Yoshimizu, M. (2006). Survival of koi herpesvirus (KHV) in environmental water. *Fish Pathol* **41**, 153-157.

Shizuya, H., Birren, B., Kim, U. J., Mancino, V., Slepak, T., Tachiiri, Y. & Simon, M. (1992). Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in Escherichia coli using an F-factor-based vector. *Proc Natl Acad Sci U S A* **89**, 8794-8797.

Sibbing, F. (1988). Specializations and limitations in the utilization of food resources by the carp, Cyprinus carpio : a study of oral food processing. *Environmental Biology of Fishes* **22**, 161-178.

Sire, J. Y. & Akimenko, M. A. (2004). Scale development in fish: a review, with description of sonic hedgehog (shh) expression in the zebrafish (Danio rerio). *Int J Dev Biol* **48**, 233-247.

Smail, D. A. & Munro, A. L. S. (2001). The virology of teleosts. In *Fish pathology*, pp. 169–254. Edited by R. J. Roberts. London: W. B. Saunders.

Smith, G. A. & Enquist, L. W. (2000). A self-recombining bacterial artificial chromosome and its application for analysis of herpesvirus pathogenesis. *Proc Natl Acad Sci U S A* **97**, 4873-4878.

Smith, J. D. (1980). An additional role for the outer nuclear membrane in the morphogenesis of herpes simplex virus. *Intervirology* **13**, 312-316.

Sodeik, B., Ebersold, M. W. & Helenius, A. (1997). Microtubule-mediated transport of incoming herpes simplex virus 1 capsids to the nucleus. *J Cell Biol* **136**, 1007-1021.

Soliman, H. & El-Matbouli, M. (2005). An inexpensive and rapid diagnostic method of Koi Herpesvirus (KHV) infection by loop-mediated isothermal amplification. *Virol J* **2**, 83.

Soliman, H. & El-Matbouli, M. (2009). Immunocapture and direct binding loop mediated isothermal amplification simplify molecular diagnosis of Cyprinid herpsvirus-3. *J Virol Methods*.

Spear, P. G. (2004). Herpes simplex virus: receptors and ligands for cell entry. *Cell Microbiol* **6**, 401-410.

St-Hilaire, S., Beevers, N., Joiner, C., Hedrick, R. P. & Way, K. (2009). Antibody response of two populations of common carp, Cyprinus carpio L., exposed to koi herpesvirus. *J Fish Dis* **32**, 311-320.

St-Hilaire, S., Beevers, N., Way, K., Le Deuff, R. M., Martin, P. & Joiner, C. (2005). Reactivation of koi herpesvirus infections in common carp Cyprinus carpio. *Dis Aquat Organ* **67**, 15-23.

Stevenson, P. G., Simas, J. P. & Efstathiou, S. (2009). Immune control of mammalian gamma-herpesviruses: lessons from murid herpesvirus-4. *J Gen Virol* **90**, 2317-2330.

Subramanian, S., Ross, N. W. & MacKinnon, S. L. (2008). Comparison of antimicrobial activity in the epidermal mucus extracts of fish. *Comparative biochemistry and physiology* **150**, 85-92.

Sunarto, A., McColl, K. A., Crane, M. S., Sumiati, T., Hyatt, A. D., Barnes, A. C. & Walker, P. J. (2011). Isolation and characterization of koi herpesvirus (KHV) from Indonesia: identification of a new genetic lineage. *J Fish Dis* **34**, 87-101.

Suzuki, Y., Tasumi, S., Tsutsui, S., Okamoto, M. & Suetake, H. (2003). Molecular diversity of skin mucus lectins in fish. *Comparative biochemistry and physiology* **136**, 723-730.

T

Takahashi, Y., Itami, T. & Konegawa, K. (1986). Enzymatic properties of bacteriolytic substances in skin mucus and intestine of carp. *Fish Pathology* **21**, 187-191.

Tu, C., Weng, M. C., Shiau, J. R. & Lin, S. Y. (2004). Detection of koi herpesvirus in koi Cyprinus carpio in Taiwan. *Fish Pathology* **39**, 109-110.

U

Uchii, K., Matsui, K., Iida, T. & Kawabata, Z. (2009). Distribution of the introduced cyprinid herpesvirus 3 in a wild population of common carp, Cyprinus carpio L. *J Fish Dis*.

Uchii, K., Telschow, A., Minamoto, T., Yamanaka, H., Honjo, M. N., Matsui, K. & Kawabata, Z. (2011). Transmission dynamics of an emerging infectious disease in wildlife through host reproductive cycles. *ISME J* **5**, 244-251.

Umbach, J. L., Kramer, M. F., Jurak, I., Karnowski, H. W., Coen, D. M. & Cullen, B. R. (2008). MicroRNAs expressed by herpes simplex virus 1 during latent infection regulate viral mRNAs. *Nature* **454**, 780-783.

V

van Beurden, S. J., Bossers, A., Voorbergen-Laarman, M. H., Haenen, O. L., Peters, S., Abma-Henkens, M. H., Peeters, B. P., Rottier, P. J. & Engelsma, M. Y. (2010). Complete genome sequence and taxonomic position of anguillid herpesvirus 1. *J Gen Virol* **91**, 880-887.

van Beurden, S. J., Leroy, B., Wattiez, R., Haenen, O. L., Boeren, S., Vervoort, J. J., Peeters, B. P., Rottier, P. J., Engelsma, M. Y. & Vanderplasschen, A. F. (2011). Identification and localization of the structural proteins of anguillid herpesvirus 1. *Vet Res* **42**, 105.

van Damme, P., Appelbaum, S. & Hecht, T. (1989). Sibling cannibalism in Koi carp, Cypvinus carpio L., larvae and juveniles reared under controlled conditions. *Journal of Fish Biology* **34**, 855-863.

Vanderplasschen, A. & Smith, G. L. (1997). A novel virus binding assay using confocal microscopy: demonstration that the intracellular and extracellular vaccinia virions bind to different cellular receptors. *J Virol* **71**, 4032-4041.

W

Wagner, E. K., Petroski, M. D., Pande, N. T., Lieu, P. T. & Rice, M. (1998). Analysis of factors influencing kinetics of herpes simplex virus transcription utilizing recombinant virus. *Methods* **16**, 105-116.

Wagner, M., Ruzsics, Z. & Koszinowski, U. H. (2002). Herpesvirus genetics has come of age. *Trends Microbiol* **10**, 318-324.

Walster (1999). Clinical observations of severe mortalities in koi carp, Cyprinus carpio, with gill disease. *Fish Veterinary Journal* **3**, 54-58.

Waltzek, T. B. & Hedrick, R. P. (2004). Koi Herpesvirus update *California veterinarian* **July-August 2004**.

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.

Waltzek, T. B., Kelley, G. O., Stone, D. M., Way, K., Hanson, L., Fukuda, H., Hirono, I., Aoki, T., Davison, A. J. & Hedrick, R. P. (2005). Koi herpesvirus represents a third cyprinid herpesvirus (CyHV-3) in the family Herpesviridae. *J Gen Virol* **86**, 1659-1667.

Wang, F. Z., Weber, F., Croce, C., Liu, C. G., Liao, X. & Pellett, P. E. (2008). Human cytomegalovirus infection alters the expression of cellular microRNA species that affect its replication. *J Virol* **82**, 9065-9074.

Warming, S., Costantino, N., Court, D. L., Jenkins, N. A. & Copeland, N. G. (2005). Simple and highly efficient BAC recombineering using galK selection. *Nucleic Acids Res* **33**, e36.

Wild, P., Engels, M., Senn, C., Tobler, K., Ziegler, U., Schraner, E. M., Loepfe, E., Ackermann, M., Mueller, M. & Walther, P. (2005). Impairment of nuclear pores in bovine herpesvirus 1-infected MDBK cells. *J Virol* **79**, 1071-1083.

Y

Yasumoto, S., Kuzuya, Y., Yasuda, M., Yoshimura, T. & Miyazaki, T. (2006). Oral Immunization of Common Carp with a Liposome Vaccine Fusing Koi Herpesvirus Antigen. *Fish Pathology* **41**, 141-145.

Z

Zinn, K. R., Chaudhuri, T. R., Szafran, A. A., O'Quinn, D., Weaver, C., Dugger, K., Lamar, D., Kesterson, R. A., Wang, X. & Frank, S. J. (2008). Noninvasive bioluminescence imaging in small animals. *Ilar J* **49**, 103-115.

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