Université de Liège

Académie Universitaire Wallonie-Europe
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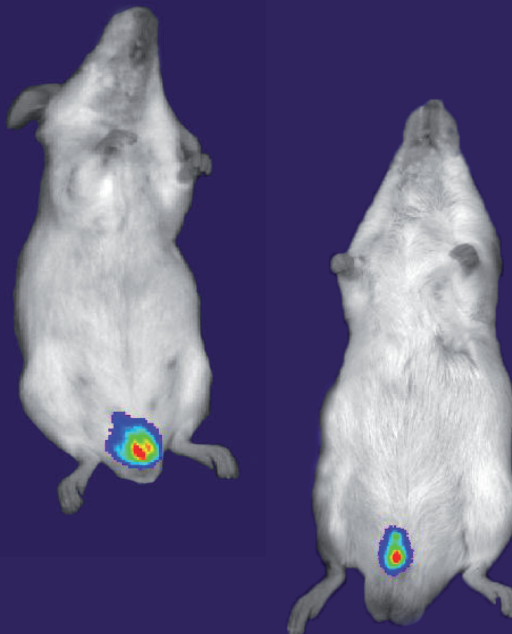
Faculté de Médecine Vétérinaire
Département des maladies infectieuses et parasitaires
Service d'Immunologie-Vaccinologie

Développement d'un modèle de transmission de l'Herpèsvirus murin 4

Development of a transmission model of Murid herpesvirus 4



FRANCOIS Sylvie

Thèse présentée en vue de l'obtention du grade de
Docteur en Sciences Vétérinaires
Année académique 2012-2013

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« Ne vous souciez pas d'être meilleur que vos
contemporains ou que vos prédécesseurs.
Essayez d'être meilleur que vous-même. »

William Faulkner

"Don't bother to be better than your
contemporaries or predecessors.
Try to be better than yourself."

William Faulkner

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A tous, Merci !

Abbreviations

AIDS	Aquired ImmunoDeficiency Syndrome
AlHV-1	*Alcelaphine herpesvirus 1*
BAC	Bacterial Artificial Chromosome
BHK-21	Baby Hamster Kidney 21
BL	Burkitt's Lymphoma
BoHV-1	*Bovine herpesvirus 1*
BoHV-4	*Bovine herpesvirus 4*
CBP	Chemokine Binding Protein
CCD	Charged Couple Device
cdk6	Cyclin Dependent Kinase 6
CMC	CarboxyMethylCellulose
CMV	Cytomegalovirus
DC	Dendritic Cell
DMEM	Dubelcco's Modified Eagle's Medium
DNA	DesoxyriboNucleic Acid
dsDNA	double strand DesoxyNibonucleic Acid
E gene	Early gene
E. coli	*Escherichia coli*
EBNA	Epstein Barr nuclear antigen
EBV	Epstein Barr Virus
EHV-2	*Equid herpesvirus 2*
ELISA	Enzyme-Linked ImmunoSorbent Assay
ER	External Repeat
FCS	Fetal Calf Serum
GAG	GlycosAminoGlycan
GFP	Green Fluorescence Protein
GPCMV	Guinea Pig cytomegalovirus
HCMV	Human cytomegalovirus
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*
HIV	Human Immunodeficiency Virus
HSV-1	Herpes simplex virus type 1
HSV-2	Herpes simplex virus type 2
HVS	Herpesvirus saimiri
ICTV	International Committee on Taxonomy of Viruses
IE gene	Immediate-Early gene
IFN	Interferon
Ig	Immunoglobulin

IL8	Interleukine 8
IR	Internal Repeat
IRBP	Interstitial Retinoid Binding Protein
IVIS	*In Vivo* Imaging System
KS	Kaposi's Sarcoma
KSHV	Kaposi's Sarcoma associated HerpesVirus
L gene	Late gene
LANA	Latency Associated Nuclear Antigen
LAT	Latency Associated Transcripts
LCL	Lymphoblastoïd Cell Line
LMP	Latent Membrane Protein
LTR	Left Terminal Repeat
MCF	Malignant Catarrhal Fever
MCMV	Murine cytomegalovirus
MHC	Major Histocompatibility Complex
MHV-60	Murine herpesvirus 60
MHV-68	Murine herpesvirus 68
MHV-72	Murine herpesvirus 72
miRNA	micro RNA
Myrs	Million years
NHANES	National Health And Nutrition Examination Surveys
NMuMG	Normal Murine Mammary Gland cells
MuHV-4	*Murid herpesvirus 4*
ORF	Open Reading Frame
OvHV-2	*Ovine herpesvirus 2*
p	Photon
p.f.u.	Plaque forming unit
p.i.	Post infection
PAMP	Pathogen Associated Molecular Pattern
PCR	Polymerase Chain Reaction
PEL	Primary Effusion Lymphoma
prDNA	Polyrepetitive DNA
PrV	Pseudorabies virus
RCMV	Rat cytomegalovirus
RNA	RiboNucleic Acid
RPMI	Roswell Park Memorial Institute
RTR	Right Terminal Repeat
SCID	Severe Combined ImmunoDeficiency
SCLN	Superficial Cervical Lymph Node
SEM	Standard Error of Mean
sr	Steradian
STD	Sexually Transmitted Disease
TLR	Toll-like Receptor
t-RNA	Transfert RNA

U.K.	United Kingdom
U.S.	United States
v-bcl2	Viral B-cell lymphoma 2
v-FLIP	Viral FLICE Inhibitory Protein
VZV	Varicella zoster virus
WMHV	Wood mouse herpesvirus
WT	Wild type

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Résumé

Les gammaherpèsvirus sont des pathogènes persistants qui ont été identifiés chez de nombreuses espèces animales dont l'homme. Jusqu'à ce jour, l'étude de la transmission des gammaherpèsvirus n'a pu être que limitée car la communauté scientifique ne dispose d'aucun modèle de transmission pour aucun de ces virus. L'établissement d'un tel modèle et sa caractérisation constituent dès lors des éléments essentiels à la poursuite d'études visant à établir des stratégies de contrôle de la transmission de ces virus. La souche MHV-68 de l'herpèsvirus murin 4 (MuHV-4) a été isolée chez le campagnol roussâtre (*Myodes glareolus*). Bien que des données sérologiques indiquent que des virus apparentés au MuHV-4 circulent au sein des populations de mulots sylvestres (*Apodemus sylvaticus*) ou de souris domestiques (*Mus musculus*), aucune transmission expérimentale du MuHV-4 n'a pu être, jusqu'à ce jour, reproduite chez la souris de laboratoire, modèle d'étude classiquement utilisé. L'objectif de ce travail était donc de combler cette lacune.

Dans une première étude, afin d'évaluer les qualités respectives des modèles « souris » et « campagnol », une caractérisation comparative de l'infection par le MuHV-4 de ces deux espèces a été réalisée. Les résultats obtenus ont montré que le processus d'infection, la pathologie et l'établissement de la latence sont comparables au sein des deux espèces, bien que la réplication soit quantitativement inférieure chez le campagnol. Il semble dès lors que la souris de laboratoire soit un bon modèle pour l'étude du MuHV-4 *in vivo*. Ces résultats ont été publiés dans la revue *Journal of General Virology* (J Gen Virol. 2010 Oct;91(Pt 10):2553-63).

Dans une deuxième étude, grâce à l'utilisation d'une méthode d'imagerie *in vivo*, nous avons été en mesure, d'effectuer la première observation de transmission du MuHV-4 chez la souris de laboratoire. Nous avons d'abord montré la ré-excrétion du MuHV-4 au niveau du tractus génital de souris femelles après que la latence ait été établie. L'imagerie *ex vivo*, l'histologie et la PCR nous ont permis de démontrer la présence de génome viral dans les tissus vaginaux et de localiser la réplication virale au niveau de la bordure vaginale externe. La présence de virus infectieux dans la cavité vaginale a également été montée. Dans un deuxième temps, nous avons montré l'implication des stéroïdes sexuels dans le phénomène observé. En effet, l'analyse de l'infection de souris non traitées, ovariectomisées, ou encore de souris ovariectomisées traitées par des œstrogènes et/ou de la progestérone nous a révélé un rôle positif des œstrogènes dans le phénomène de ré-excrétion. Enfin, différents modes de transmission du MuHV-4 chez la souris de laboratoire ont été testés. Dans les conditions testées, nous n'avons pas observé de transmission verticale ou de transmission horizontale du virus entre individus de même sexe. Par contre, la transmission par voie sexuelle a quant à elle pu être observée tant par des méthodes sérologiques, que par imagerie *in vivo* ou par PCR quantitative.

L’ensemble de ce travail a donc permis d’une part de démontrer la qualité du modèle souris dans l’étude du MuHV-4, et d’autre part de montrer pour la première fois un site de ré-excrétion du MuHV-4 chez la souris de laboratoire. Cette dernière observation a par ailleurs conduit à la mise en place du premier modèle de transmission, par voie sexuelle, du MuHV-4 en condition de laboratoire. Les résultats obtenus au cours de ce travail devraient avoir, dans le futur, des implications pour l’étude des gammaherpesvirus en particulier, mais également plus largement pour l’étude d’infections sexuellement transmissibles.

Abstract

Gammaherpesviruses are the archetype of persistent viruses that have been identified in a series of animals ranging from mice to man. To date the study of transmission of these viruses in natural condition has been limited by the fact that no experimental transmission model exists. Establishment and characterization of a model of transmission are therefore critical points to evaluate strategies of interference with the epidemiological cycle of gammaherpesviruses. We are studying Murid herpesvirus 4 (MuHV-4) which has originally been isolated from naturally infected bank voles (*Myodes glareolus*). Although serological data indicate that closely related strains are present in wood mice (*Apodemus sylvaticus*) and domestic mice (*Mus musculus*), no experimental transmission of MuHV-4 has been demonstrated in laboratory mice, the classically used *in vivo* model. The objective of this work was therefore to fill this gap.

In a first study, we performed a comparative characterization of the infection by MuHV-4 in mice and bank voles. Our results showed that the infectious process, the pathology and the latency establishment are similar in the two species, even if replication is quantitatively lower in bank voles than in mice. It therefore appeared that, *Mus musculus* represents a suitable host for studying gammaherpesvirus pathogenesis with MuHV-4. These results have been published in *Journal of General Virology* (J Gen Virol. 2010 Oct;91(Pt 10):2553-63).

In a second study, thanks to *in vivo* imaging, we have been able to observe, for the first time, transmission of MuHV-4 in mice. We firstly showed that MuHV-4 reexcretion occurs in the genital tract of female mice at a period by which latency is considered as established. *Ex vivo* imaging, histology and PCR allowed us to demonstrate the presence of viral genomes in vaginal tissues and to localize viral replication at the external border of the vagina. We also demonstrated the transient and repetitive presence of infectious viruses in the vaginal cavity. Secondly, we demonstrated the implication of sexual steroid hormones in this re-excretion process. Indeed, we analyzed the infection of untreated mice, ovariectomized mice and ovariectomized mice complemented with estrogens and/or progesterone. These analyses revealed a positive role of estrogens in the observed re-excretion. Finally, based on these results, we tested MuHV-4 transmission in mice by creating different epidemiological conditions. In the conditions tested, vertical transmission did not occur, nor did horizontal transmission between individuals of the same gender. In contrast, we were able to observe sexual transmission to naïve males by serology, *in vivo* imaging and quantitative PCR.

In conclusion, this work has on one hand demonstrated the quality of mice as an *in vivo* model for MuHV-4 studies and, on the other, it has shown for the first time the existence of re-excretion and

sexual transmission of MuHV-4 amongst laboratory mice. The results of this work should therefore have implications for the study of gammaherpesviruses, but also more generally for the study of sexually transmissible infections.

General preamble

General preamble

The *gammaherpesvirinae* are a subfamily of the order *Herpesvirales*. They are ubiquist viruses, able to infect a wide range of hosts. Amongst *gammaherpesvirinae*, Epstein-Barr virus (EBV or HHV-4) and Kaposi's sarcoma associated virus (KSHV or HHV-8), the two viruses infecting humans, are of particular interest. Seropositivity for EBV is of more than 90% around the world (Henle *et al.* 1969) and can locally reach 50% for KSHV (Butler *et al.* 2011). Although the infection by these viruses is generally asymptomatic, latency is systematically established following primo-infection in certain subsets of cells in the host and this despite immune response. Viral reactivation and re-excretion of infectious viral particles can take place sporadically, leading to transmission and viral spread among the host population.

The interest dedicated to these viruses is not trivial in terms of public health. Indeed, their persistence in the host and their capacity of escaping the immune response, associated with the presence of oncogenes in the viral genome, can lead to neoplasic disorder, especially lymphoproloferative, and through this pathway to the development of some cancers. It can therefore be said that the infection by gammaherpesvirus can be a major problem in every situation in which the host's immune system is deficient. The major examples for this are co-infection by HIV (Human Immunodeficiency Virus) and treatment in the context of grafts.

Interfering with the epidemiological cycle of these viruses is consequently a major challenge. However, studying EBV and KSHV is very difficult if not impossible because of their limited *in vitro* growth and the impossibility of *in vivo* studies in the natural host. In this context, Murid herpesvirus 4 (MuHV-4), a gammaherpesvirus isolated in a naturally infected bank vole, is a particularly interesting model. For about 30 years, MuHV-4 infection of laboratory mice has been a major model for studies concerning pathogenicity and biology of gammaherpesviruses even though transmission and viral spread in mice population has never been observed. It remains, however, that having a model of transmission is of particular importance when thinking about testing vaccinal strategies.

This manuscript presents, besides an introduction reviewing the current literature concerning gammaherpesviruses, two original studies designed to develop a transmission model of MuHV-4. The first one consists in a comparison of the infection in mice and bank voles, the evaluation of the quality of the mouse model and of an alternate model for *in vivo* studies. The second one is devoted to the characterization of the first phenomenon of re-excretion of MuHV-4 observed in infected mice. Finally, a general discussion and the perspectives generated by the work are presented.

1. Introduction

1. The *Herpesviridae*

1.1. Nomenclature and classification

The order *Herpesvirales* contains three subfamilies: the *Alloherpesviridae*, the *Malacoherpesviridae* and finally the *Herpesviridae*. The family *Herpesviridae* is the largest and is composed of three subfamilies: the *alpha-*, *beta-* and *gammaherpesvirinae.* This viral family contains at present more than 130 species sharing common features (Ackermann 2004; Davison *et al.* 2005; Davison *et al.* 2009). Indeed, they are spherical viral particles sizing from 150 to 300 nm. They all have a linear double strand DNA (dsDNA, double strand DNA) accompanied by some rare RNA molecules (Bresnahan and Shenk 2000; Bechtel *et al.* 2005; Jochum *et al.* 2012). These nucleic acids are associated with an icosaedral nucleocapsid composed of 162 capsomers, surrounded by a tegument containing proteins with regulatory functions. Lastly, a lipidic viral envelope, made from cellular membranes and containing viral glycoproteins, surrounds this assembly (Figure 1). Besides these morphological properties, several biological properties (Ackermann 2004) are also shared by all the *Herpesviridae*. Assuredly, they all have their own enzymatic machinery dedicated to the synthesis of their nucleic acids, which occurs in the nucleus of the infected cell, as well as the assembly of viral capsids. In addition, all the viruses belonging to this family produce a lytic cycle, often leading to the death of the infected cell, before establishing latency in certain cell types (Roizman and Pellet 2007). The term latency is used to describe the state of infection both at cell-level and at host-level. Indeed, a host is considered latently infected when acute primary infection is resolved (Barton *et al.* 2011).

The classification into three subfamilies was mainly established on the basis of host range, but also on the spectrum of cells capable of supporting viral latency *in vivo* (Roizman 1996), as only some cell types can support latency. Thus, *alphaherpesvirinae* are neurotropic viruses, establishing latency in specific neuronal populations while *beta-* and *gammaherpesvirinae* will establish latency essentially in lymphocytes and thus persist in lymphoid organs (Roizman and Pellet 2007). *Gammaherpesvirinea* are particular in the sense that, contrary to *alpha-* and *betahepresvirinae*, they establish early latency preferably to lytic infection. Moreover, *gammaherpesvirinae* are often associated with lymphoproliferative diseases and/or other non lymphoid cancers (Roizman 1996).

The classification is now completed by more objective molecular criteria and was largely confirmed by phylogenetic analyses (McGeoch *et al.* 1995; McGeoch *et al.* 2000; McGeoch *et al.* 2005; Davison *et al.* 2009). The *gammaherpesvirinae* subfamily classification was recently updated by the *International Committee on Taxonomy of Viruses* (ICTV; http://www.ictvonline.org), and now contains 4 genera (Table 1):

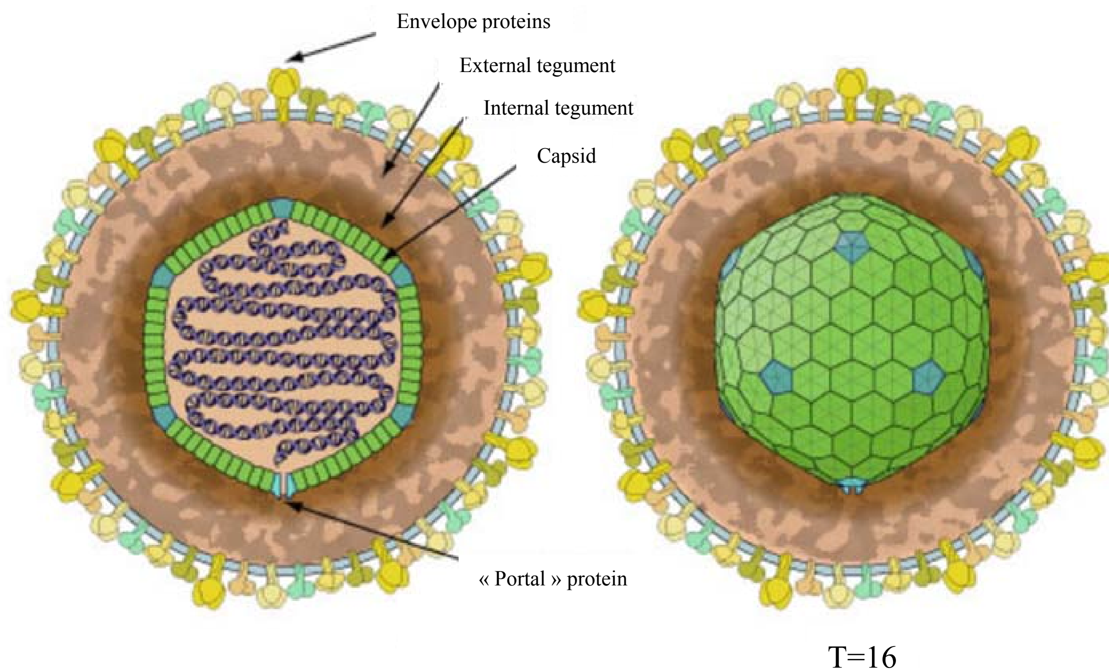


Figure 1. Morphology of herpesviruses. The viral particle is made of a capsid containig the viral genome, an intermediate proteic layer named tegument and a viral envelope with glycoproteins. The capsid is made of 150 hexons (green) and 11 pentons (blue) plus the portal protein to form an isocaedral structure (T=16) with pentons at the tops of the structure. One of the 12 tops is a pore by which DNA is enclosed in the capsid. From http://viralzone.expasy.org/all_by_species/181.html

- the *lymphocryptovirus* with EBV or *human herpesvirus 4* and some viruses infecting primates of the Old World;
- the *rhadinovirus* including KSHV or *human herpesvirus 8*, several viruses infecting primates of the Old and New Worlds but also other mammals;
- the *Percavirus* (standing for **pe**rissodactyl and **ca**rnivore) with the type-species *Equid herpesvirus 2* (EHV-2);
- the *Macavirus* (standing for **Ma**lignant **ca**tarrhal fever) with the type-species *Alcelaphine herpesvirus 1* (AlHV-1).

The newly introduced designations, *Percavirus* and *Macavirus,* take into account the recent characterization and the natural host specificities (artiodactyls versus perissodactyls) of the following viruses: *Bovine herpesvirus 6, Caprine herpesvirus 2, Suid herpesvirus 3, Suid herpesvirus 4* and *Suid herpesvirus 5* (Chmielewicz *et al.* 2001; Chmielewicz *et al.* 2003; Davison *et al.* 2009).

1.2. The viral cycle

1.2.1. The lytic infection

The multiplication cycle of herpesviruses is globally constant among members of the group and represented in figure 2A. The attachment of the viral particle on the cellular surface results from the interaction between one or more viral glycoproteins and cellular receptor(s). Frequently, primary interactions between the virus and the cell are of low specificity and imply the presence of glycosaminoglycans (GAG) at the surface of the cell. It was demonstrated that the ubiquitous presence of GAG at the cellular surface is an important co-factor for the entry of several herpesviruses into the cells, allowing the establishment of the initial contacts between the virus and the target cell (Shukla and Spear 2001). After these low affinity interactions, higher affinity contacts are established, implying one or more cellular receptors and viral envelope glycoproteins. These specific interactions are an important determinant of the sensitivity of the cell to infection and therefore of the viral tropism and host range. As shown for other viral families (Helenius 2007), herpesviruses are also able to use different cellular receptors and several cell surface molecules can be necessary for viral attachment. This has particularly been documented with HSV-1.

When the virus is attached, the viral envelope will fuse with the cellular membrane, leading to the release of proteins from the tegument and from the nucleocapsid into the cytoplasm (Roizman 1996). Two mechanisms of entry are described: on one hand, the entry can be performed by fusion of

Order	Family	Subfamily	Genus	Species
Herpesvirales	*Malacoherpesviridae*			
	Alloherpesviridae			
	Herpesviridae	*Alphaherpesvirinae*		
		Betaherpesvirinae		
		Gammaherpesvirinae	*Lymphocryptovirus*	*Callitrichine herpesvirus 3*
				Cercopithecine herpesvirus 14
				Gorilline herpesvirus 1
				Human herpesvirus 4
				Macacine herpesvirus 4
				Panine herpesvirus 1
				Papiine herpesvirus 1
				Pongine herpesvirus 2
			Macavirus	***Alcelaphine herpesvirus 1***
				Alcelaphine herpesvirus 2
				Bovine herpesvirus 6
				Caprine herpesvirus 2
				Hippotragine herpesvirus 1
				Ovine herpesvirus 2
				Suid herpesvirus 3
				Suid herpesvirus 4
				Suid herpesvirus 5
			Percavirus	***Equid herpesvirus 2***
				Equid herpesvirus 5
				Mustelid herpesvirus
			Rhadinovirus	*Ateline herpesvirus 2*
				Ateline herpesvirus 3
				Bovine herpesvirus 4
				Human herpesvirus 8
				Macacine herpesvirus 5
				Murid herpesvirus 4
				Saimiriine herpesvirus 2

Table 1: Nomenclature and classification of gammaherpesviruses. Murid herpesvirus-4 classification is mentioned by grey areas and type-species are indicated in bold. From http://ictvdb.org/Ictv/index.htm.

the viral envelope with the cellular plasmic membrane and on the other hand, an entry by endocytosis followed by the fusion of the endosomal membrane with the viral envelope is also possible (Roizman 1996). Fusion is mediated by, at least, glycoproteins B, H and L that form the core entry machinery conserved in all the *Herpesviridae*. gB is the fusion protein (Pertel 2002; Sharma-Walia *et al.* 2004; Backovic *et al.* 2007; Vanarsdall *et al.* 2008; Atanasiu *et al.* 2010). The roles of gH and gL are less clear and still controversial.

Once into the cytoplasm, free capsids follow the network of tubulin microtubules to reach a nuclear pore (Granzow *et al.* 1997; Sodeik *et al.* 1997). This phenomenon implies the « dynein/dynactin » proteic motor (Dohner *et al.* 2002). Once transported to the nucleus, the capsid releases nucleic acids at a nuclear pore (Sodeik *et al.* 1997; Peng *et al.* 2010). After its entry into the nucleus, the viral genome is rapidly circularized in the absence of any viral protein synthesis, suggesting a mechanism under dependence of cellular proteins and/or structural viral proteins (Poffenberger and Roizman 1985). The transcription of the herpesviruses' genome then proceeds, following a series of events that are strictly regulated by viral proteins. We can distinguish three transcriptional phases. The first wave of transcription is initiated by the regulatory tegument proteins and allows the transcription of genes called "immediate-early" (IE) or α. The proteins synthesized at this stage essentially act as activators of transcription. Subsequently, the genes "early" (E) or β, including the viral DNA polymerase, are transcribed. The last phase, called late (L) or γ, allows the synthesis of structural proteins including the envelope glycoproteins and the capsid proteins. Replication of viral DNA marks the separation between early and late phases (Honess and Roizman 1974; Honess and Roizman 1975; Jones and Roizman 1979).

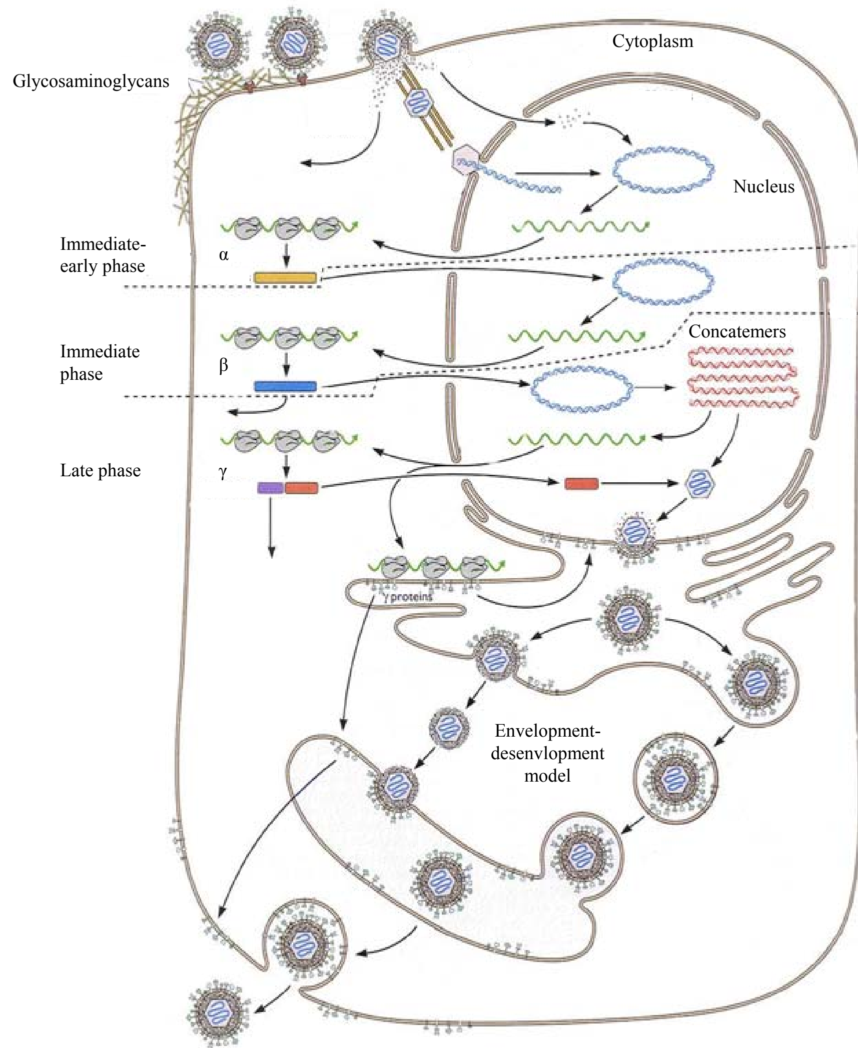
Once the circularized viral genome is inside the nucleus of the host cell, the tegument proteins that are present will interact with various cellular components to induce IE gene transcription. This first transcriptional step is carried out by the cellular RNA polymerase II. Protein synthesis occurs in the cytoplasm and some of these proteins are then imported into the nucleus to stimulate transcription of E and L genes, but also to inhibit the transcription of IE genes. E genes show a peak of expression 4-9 hours after infection of the cell, while the L gene expression is maximal after the beginning of the synthesis of viral DNA. These genes can be classified into two categories: the expression of partial late genes (or γ1) is increased by the synthesis of viral DNA, whereas the expression of real late genes (or γ2) is entirely dependent on viral DNA synthesis (Roizman 1996). Viral DNA replication is a critical step in the replication cycle of herpesviruses. It is placed under the control of the viral DNA polymerase synthesized during the early phase and starts at one or more origins of replication. The synthesis of viral genomes occurs through the mechanism of "rolling circles" (Jacob *et al.* 1979; Ackermann 2004), generating units consisting of concatemeric structures separated by sequences that

A

Attachement of the virus at cellular surface and fusion with the plasma membrane

Viral proteins sythesis and viral DNA replication

Viral membrane acquisition



B

Viral genome is under episomal form

LANA (maintenance protein encoded by ORF 73) is expressed

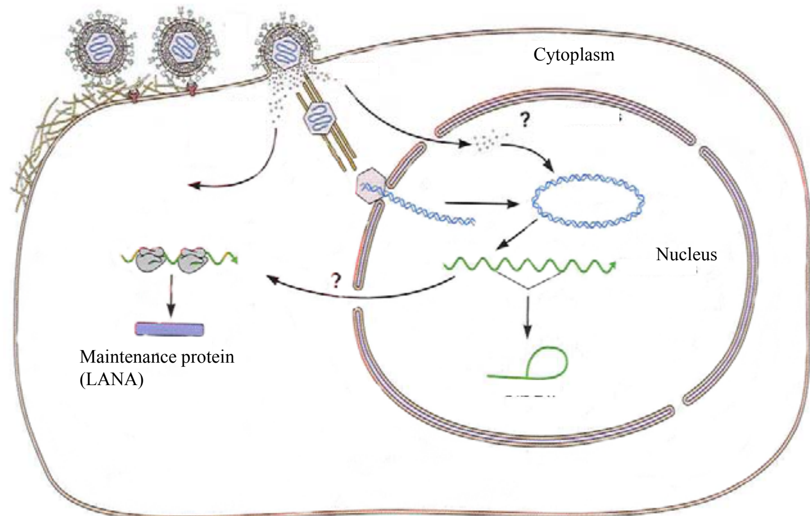


Figure 2. Representation of the general cycle of herpesviruses, comprising the lytic (A) and the latent (B) state. From Flint *et al.*, 2000.

are targets for enzymatic cleavage. Indeed, during the encapsidation of the viral genome, these enzymes recognize and cleave target sequences in order to ensure the encapsidation of one genome by a virus particle (McVoy *et al.* 2000).

Herpesvirus morphology is highly constant, suggesting similar morphogenesis processes (Mettenleiter *et al.* 2009). The proteins of the capsids are synthesized in the cytoplasm and are translocated from the cytoplasm to the nucleus where the capsids are assembled by an autocatalytic process (Homa and Brown 1997). In the structure of the capsids, a portal complex (Chang *et al.* 2007) by which viral DNA is encapsidated is found (Newcomb *et al.* 2006). Mechanisms ruling the egress of the nucleocapsids from the nucleus to the cytoplasm are not well known. However, several mechanisms, presented in figure 3, were proposed: the model of nuclear pore egress, the “luminal” model and finally the “envelopment/deenvelopment” model (Wild *et al.* 2005; Mettenleiter *et al.* 2006). The nuclear pore egress model was proved for Bovine herpesvirus 1 (BoHV-1) (Wild *et al.* 2005). According to this model, the capsids that are present in the nucleus can attain the cytoplasm *via* a previously enlarged nuclear pore. These free cytoplasmic capsids bud in vesicles derived from the Golgi apparatus and the enveloped virions are released at the surface of the cell (Wild *et al.* 2005). The second model proposed, the « luminal » model, suggests the transport of the enveloped virions from the nucleus by a secretion pathway which maintains the integrity of the envelope acquired earlier at the level of the inner nuclear membrane. This model implies *in situ* modifications of the envelope’s proteins (Darlington and Moss 1968; Johnson and Spear 1982; Campadelli-Fiume *et al.* 1991). The enveloped viruses are finally released in the extracellular environment (Roizman and Taddeo 2007). However, the most likely model is the envelopment/deenvelopment one, first suggested by Siminoff and Menefee in 1966 as a part of HSV-1 (Herpes simplex 1) morphogenesis and then confirmed by electronic microscopy (Stackpole 1969). According to this model, viral capsids in the nucleus bud at the internal nuclear membrane. Pre-enveloped viruses are then localized between the inner and the outer nuclear membrane. It was demonstrated that nuclear actin filaments are used to mobilize HSV-1 viral capsids (Forest *et al.* 2005). The primary envelope acquired during budding through the inner nuclear membrane is then lost because of the fusion with the external nuclear membrane. This leads to the release of free nude capsids into the cellular cytoplasm (reviewed in: Mettenleiter 2002; Mettenleiter 2004; Mettenleiter *et al.* 2006; Mettenleiter *et al.* 2009). Capsids then transit in the cytoplasm to acquire, on one hand, tegument proteins and, on the other hand, envelope glycoproteins, by budding in Golgi apparatus vesicles. Mature virions are then released at the surface of the cell by exocytosis (Mettenleiter 2006; Mettenleiter *et al.* 2006).

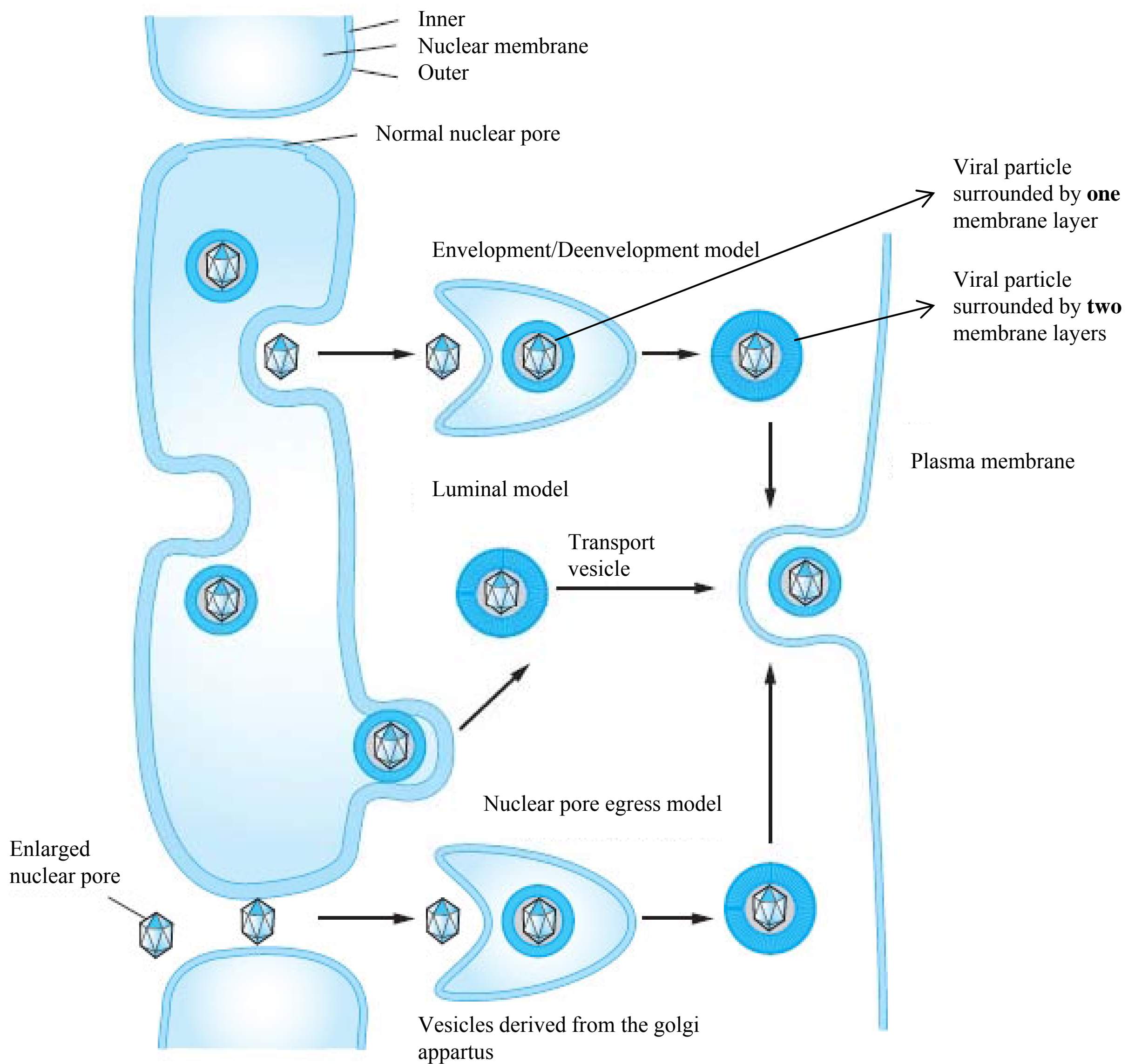


Figure 3: Models for herpesviruses egress from the host cell. 3 pathways are proposed. For the envelopment/deenvelopment model, capsids undergo primary envelopment at the inner nuclear membrane, are deenveloped at the outer nuclear membrane, reenveloped at cytoplamics membrane and then transportated to plama membrane by vesicles. The vesicular memebrane fuses with the plasma membrane to release the virion in the extracellular space. According to the luminal model, capsid is enveloped at the inner membrane and enters a vesicle at the outer nuclear memebrane. The vesicle traffics in the cytaplasm to reach plasma membrane where the virion is released. Finally, in the nuclear pore egress model, capsids exit the nuclei by a nuclear pore, bud into cytoplasmic vesicles to acquire the envelope and are released at the plasma membrane. Adapted from Roizman *et al*., 2007.

1.2.2. The latent infection

The ability to establish a latent state of infection is a fundamental and common characteristic of all herpesviruses (Roizman 1996). This state is described as the maintaining of the virus in the host cell in absence of a productive cycle (Figure 2B). During latency, the viral genome is maintained in the cellular nucleus in a circular form called episome. For the gammaherpesviruses, the viral episome is associated with the cellular genome (Vogel *et al.* 2010). For the rhadinoviruses, the mechanism implies LANA protein or orthologs (Garber *et al.* 2002; Fejer *et al.* 2003). This protein is highly preserved and bifunctional: the N-terminus links to cellular chromatin, whereas the C-terminus interacts with different sequences present in the polyrepetitive DNA (prDNA) units located at the ends of the viral genome (Griffiths *et al.* 2008). This interaction allows the initiation of the viral DNA replication by cellular enzymes and the anchoring of the viral episome to the cellular chromosome (Piolot *et al.* 2001; Ohsaki and Ueda 2012). When the cell is dividing, this anchoring allows the random distribution of episomes between daughter cells, but also avoids their loss in the cellular cytoplasm.

The molecular mechanisms governing the initiation of latency are not well known (Flint *et al.* 2000). However, the establishment of latency always induces a drastic limitation of viral gene transcription associated or not with the production of viral proteins. Thus, a low level of α or β genes can occur but is not sufficient to initiate a productive infection. Alphaherpesviruses only express LATs transcripts (*latency associated transcripts*) (Jones 2003), but beta- and gammaherpesviruses express latency proteins (Lee *et al.* 1999; Ballestas and Kaye 2001; Cardin *et al.* 2009). Maintaining this state on a long term requires the existence of specific and evolved immunoevasion mechanisms allowing the virus to escape the host immune surveillance and persist. The mechanisms implied will be addressed later in this chapter. Lastly, recent studies have demonstrated the existence of miRNA (micro RNA), produced from latency-associated transcripts, for members of all the three subfamilies of herpesviruses (Pfeffer *et al.* 2005). Essentially, their role seems to be in the helping of the maintenance of latency by modulation of cellular immunity and cellular apoptotic pathways, but also by the restraining of the viral lytic cycle (Cai *et al.* 2005; Burnside *et al.* 2006; Lu *et al.* 2008; Umbach *et al.* 2008; Wang *et al.* 2008)

Following an exogenous stimulus, the latency state can be interrupted. Indeed, physiological changes in the cell may provide the needed permissiveness to the cell, allowing it to support a productive infection. The viral genome is then transcribed with more efficiency and is replicated leading to the production of new virions. To date, little is known about mechanisms and stimuli causing efficient viral reactivation of gammaherpesviruses. However, several studies suggest a

role for TLR (*Toll-like* receptor). Indeed, these receptors have a crucial role concerning innate immunity and are able to recognize specific patterns, named PAMP's (pathogen associated molecular patterns), and to provoke a rapid immune response toward pathogens that have induced their activation. Several recent studies highlighted the reactivation of herpesviruses following the activation of TLR's. For example, in 2009, Gargano *et al*. demonstrated that the stimulation of the TLR 3, 4, 5 and 9 by their specific ligands increases the MuHV-4 viral load in mice at 42 days post infection (Gargano *et al.* 2009). In the same way, the activation of the KSHV's lytic genes transcription was observed when the TLR 7 and 8 were stimulated (Gregory *et al.* 2009). Although this mechanism is probably not the only one implied, this data suggests a strong link between innate immunity stimulation and the reactivation of the viral lytic cycle of the gammaherpesviruses. Moreover, the final differentiation of B cells infected by MuHV-4 or EBV into plasmocytes seems to be a signal for the efficient reactivation of these two viruses (Laichalk and Thorley-Lawson 2005; Liang *et al.* 2009).

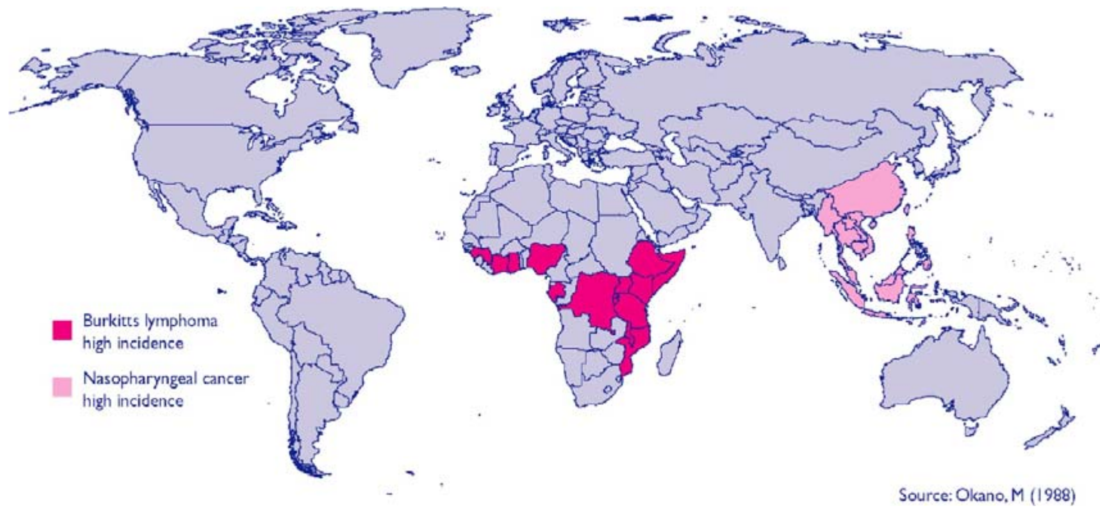
2. The *gammaherpesvirinae*

The *gammaherpesvirinae* are able to infect a wide range of mammals and birds. Among these viruses, some are particularly interesting in terms of animal health, human health or fundamental research. *Alcephaline herpesvirus 1* (AlHV-1), *Ovine herpesvirus 2* (OvHV-2), *Equine herpesvirus 2* (EHV-2), *Bovine herpesvirus 4* (BoHV-4), *Murid herpesvirus 4* (MuHV-4), as well as the human gammaherpesviruses, EBV and KSHV, are certainly the most studied.

AlHV-1 and OvHV-2 are particularly interesting viruses in terms of host specificity. Indeed, even if apparently apathogenic when infecting their natural host, respectively wildebeest and sheep, they are able to infect species as diverse as cattle, swine, but also lagomorphs, rats and hamsters, inducing in these dead-end hosts profound dysregulation of the immune system. (Russell *et al.* 2009). Thus, these two viruses cause a syndrome that is often lethal, known as "MCF" or "malignant catarrhal fever" in sensitive species such as cattle. This clinical entity, with a mortality rate of over 50%, is characterized by high fever and persistent lymphoproliferative lesions reaching all the mucous membranes of the anterior respiratory and digestive tracts, blood vessels and lymphoid organs. Represented by two similar forms of the disease (the African form (AlHV-1) and the European form (OvHV-2)), malignant catarrhal fever has been described clinically in 33 species of domestic and wild ruminants (Metzler and Burri 1990) and occurs on all continents (Mushi and Rurangirwa 1981).

EHV-2 (from the genus *Percavirus*) is also a very well host-adapted virus. With high prevalence, the infection by EHV-2 is distributed worldwide and was isolated from healthy individuals as well as from sick animals, raising the controversial issue of its pathogenicity. While EHV-2 may

A.



B.

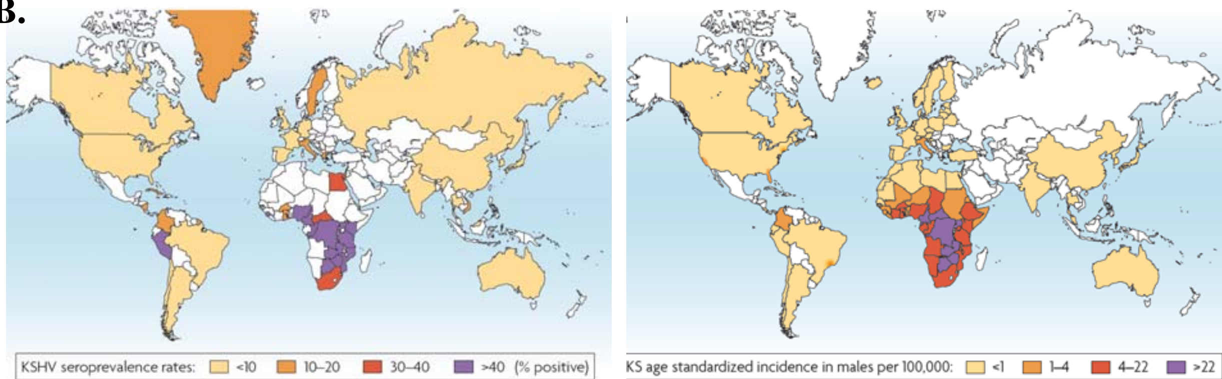


Figure 4: (A) Incidence of Burkitt's lymphoma and nasopharygeal carcinoma associated with EBV infection. From: http://www.cancerresearchuk.org/ (B) Seroprevalence of KSHV and incidence of the associated Kaposi's sarcoma**.** From Mesri *et.al*, 2010.

play a role as reactivator of other equine herpesviruses, this virus has been associated with some upper respiratory diseases, hyperthermia, an overall decline of general health and some forms of keratoconjunctivitis (Dunowska *et al.* 2002; Ruszczyk *et al.* 2004). EHV-2 infection is not strictly limited to domestic horses since the virus was isolated from two other species within the genus Equus, the Przewalski horse and mountain zebra (Borchers *et al.* 1999) while the experimental infection of mice has highlighted the persistence of the virus in the lungs and spleen cells (Rizvi *et al.* 1997; Borchers *et al.* 2002).

BoHV-4 was isolated from diverse samples from healthy cattle presenting various pathologies such as endometritis, abortion, respiratory or digestive troubles and mammary skin lesions (Donofrio *et al.* 2005). It was also demonstrated that African buffaloes can be infected, reflecting the possibility of inter-species transmission (Dewals *et al.* 2005). However, little studies allowed the experimental reproduction of the clinical disease and the role of BoHV-4 as a pathogenic agent remains, for now, uncertain (Thiry 1989). Although cattle have always been considered as the natural host of BoHV-4, the virus probably originates from African buffalo (Dewals *et al.* 2005). However, the virus is able to infect a large diversity of species such as guinea pigs and rabbits. The latter of these species is actually considered as the best model in experimental *in vivo* studies of BoHV-4

The two important gammaherpesviruses in human health are EBV and KSHV. EBV belongs to the *lymphocryptovirus* genus and was indentified about fifty years ago in cells isolated from Burkitt’s lymphoma (BL) that are B cell derived tumors (Epstein *et al.* 1964). From an epidemiological point of view, it is estimated that about 90% of the adult population is infected (Henle *et al.* 1969; Andersson 2000) (Figure 4A). Primary infection can be asymptomatic, but can also lead, to a clinical entity named infectious mononucleosis (IM). The clinical signs and the gravity of IM can be highly inconstant (Thorley-Lawson and Gross 2004). The acute phase is characterized by cervical lymphadenopathy, fever and pharyngitis followed by general sickness and acute fatigue that can last several months (Callan *et al.* 1996). The host immune system is then able to control the infection, essentially by $CD4^+$ and $CD8^+$ T cells. Therefore, a homeostasis state between the host and the virus is set up and the virus will persist in the host organism in a latent state throughout its life. During this period, T cell surveillance is necessary for the control of the infection as evidenced by the development of lymphoproliferative disorders in patients receiving immunosuppressive therapies (Rickinson and Kieff 2001). Moreover, in these patients, but also in those with AIDS (Aquired ImmunoDeficiency Syndrom), neoplasic diseases such as Burkitt’s lymphoma, Hodgkin’s lymphoma or nasopharyngeal carcinoma can be developed (Rickinson and Kieff 2001).

In vitro, EBV infects B lymphocytes and almost always causes transformation of these cells into proliferative lymphoblasts (LCL, lymphoblastoïd cell lines) (Diehl *et al.* 1968; Pope *et al.* 1968). This effect depends on the expression of viral proteins during latency. The expression profile of the genes responsible of malignant transformation is called the « *growth program* ». The study of LCL cells has allowed the evidencing of a limited set of genes expressed during the infection *in vitro*. Thus, six nuclear proteins are described: *Epstein Barr nuclear antigen 1*, (EBNA-1), EBNA-2, EBNA3A, EBNA3B, EBNA-3, EBNA-LP. Four membrane proteins are also described : *Latent membrane protein 1* (LMP1), LMP2A, LMP2B, BHRF1 and a variable number of non-coding RNA (Rowe *et al.* 2009). Moreover, besides the characterization of the EBV infection in LCL cells, the study of the expression of genes associated with latency in tumoral tissues has shown the complexity of the EBV cycle *in vivo*. Indeed, several transcription programs are used by the EBV to infect cells, but also to maintain a long-term infection (Thorley-Lawson and Gross 2004). These programs are characterized by distinct expression of viral and cellular genes.

KSHV was identified in 1994 in very characteristic tumoral lesions, known as Kaposi's sarcoma (KS) and in HIV (Human Immunodeficiency Virus) seropositive patients (Chang *et al.* 1994). Later, it was demonstrated that KSHV is also associated with the development of other lymphoproliferative malignancies such as Castelman's disease and primary effusion lymphoma (PEL), that are rare B cell lymphomas essentially observed in patients with AIDS (Staskus *et al.* 1997; Ensoli *et al.* 2001; Schulz 2001). Cancerous lesions associated with KSHV are at this time the most frequent tumours in patients infected by HIV (Mesri *et al.* 2010). Seroprevalence can reach 50% (Butler *et al.* 2011) in some Sub-Saharan African regions and 10 to 25% in the Mediterranean area. The rest of the world is at low risk with prevalence ranging from 2 to 5% (Chatlynne and Ablashi 1999) (Figure 4B). Historically, four forms of KS are distinguished: the classical form in the Mediterranean region, the epidemic form or the form associated with AIDS, the endemic form in Africa (Oettle 1962) and finally the iatrogenic form in patients under immunosuppressive treatment following transplant (Siegel *et al.* 1969). These forms are different in terms of clinical etiology, with variations in aggressiveness, injured anatomical sites, mortality and morbidity. However, in all of the four forms, individuals are co-infected with HIV and KSHV (Dourmishev *et al.* 2003). In KS lesions, HHV-8 was detected at the level of vascular endothelial cells and in « *spindle cells* » constituting a histological signature of pathology (Staskus *et al.* 1997; Ensoli *et al.* 2001). Other characteristics of the KS lesions are the large diversity of cell types that can be transformed (Regezi *et al.* 1993; Herndier and Ganem 2001) as well as the early and high level of neovascularization (Hanahan and Folkman 1996). Clinical signs evolve from dermal flat lesions to edematous lesions, finally becoming purplish nodules. Although the virus is proven present in transformed endothelial cells, initial target cells are B lymphocytes (Ambroziak *et al.* 1995) as it is the case for the majority of gammaherpesviruses.

During latency, KSHV expresses a small number of genes: the genes coding for the latency-associated nuclear antigen (LANA), for the proteins v-cyclin and v-FLIP (*viral FLICE inhibitory protein*), for Kaposines A, B, C and finally 18 miRNAs (Cai *et al.* 2005; Samols *et al.* 2005). The majority of these proteins are implied in pathogenesis and malignant transformation associated with the infection by KSHV through diverse mechanisms including inhibition of apoptosis, interferrence with the host immune system, angiogenesis and cell cycle manipulation (Moore and Chang 1998; Moore and Chang 2003).

Lastly, while the oncogenic potential of both human gammaherpesviruses is now clearly established, the impact of this process may be underestimated. Indeed, several human tumors, in which no virus is yet isolated, potentially have a viral origin (Shimizu *et al.* 1994; Srinivas *et al.* 1998). This "hit and run" effect suggests that infection with an oncogenic virus can induce genetic instability and/or epigenetic dysregulation responsible for initiation and maintenance of the transformation of the infected cell (Niller *et al.* ; zur Hausen 1999; Pagano *et al.* 2004). Subsequently, the loss of the viral genome does not affect neoplasic progression following the alteration of cellular functions (Niller *et al.* ; Shen *et al.* 1997).

3. The *Murid herpesvirus 4*

3.1. Host range

The MHV-68 strain of MuHV-4 has been isolated from bank voles (*Myodes glareolus*) caught in Slovakia in 1980, and this, concomitantly to the strains 60 and 72 (Blaskovic *et al.* 1980). During the same study, the strains 76 and 78 were isolated in yellow neck mice (*Apodemus flavicollis*) (Blaskovic *et al.* 1980). Recently, very close viruses were isolated in other species such as field voles (*Microtus agrestis*), field mice (*Apodemus sylvaticus*) (Blasdell *et al.* 2003; Hughes *et al.* 2009) and shrew (*Crocidura russula*) (Chastel *et al.* 1994). To date, no consensus exists with regards to the determination of the natural host(s) of MuHV-4. However, from an epidemiological point of view, several field studies were conducted to determine the species which can be naturally infected. In 2003, a study conducted in England showed that seroprevalence was much higher in populations of wood mice in this country (Blasdell *et al.* 2003). Moreover, these results were confirmed in 2007 (Telfer *et al.* 2007) and studies concluded that the most probable natural host for MuHV-4 could be the wood mouse but that this does not exclude that other species could also be natural hosts for MuHV-4. In addition, in 2009, Hughes *et al.* isolated WMHV (Wood mouse herpesvirus) in a wood mouse (Hughes *et al.* 2009). This newly isolated virus is very close to MuHV-4. Consequently it cannot be excluded that natural populations tested during the two studies mentioned above were infected by

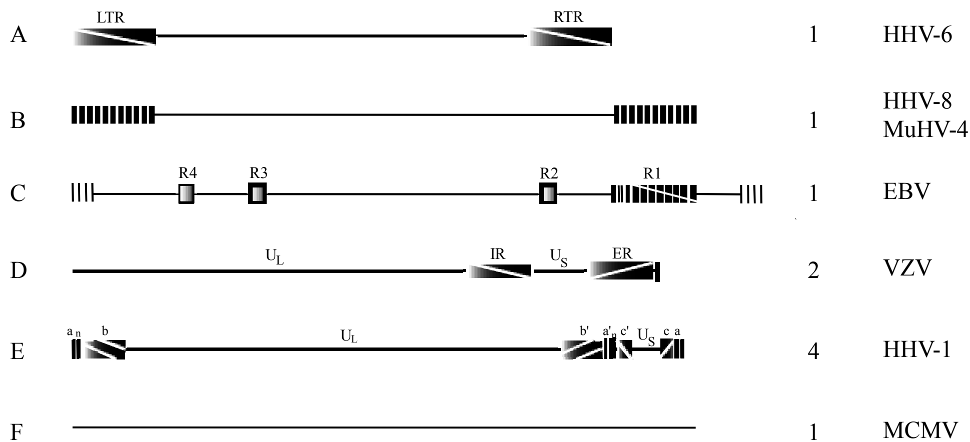


Figure 5: A schematic representations of the six genomic organizations of the viruses of the family *Herpesviridae*. The horizontal lines represent unique or quasi-unique regions. The repeated domains are shown as rectangles and are designated as left and right terminal repeats (LTR and RTR) for group A. In group B, the terminal repeats are reiterated numerous and variable times at both termini. In the case of the group C, the R1 to R4 repeats are internal to the sequence and internal and terminal (IR and ER) repeats are observed for group D. For the group E, the termini is composed of two elements. One terminus contains *n* copies of sequence a next to a larger sequence designated as *b*. The other terminus has one directly repeated *a* sequence next to a sequence designated *c*. The terminal *ab* and *ca* sequences are inserted in an inverted orientation separating the unique sequences into a long (U_L) and short (U_S) domains. Group F genomes do not contain repeat. The components of the genomes in classes D and E invert. In class D, the short component inverts relative to the long. Although (rarely) the long component may also invert, most of the DNA forms two populations differing in the orientation of the short component. In the class E genomes, both the short and long components can invert and viral DNA consists of four equimolar isomers. Adapted from Roizman and Pellet, 2007

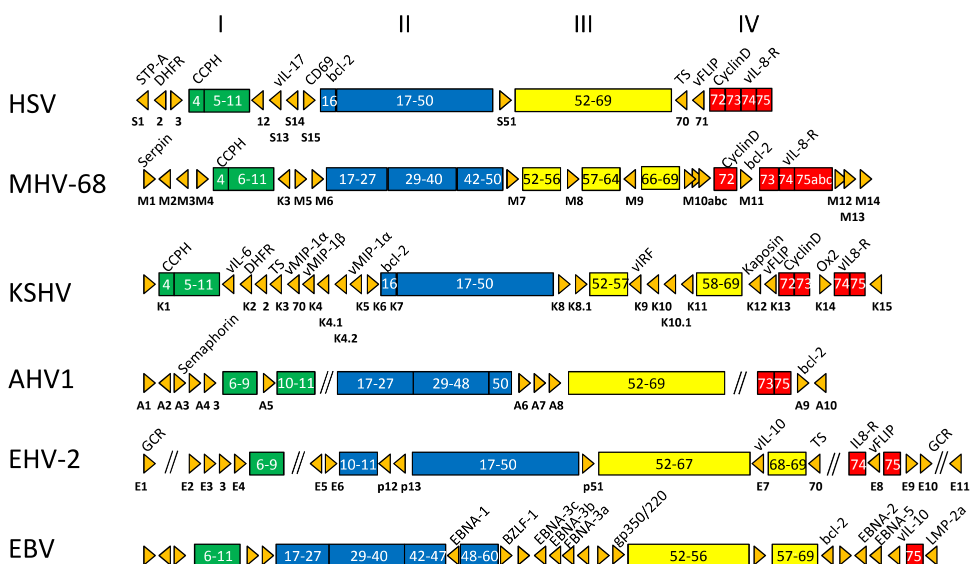


Figure 6: A comparison of the genomic organization of some fully sequenced gammaherpesviruses. As examples, HSV (Herpesvirus saimiri), MHV-68 (*Murid herpesvirus 4*), HHV-8 (*Humam herpesvirus 8* or KSHV), AlHV-1 (*Alcephaline herepsvirus 1*), EHV-2 (*Equid herpesvirus 2*) and EBV (Epstein-Barr virus), are shown. The conserved genome blocks are represented in green, blue, yellow and red and the numbering of the ORF's is relative to the one of HSV. Adapted from Simas and Efstathiou, 1998.

WMHV rather than by MuHV-4. Finally, in 2007, a study designed to identify new herpesviruses in natural rodent populations using PCR (polymerase chain reaction) was conducted by Ehlers *et al.* (Ehlers *et al.* 2007). More than 1,100 samples from rodents caught in the UK, Germany and Thailand were tested, allowing the identification of several new *beta-* and *gammaherpesvirinae*, among which the first gammaherpesvirus naturally infecting house mice (*Mus musculus*), the species from which laboratory mice are derived (Ehlers *et al.* 2007). Although the identification of this new virus opens interesting perspectives concerning the study of a gammaherpesvirus in its natural host, the absence of virus isolation makes it impossible to present any experimental perspective.

3.2. Molecular biology

3.2.1. The viral genome

As already mentioned, MuHV-4 is a gammaherpesvirus belonging to the *rhadinovirus* genus. Rapidly after its isolation, this virus became an essential tool for the study of gammaherpesvirus biology. The genome of MuHV-4 has a B-type structure (Figure 5), and was entirely sequenced in 1997 (Virgin *et al.* 1997). With a length of about 120 kb, the genome of MuHV-4 is composed of a long unique region (118 237 kb) flanked by a variable number of 1.2 kb direct terminal repeats (Efstathiou *et al.* 1990b). The sequence analysis allowed the identification of 80 open reading frames (ORF), 63 of which are homologues of HVS (herpesvirus saimiri), the type species of the genus *rhadinovirus* (Figure 6) (Virgin *et al.* 1997). These 63 ORF's are also present in the genome of KSHV and the majority is present in the genome of EBV. The genome is composed of large blocks of genes conserved among all the gammaherpesviruses between which MuHV-4-specific ORF's are interspersed. These specific ORF's seem to participate in specific biological properties of the virus. This genomic organization, the high positional homology between MuHV-4 and KSHV and the lack of conservation of many proteins involved in the tumoral transformation following EBV infection (Virgin *et al.* 1997) led to the classification of MHV-68 within the *rhadinovirus* genus (Efstathiou *et al.* 1990a). This classification was moreover confirmed later by phylogenetic evidences (Ehlers *et al.* 2008).

The MuHV-4 genome contains 14 unique genes named M1 to M14. The majority of these are only accessory for lytic infection. As an example, the left end of the MHV-68 genome contains the M1 to M4 genes and sequences coding for t-RNAs and micro RNAs (see later), that are absent in the strain 76 isolated simultaneously with the 68 strain. The analysis of the infectivity of the strain 76 in mice has demonstrated that this region is essential for viral pathogenesis as the infection by the strain lacking this region is more rapidly controlled in the lungs and latency is less efficient. Moreover, a

recombinant virus made by the 76 strain complemented with the left end of the 68 strain shows similar infectivity to 68 strain (Macrae *et al.* 2001).

As for all the gammaherpesviruses, numerous cellular homologues are found in the MHV-68 genome. It seems that these DNA sequences were recently acquired at the scale of evolution and are implied in the manipulation of the cellular cycle and the regulation of apoptosis by the virus during the infectious process. For the KSHV, at least 12 ORF's encode proteins with cellular homologues. Concerning MuHV-4, this number is reduced to 4 proteins : a homologue (ORF 4) of a complement regulatory protein whose role could be the inhibition of complement-dependent lysis; a D-cyclin homologue (ORF 72) able to interact with the cyclin dependent kinase 6 (cdk6) and to a lesser extend with cdk4, leading to the alteration of the cell cycle; a receptor for the interleukine 8 (IL8) whose KSHV homologue is known to be implied in tumourigenesis and neoangiogenesis and finally a homologue of the cellular gene *bcl-2* (M11 gene) whose function would be to improve the survival of infected cells, ensuring the maintenance of a pool of latently infected cells (Virgin *et al.* 1997).

The genome also contains eight t-RNA (transfert RNA) type sequences localized at the 5' terminus (Bowden *et al.* 1997; Virgin *et al.* 1997). The role of these RNA is not clearly defined but it is interesting to note that they are abundant in germinal centers when viral latency is established. The expression of the t-RNA is therefore a marker of viral latency (Bowden *et al.* 1997). It is however speculated that those vt-RNA sequences are evolutional remnants serving as promoter for viral microRNA (miRNA) sequences (Zhu *et al.* 2010). Indeed, the expression of viral microRNA during latency was recently demonstrated for members of the three families of herpesviruses (Pfeffer *et al.* 2005). These were proposed to be implied in both lytic and latent infection, possibly acting on viral/host interaction (Sullivan and Ganem 2005; Nair and Zavolan 2006). Pfeffer et al. have predicted 17 miRNAs encoded by MuHV-4, 9 of which were experimentally confirmed (Pfeffer *et al.* 2005). More recently, Zhu *et al*. have systematically analyzed the expression profile of RNAs in lytically and latently MuHV-4 infected cells (Zhu *et al.* 2010). Their results show increased level of miRNAs in latently infected cells in comparison with levels observed in lytically infected cells. Thus these RNAs seems to act positively on viral latency by modulating apoptosis cellular pathways, immunity and repression of viral lytic cycle (Cai *et al.* 2005; Burnside *et al.* 2006; Lu *et al.* 2008; Umbach *et al.* 2008; Wang *et al.* 2008; Lei *et al.* 2010; Forte and Luftig 2011).

3.2.2. Tools

Making great advances in fundamental and applied research about herpesviruses often requires genetic manipulations such as transgene insertion or ORF deletion. While these two types

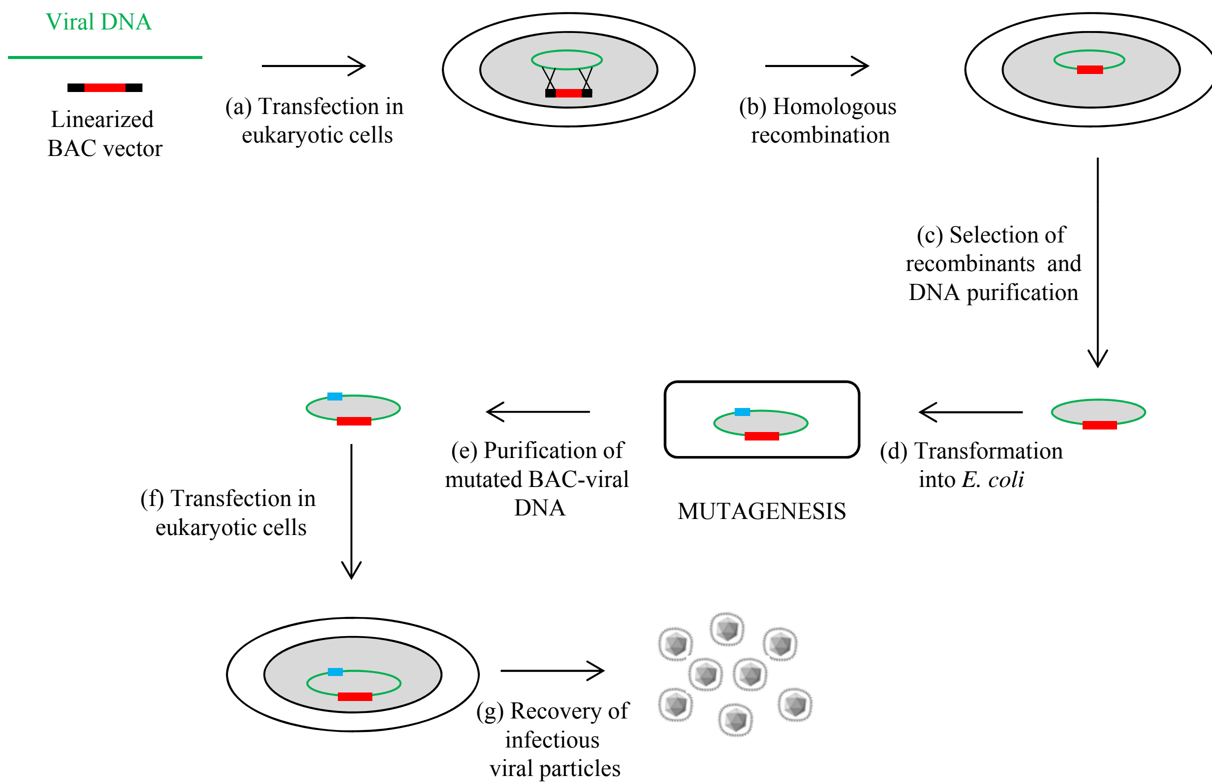


Figure 7: Schematic representation of the generation of a viral BAC-genome. (a) The viral genome and a linearized baterial plasmid containing the BAC cassette surrounded by homologues sequences to viral genome are co-transfected in eukaryotic cells. (b) Homologous recombinaison occurs and viral genomes containig BAC cassette are naturally circularized. (c) Viral genomes containing BAC cassette are selected using a selection marker and viral DNA is extracted from selected plaques. (d) BAC-containing viral genome is used to transform baterial cells. (e) BAC-genome can be mutated by various methods (here the mutation is represented in blue) and isolated from bateria. (f) Viral BAC-DNA may be tranfected in eukaryotic cells and (g) infectious viral particles are recovered.

of modifications can be made by homologous recombination in eukaryotic cells, the approach is extremely long and difficult due to the large genome of herpesviruses and their relatively slow kinetics of replication (Adler *et al.* 2003). Furthermore, with this strategy, genome analysis of the mutant virus is only possible after the experimental procedure, revealing only later accidents such as unwanted genetic recombination, deletions or rearrangements. On the other hand, the selection of target mutants is also difficult. To overcome all these constraints, a new approach for constructing herpesvirus mutants has been developed. The principle of it is based on the cloning of the viral genome as a bacterial artificial chromosome (BAC) in *Escherichia coli* (*E. coli*). The maintenance of the viral genome under the form of a bacterial chromosome in prokaryotic cells allows the researchers to apply well known and controlled mutagenesis methods, using bacterial or phage recombinases, and greatly simplifies genetic modification and selection of recombinants.

To generate a BAC-cloned genome (Figure 7), a BAC cassette composed of genetic sequences needed for DNA replication and segregation in prokaryotic daughter cells as well as selection markers and often *loxP* sites surrounding the cassette is inserted in the viral genome. Infectious viral particles are obtained following transfection of the BAC genome in eucaryotic cells able to support viral lytic infection. The virions therefore contain the BAC cassette which can be removed, if wanted, by the action of *Cre* recombinase on the specifically recognized *loxP* sites (Zhang *et al.* 1998; Wagner *et al.* 2002; Warden *et al.* 2011). Alternatively, the BAC cassette can be excised by the presence of homologous sequences, promoting recombination. This technique of self-excision leads early in the process of virus-reconstitution to viral genomes rid of the BAC cassette thus shorter and therefore preferentially encapsidated (Wagner *et al.* 1999). Excision of the BAC cassette can be an important step, particularly in the context of *in vivo* experiments. Indeed, the presence of these complementary sequences but also the transcription of encoded proteins, mainly the selection markers, may alter viral infectivity in mice by eliciting specific immune response againgt the transgene (Adler *et al.* 2001; El-Gogo *et al.* 2008).

The first BAC cloning of the genome of a herpesvirus was made in 1997 with the cloning of murine cytomegalovirus by Messerle *et al.* (Messerle *et al.* 1997). Today, numerous genomes of herpesviruses have been cloned as BAC (reviewed in Warden *et al.* 2011) : all the human herpesviruses except HHV-7, and also a lot of animal herpesviruses such as BoHV-4 (Gillet *et al.* 2005) or AlHV-1 (Dewals *et al.* 2006a). MuHV-4 was also BAC-cloned in 2000 (Adler *et al.* 2000). Today, a lot of recombinant MuHV-4 viruses are available and the majority has been obtained from the BAC-cloned genome. Subsequently, many studies, both *in vitro* and *in vivo,* were permitted by this molecular tool that has now become indispensable for the specific study of the involvement of specific genes in the pathogenesis of the herpesviruses.

Having this molecular cloning tool has also opened many perspectives in terms of gene therapies and vaccination strategies. In this context, BAC-cloning and mutagenesis methods are constantly evolving with the development of new strategies allowing the rapid construction of vaccinal and therapeutic vectors. For the purpose of gene therapies, viral vectors contain all genes needed for viral replication but lack those needed for virulence. Such HSV-1 (Marconi *et al.* 2009) based vectors have been developed with hopes in treating cancers, (Kuroda *et al.* 2006; Terada *et al.* 2006) and osteoporosis (Xing *et al.* 2004). EBV vectors have also been developed in the context of gene therapies (Magin-Lachmann *et al.* 2003; Hettich *et al.* 2006). In addition, these methods are very useful in fundamental research.

3.3. Pathogenesis of the MuHV-4 infection

MuHV-4 infects and establishes a chronic life-long infection in laboratory mice. The natural way of infection is not known but it is usually considered that the upper respiratory tract should be the most probable entry site. Indeed, a comparative study of intranasal and intravenous infections has proven that the intranasal infection was the most likely to produce clinical signs associated with the infection while representing a more natural way of contamination (Sunil-Chandra *et al.* 1992). Other studies have tested the potential of intraperitoneal, subcutaneous, intracerebral and oral infections. By all these routes, MuHV-4 is able to infect the host, proving the ability of the virus to infect several anatomical sites due to a large tropism for diverse epithelial and fibroblastic cell lines. Regardless of the route of inoculation, B cell infection is a common feature but bypassing the epithelial barrier (as with intraperitoneal infection) leads to more severe disease and seems to make some genes accessory (Jacoby *et al.* 2002). Circulating latently infected cells can theoretically lead to the infection of any other anatomical site explaining the relative equivalence of all routes of infection. The classical experimental infection is intranasal and leads to viral replication in the nasal mucosa during primary infection (Milho *et al.* 2009). The replication then continues in the lungs, causing severe interstitial pneumonia associated with leukocytes in perivascular and peribroncheolar infiltrates. The primo-infection is largely controlled within 10 to 14 days post-infection (Sunil-Chandra *et al.* 1992). Interestingly, no replication is observed in the lungs when the animals are infected without anesthesia (Milho *et al.* 2009). This strongly indicates that replication in the lungs should probably not be considered as a part of natural infection. Simultaneously to the productive infection, latency is established in lymphoid organs, essentially in the spleen and the superficial cranial lymph nodes (SCLN) (Milho *et al.* 2009). Acute infection of these organs is controlled within 14 to 16 days and latency is considered established within 16 to 18 days post-infection. Clinical signs are similar to those observed in the case of an IM following the infection by EBV: lymphadenomegaly, splenomegaly, weight loss, and in the case of mice, dorsal curvature and ruffled fur (Sunil-Chandra *et al.* 1992).

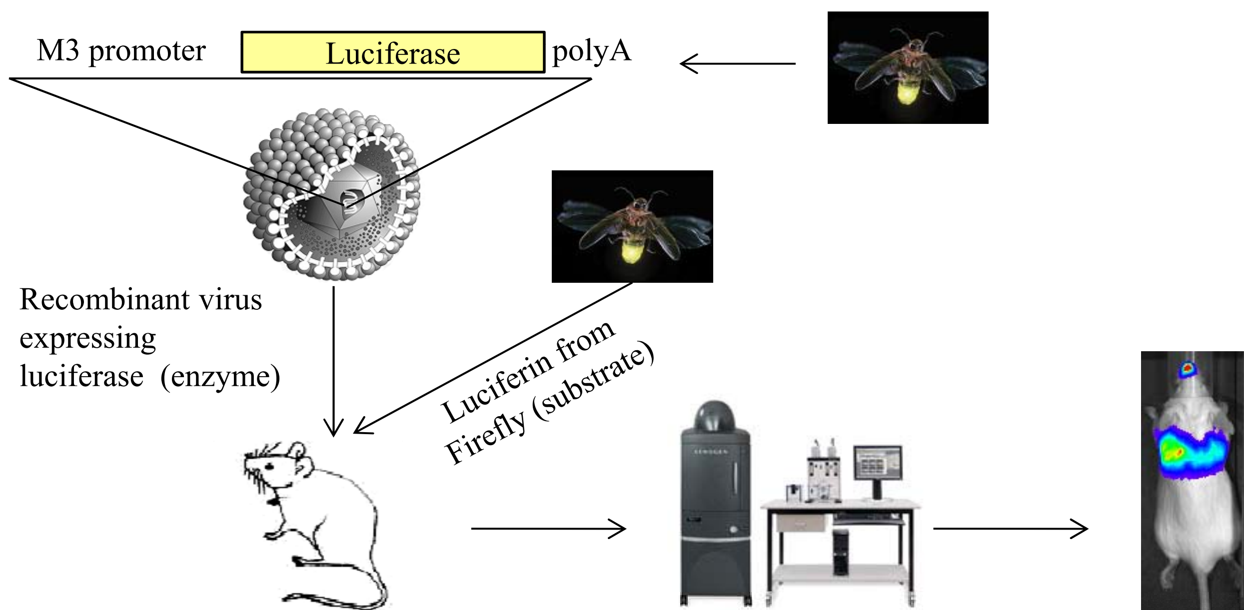


Figure 8: *In vivo* imaging principle (IVIS, *In vivo* imaging system). A recombinant virus expressing luciferase from *Photinus pyralis* under the control of the promoter of a gene associated with the replicative cycle is used to infect animals. Following intaperitoneal injection of luciferase substrate, luciferin, this substrate is oxydized with emission of photons which are detected by a CCD camera. A photagraph and a luminescence image are acquired and superimposed by the software. A color scale is determined to render intensities and quantitative analyzes can be made.

Although many studies using classical virology methods were performed to bring out MuHV-4 pathogenesis, new insights were made by the use of *in vivo* imaging. Indeed, the existence of the MuHV-4 genomic BAC-clone has allowed the construction of a recombinant virus expressing firefly (*Photinus pyralis*) luciferase (Milho *et al.* 2009). This enzyme oxidizes its specific substrate, luciferin, with emission of a photon during the reaction (Figure 8). This mechanism, causing the luminescence of fireflies, is now advantageously used in biology, among others, in order to study infectious processes (Hutchens and Luker 2007). Another important advantage of this method is that the substrate needed for the enzymatic reaction is easily available and can be injected *in vivo* without damaging the animal (Hutchens and Luker 2007) but with an excellent biorepartition and bioavailability in the organism. Also, this method drastically limits the number of animals needed for experiments while minimizing experimental bias caused by the analysis of different groups of animals during kinetics experiments. However, the oxidation reaction needs oxygen and is therefore limited in most anaerobic organs such as the gut. For the MuHV-4 recombinant, the luciferase coding gene was cloned under the dependence of the promoter of the M3 gene (Milho *et al.* 2009), associated with the lytic phase of the viral cycle. Is has to be noted that a similar recombinant was made by another group (Hwang *et al.* 2008). After infection with a luciferase expressing recombinant virus, the infectious process can be monitored continuously using an imaging system composed of a charged coupled device (CCD) camera able to detect photons emitted from the animal. This technique permitted further elucidation of the lytic infection in model animals allowing for example the observation of replication in the nasal mucosa (Milho *et al.* 2009).

3.4. The immune response and the control of the infection

Gammaherpesviruses are the archetype of persistent viruses. The co-evolution of these viruses with their natural host(s) led them to develop near-perfect adaptation between infectivity and long-term persistence. Thus gammaherpesviruses have developed immune evasion strategies from the innate response but also from the adaptative response that allow their persistence in the host and their re-excretion despite the presence of specific antibodies. This ability to evade the immune response is also responsible for the concomitant presence of several related viral strains within the same host organism. Indeed, the host immune response is so limited and circumvented that a close second strain may be the source of infection within an immunized host (Sitki-Green *et al.* 2003; Gorman *et al.* 2006; Muylkens *et al.* 2009). This highlights the difficulty in developing efficient vaccines which induce a sufficient immune response, *in fine* allowing the control of the herpesvirus infection.

Moreover, it is recognized today that the induction of long-term neutralizing response is one of the main antiviral mechanism (Burton *et al.* 2005; Hangartner *et al.* 2006). Therefore, the development

of vaccines against persistent viruses proved to be a major challenge as these viruses have evolved by co-speciation, adapting to their host in order to coexist with the antibody response and resist neutralization (Burton *et al.* 2005). At present, the understanding of the mechanisms of evasion of gammaherpesviruses toward the neutralizing response is very limited. The development of effective vaccine strategies is also complicated by other factors such as low-antigen expression during latency, the manipulation of the elements involved in antigen presentation to the immune system, a tropism that is not confined to a single cell type, the establishment of latency within immune cells in the case of gammaherpesviruses and finally the few available *in vivo* models allowing a detailed study of mechanisms of interaction between the virus and the host immune system. These elements will be discussed in this section.

In this context, the study of MuHV-4 in the laboratory mouse is very useful. Indeed, we can say that the immune response of laboratory mice has many similarities with that of humans and secondly, a majority of the genes involved in gammaherpesvirus latency was found in MuHV-4. However, it has unique genes but their function appears to be preserved. Thus, the assumption of common strategies for pathogenesis and escape of the immune system can be emitted (Barton *et al.* 2011).

The control of the gammaherpesvirus lytic infection is primarily provided by the cell-mediated immunity and particularly by CD8 + cytotoxic T lymphocytes. Indeed, the end of pulmonary lytic infection coincides with a peak of virus-specific CD8 + T cells (Stevenson and Doherty 1998) and depletion of these cells prior to infection leads to uncontrolled lytic infection and death (Ehtisham *et al.* 1993). However, the gammaherpesviruses have evolved to considerably limit the recognition of their essential epitopes, lytic as well as latent, by the host immune system. In the case of MuHV-4, it was shown that CD8 + T cells, although able to proliferate massively following the viral challenge after previous infection, are unable to control chronic lytic infection (Belz *et al.* 2000). Tests of pre-exposure vaccination designed to increase the efficiency of these CD8 + T cells and to limit the colonization of the infected host have failed (Liu *et al.* 1999; Stevenson *et al.* 1999a). This shows the high capacity of the virus in the escape of the immune system in order to establish an effective state of latency. At a molecular level, there are several genes involved in this process. Among these, the K3 gene product, acts predominantly during the lytic phase to inhibit the presentation of viral antigens in the context of class I major histocompatibility complex (MHC) thus limiting the recognition by CD8 + T cells and limiting the effect of the cytotoxic response (Stevenson *et al.* 2000). This effect is related to the ability of the K3 protein to bind to neoformed molecules of the MHC class I in the endoplasmic reticulum, inducing an ubiquitination complex and its subsequent degradation by the proteasome (Boname and Stevenson 2001). Escaping the CD8 + T cell response is also important during latency.

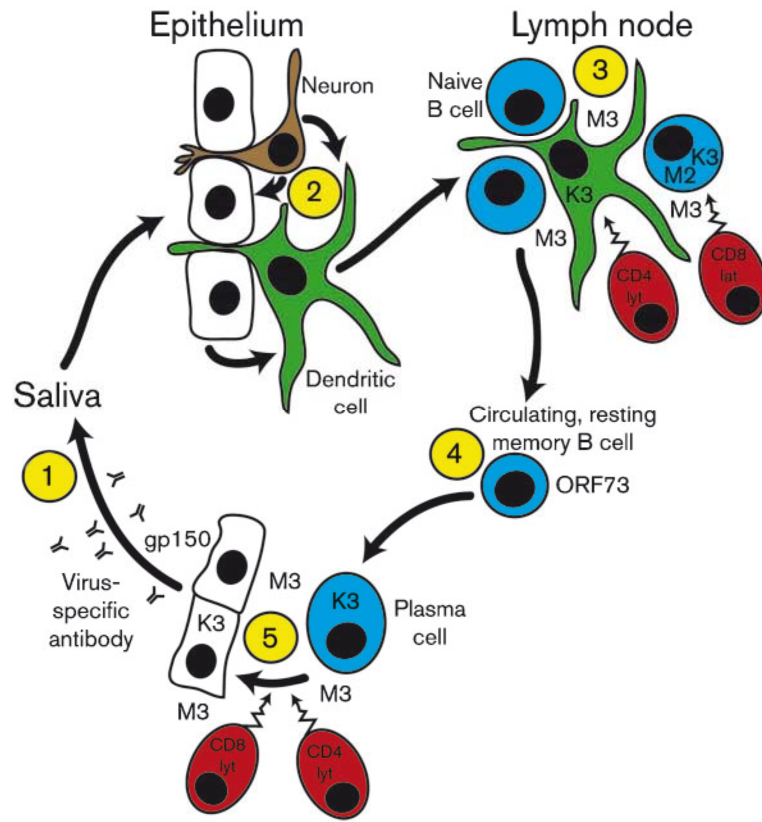


Figure 9: A schematic view of the gammaherpesvirus life cycle for MuHV-4. T cells are shown in red, B cells in blue, epithelial cells in white, neuronal cells are brown and relevant others are in green. Arrows show the movement of virus. Numbers 1–5 indicate definable steps. Key intervention points are latency establishment in naive hosts (steps 2 and 3) and antibody binding to the virions shed by carriers (step 1). Step 1: virions enter a naive host via secretions such as saliva. Since virions come from immune carriers, they are likely to have attached antibody. The virions are not normally neutralized, but neutralization may be possible through boosting fusion-complex-specific antibodies in virus carriers. Nonneutralized MuHV-4 could first infect epithelial cells, IgG Fc receptor-bearing dendritic cells or olfactory neurons. There is no good evidence for direct B cell infection after a non-invasive inoculation. Step 2: local lytic spread could potentially be targeted by antiviral drugs or antibody. Latency establishment seems to occur mainly in draining lymph nodes. Infection may reach these via dendritic cells or via cell-free virions captured by subcapsular sinus macrophages. Step 3: infection next spreads to B cells, which proliferate. Lytically infected myeloid cells secrete evasion proteins such as the M3 chemokine binding protein to protect B cells against CD8+ T-cell attack. K3 protects myeloid cells and perhaps also B cells against CD8+ T-cell recognition. IFN-γ produced by CD4+ T cells may limit M3 production and thereby help latent antigen-specific CD8+ T cells to attack B cells. Step 4: B cells exit germinal centres and differentiate into long-lived, resting memory B cells. Episome maintenance by ORF73 remains below the threshold of antigen presentation. Step 5: virus reactivation probably occurs in submucosal sites, accompanied by B-cell differentiation to a plasma cell phenotype (Sun & Thorley-Lawson, 2007; Wilson et al., 2007). There may be further lytic replication in epithelial cells prior to virion shedding. Lytic antigen-specific CD8+ T cells can potentially inhibit reactivation, but K3 and M3 limit their impact. Consequently, CD4+ T cells seem to protect better against lytic spread. Gp150 promotes virion release and helps to limit neutralization. From Stevenson *et al.,* 2009.

Hence, the M3 protein of MHV-68, that has no counterpart in other gammaherpesviruses, is a chemokine-binding protein (CBP) with a wide spectrum and whose role would be to limit CD8 + T cell migration to sites of latency-antigen expression (Parry *et al.* 2000; van Berkel *et al.* 2000). M3 is expressed during the lytic phase of the infection (Martinez-Guzman *et al.* 2003). In the absence of the M3 protein, amplification of latency is dramatically reduced but can be largely restored after depletion of CD8 + T cells (Bridgeman *et al.* 2001). M3 acts to protect infected B cells from the host's immune system but the way by which this is realized remains unclear as of yet (Stevenson and Efstathiou 2005). We can also mention the LANA protein, present in the MuHV-4 (ORF 73) and in the KSHV, which has a functional homologue in EBV: EBNA-1. This protein, besides its crucial role in maintaining the viral genome as an episome in latently infected cells, significantly limits the expression of viral genes, slows the lytic phase and inhibits epitope presentation during episome maintenance (Bennett *et al.* 2005; Li *et al.* 2008; Wen *et al.* 2009).

CD4 + T cells are also important for the control of infection. Indeed, CD4 + T cells also display a role in the control of the acute phase of infection by MuHV-4. Assuredly, these cells can act as a weapon for the development of a cytotoxic immune response type, but also have a supervisory role by themselves. Therefore, infection of mice deficient in B cells and depleted of CD8 + T cells may be controlled by the action of CD4 + cells. This mechanism appears to depend on the induction of interferon (IFN) production, IFN-γ mainly. Indeed, inhibition of this molecule in these same animals led to a complete loss of control of infection by the organism (Christensen and Doherty 1999). Furthermore, infection of animals that were deficient for the production of IFN-γ led to a chronic lytic infection. Thus, IFN-γ is crucial in the limitation of replication and potentially in that of viral reactivation (Dutia *et al.* 1997; Weck *et al.* 1997).

Finally, a model was suggested to explain the balance between the host's immune system and viral evasion (Figure 9). In this model, it is put forward that tissue damage caused by the lytic phase leads to an acute inflammatory process and therefore to a response during which the CD8 + T cell response is predominant for lytic infection control. During this phase, the host's immune response dominates. Consequently, during the establishment of latency, and during the latency itself, tissue damage is severely limited, as is the inflammation reaction, leading to a dominance of the evasion mechanisms which act by the expression of viral proteins M3 and K3 (Stevenson *et al.* 2002).

4. Epidemiology and transmission of the *gammaherpesvirinae*

Knowledge and understanding of the mechanisms of transmission of herpesviruses in populations are essential to implement large scale antiviral strategies. Indeed, the development of

strategies and/or fully adequate vaccination requires in-depth knowledge not only of the molecular mechanisms governing replication, viral latency, reactivation and re-excretion in a single individual, but also of the mechanisms involved in the dispersion of these viruses at population level, whether human or animal. The association of these diseases with other pathogenesis may also have significant repercussions on prevention of the illness.

In humans, the determination of the mechanisms of transmission at population level is essentially based on sero-epidemiological studies that often involve the interrogation of individuals included in studies. Although this method is probably the oldest source of epidemiological information, many biases may appear in this type of study such as an incomplete questionnaire, the inability to check the accuracy of the answers or a limited or inhomogeneous population sample. At present, molecular biology reinforces these methods allowing for example the assessment of the presence of a pathogen at different anatomical sites. However, access to samples is limited, at least in humans, to body fluids and biopsies.

This section presents a review of current knowledge about the epidemiology and mechanisms of transmission of some gammaherpesviruses of interest in human or veterinary medicine. Indeed, the different gammaherpesviruses share many common properties, whether at a molecular or an evolutionary level, and knowledge of transmission mechanisms involved in the epidemiology of these viruses may therefore be important in the context of the study of another gammaherpesvirus, especially when developing a model of transmission under experimental conditions.

As previously mentioned, two gammaherpesviruses are important in human medicine, namely EBV and KSHV. Associated diseases, but also some epidemiological data have already been mentioned in section 2 of this introduction. This section therefore aims to further explore currently known data concerning the transmission of these two infections.

There are now several studies that tend to demonstrate the presence of EBV in the genital tract and therefore to hypothesize potential sexual transmission. Indeed, by 1986 the presence of EBV in cervical secretions, whether in the form of virions associated with epithelial cells or as free infectious viral particles, was shown (Sixbey *et al.* 1986). Subsequently, many studies have been conducted and have disclosed some interesting elements. Thus, about thirty cases of ulcerative vaginal manifestations associated with EBV have been reported (Halvorsen *et al.* 2006; Leigh and Nyirjesy 2009). Some of these cases were certainly associated with a primary infection by EBV. Indeed, for one, the seroconversion was observed in the month following detection of the ulcerative lesions and secondly, because of the detection by PCR of viral genomes in biopsies of the lesions (Halvorsen *et al.* 2006).

Moreover, a study conducted among women of a rural area of India showed that EBV is detected in genital secretions of about 20% of these women and that it is frequently associated with poor hygiene (for instance, they have no running water), cervical inflammation and genital cancer (Silver *et al.* 2011). Further studies have shown EBV viral particles in male and female genital secretions (Sixbey *et al.* 1986; Israele *et al.* 1991; Naher *et al.* 1992; Thomas *et al.* 2006b). In addition, sero-epidemiological studies conducted on cohorts of university students have correlated EBV seropositivity, history of infectious mononucleosis and the number of sexual intercourses and partners reported (Crawford *et al.* 2002; Woodman *et al.* 2005; Crawford *et al.* 2006). It was also shown that members of a couple often share the same virus isolate (Thomas *et al.* 2006b). However these latest studies do not actually demonstrate sexual transmission as it is not possible to discriminate direct sexual transmission from transmission due to practices associated with sexual intercourse such as kissing. In conclusion, although sexual transmission should be considered as a way of transmission because of the presence of infectious virus in vaginal and urethral secretions, this route does not seem to be the most important, but its incidence remains unclear to date.

KSHV is less prevalent than EBV. Sero-prevalence rates vary greatly depending on the geographical areas considered (see section 2) and are higher among low socio-economic populations, but also among HIV infected individuals (Cannon *et al.* 2001).

A PCR-based detection study of KSHV in body fluids allowed to evidence the presence of the virus essentially in saliva with high titers of viral DNA, but also in the blood, the semen, the skin injured by KS and even in healthy skin (LaDuca *et al.* 1998). The virus was also isolated in the oropharyngeal secretions of homosexuals, but only rarely in samples harvested at the anal and genital regions of the same individuals (Pauk *et al.* 2000). During the studies conducted on biopsies from AIDS patients, prostatic tissues were positive for viral KSHV genomes (Corbellino *et al.* 1996), suggesting that the prostate could be a replication site. KSHV was also shown to be present in the semen and this, often in patients with KS (Diamond *et al.* 1997), sometimes associated with epithelial cells and never associated with spermatozoa (Pellett *et al.* 1999; Ablashi *et al.* 2002). KSHV was also detected in the cervix of some women (Whitby *et al.* 1999). This biological data suggests that sexual transmission should be considered for KSHV. Sero-epidemiological studies were elaborated taking this into account. In 1999, a study conducted on more than 3000 people monitored for more than a year linked the risk of KSHV acquisition to a homosexual or bisexual way of life but not to that of heterosexuals (Smith *et al.* 1999). This was even true in high prevalence areas such as Zimbabwe (Campbell *et al.* 2009). However, in France and Italy, studies tend to show an increased risk of seroconversion of an uninfected partner in a heterosexual relationship with an individual suffering from KS (Brambilla *et al.* 2000; Dupuy *et al.* 2009). Another study has shown that the risk is also

correlated with the number of partners and with infection by HIV (Martin *et al.* 1998). Finally, KSHV was frequently detected in oropharyngeal and cervical secretions of a sample of Zimbabwean women presenting classical KS lesions, but not in the control sample population without KS disease, including women seropositive for KSHV (Lampinen *et al.* 2000). This suggests that sexual or perinatal transmission in high prevalence populations could be linked to the immunodeficient status that allows the shedding at these anatomical sites. This would mean that these transmission routes are probably very limited in immuno-competent individuals (Lampinen *et al.* 2000). High oral and cervical shedding is also correlated with HIV co-infection in Kenya (Taylor *et al.* 2004). In conclusion, despite the highly controversial aspect of the results presented here, it seems that sexual transmission is a major risk of KSHV acquisition among certain populations particularly at risk, but should be very limited in heterosexual and immuno-competent populations (de Sanjose *et al.* 2009).

Mother-to-child transmission, both transplacental or perinatal, seems to be very limited, but could occur in high prevalence areas (Pica and Volpi 2007). However, in women co-infected by HIV, viral reactivation seems to be increased during pregnancy, and this also appears to be the case for perinatal shedding (Lisco *et al.* 2006), but no influence of the infection on the outcome of the pregnancy was observed. Mother-to-child infection exists but seems to be essentially caused by an increase of the mother's perinatal salivary shedding (Dedicoat *et al.* 2004). Additionally, the acquisition of infection during childhood would imply the transmission between young children who shed high levels of viruses in saliva (Plancoulaine *et al.* 2000; Mbulaiteye *et al.* 2006).

The high frequency of association of KSHV and HIV infections, as well as the much more frequent appearance of KSHV-associated diseases such as KS in individuals with AIDS naturally led to evaluate the impact of some factors known to be implied in HIV transmission on the epidemiology of KSHV. Thus, the potency of blood-borne transmission was considered. A study conducted on samples from about 30 infected donnor/healthy patient pairs in West Africa led to the detection of only one case of transmission following the transfusion of a blood sample with high viral load (Gobbini *et al.* 2012). In Uganda, it was shown that the risk of blood-borne transmission from infected donors decreased after 4 days of blood storage (Hladik *et al.* 2006). A similar study but of larger scale was conducted in the U.S.A. revealing no transmission by blood products (Cannon *et al.* 2009). However, another study of lesser extent that used a more sensitive immunofluorescence method showed occasional cases of transmission following blood transfusion in the U.S.A. (Dollard *et al.* 2005). Moreover, the possibility of transmission through needle sharing among populations of injection drug users was also evaluated. A study comparing the seroprevalence of KSHV in a group of intravenous drugs users and a control group has shown that such practices constitute a risk of KSHV acquisition (Sosa *et al.* 2001), regardless of sexual orientation (Atkinson *et al.* 2003). However, another similar

study did not reveal such a correlation and concluded in the dominance of sexual orientation as an important factor regarding the prevalence of KSHV in populations considered at risk (Bernstein *et al.* 2003). Finally it appears that the presence of infectious viral particles in the blood stream greatly depends on the stage of the infection as the massive presence of IgG correlates with a high viral load (Ablashi *et al.* 2002). Globally, this data indicates that the spread by way of blood transmission or needle sharing by drug users can exist but remains of low importance, at least in immune-competent individuals.

AlHV-1 and OvHV-2 are two important gammaherpesviruses in veterinary medicine. As previously mentioned, they are the causative agents of the disease named MCF or malignant catarrhal fever. AlHV-1 is the source of a particularly important epidemiological problem concerning cattle in Africa, but also in the context of zoos hosting many exotic animals. Although the virus and its associated diseases should have been studied for many years, little data actually exists concerning viral transmission. For AlHV-1, it seems that in the natural host, transmission occurs by direct contacts between healthy and infected animals during the first weeks of life. The virus is mostly re-excreted in the nasal and ocular mucosa (Mushi *et al.* 1980; Pretorius *et al.* 2008). An epidemiological study has concluded that the infection is often acquired *in utero* or soon after birth (Pretorius *et al.* 2008). Some serological studies in which geographical elements were taken into account were conducted, mainly in South Africa, over a period of 80 years. The results show that transmission occurs primarily between animals grazing in the same areas, probably *via* viral particles deposited by an infected animal, during parturition as an example, on the grass grazed by a healthy animal. Moreover, it seems that the shedding needed for transmission occurs mainly in young animals that have been recently infected (Barnard *et al.* 1989). However, transmission in the absence of any possibility of direct contact was observed in South Africa, leading the authors to hypothesize transmission through a fly species (Barnard and Van de Pypekamp 1988).

OvHV-2 seems to be predominantly transmitted by nasal secretions from young infected animals (Kim *et al.* 2003). Indeed, a transmission study of the virus was conducted to evaluate the infection ability of samples harvested at the nasal mucosa of sheep, with on one hand recently infected animals and on the other, individuals that had been infected a long time ago (Nishimori *et al.* 2004). The inoculation of naïve animals was realized at the level of nostrils as the virus is thought to infect its host by respiratory route following close contact. Results showed that the efficiency of transmission is a lot higher with nasal swabs from recently infected animals than with those from long-term infected animals, demonstrating shedding in the nasal mucosa of animals undergoing primary infection. Moreover, these results were confirmed during a similar study which also demonstrated that transitory shedding episodes occur in the nasal mucosa of infected sheep (Li *et al.* 2004). All this data indicates

that transmission is primarily executed *via* nasal secretions containing infectious viral particles, often from young and recently infected animals.

BoHV-4 infects cattle and was isolated from various samples harvested from both healthy animals and animals presenting various diseases such as endometritis, abortion, respiratory and digestive problems or even mammary skin lesions (Donofrio *et al.* 2005). This virus is present in European as well as in African, North American or Asian cattle without causing major health damage. Furthermore, experimental infection of cattle causes no clinical signs. The presence of the virus in healthy animals as well as in animals undergoing other pathologies seems to place this virus into the category of secondary pathogens, generating clinical symptoms only in cases of co-infection with other pathogens. These factors result in the low availability of data on the mechanisms of transmission of BoHV-4. However, the virus could be detected in the milk of cows suffering from bacterial mastitis (Kalman et al. 2004), leading to the hypothesis of food-borne transmission to newborn calves. The presence of cell-associated viruses in the milk from infected cows has been confirmed (Donofrio *et al.* 2000). Another study suggests the possibility of *in utero* transmission, as viral DNA was detected in blood samples of calves before colostrum intake. However, during this study, calves were seronegative at birth and showed no symptoms (Egyed *et al.* 2011).

There is less data available concerning the epidemiology and the transmission of MuHV-4. Natural epidemiology has already been evoked (see section 3.1). Regarding transmission, the literature currently available is very limited. However, it was suggested that the virus could be detected in many biological fluids including breast milk and urine (Hricová and Mistríková 2008). This study suggests the possibility of transmission from mother to offspring but also the possibility of transmission through territorial marking behavior. Transplacental transmission was also suggested and Stiglincova et al. published a study in 2011 strengthening this hypothesis (Stiglincova *et al.* 2011). This study also seems to show that the infection, even if it is latent, is the cause of a delay of fetal development as well as of shorter duration of gestation, resulting in fewer births. This study also provided further indications of the presence of virus in breast milk (Stiglincova *et al.* 2011). Globally, available data concerning MuHV-4 transmission are poor and the essential of the epidemiological cycle of the virus in natural population remains unknown.

The diversity of the routes of transmission and the frequent co-existence of multiple routes of dissemination of herpesviruses within their host population makes it relatively difficult to elucidate the preferred mode of transmission of a newly studied herpesvirus. Thus, although assumptions can be made by comparison with other known viruses belonging to the same viral genus, experimental evidence and/or epidemiological studies are needed to determine the existence of a specific mode of

transmission. Indeed, we have evoked that the human gammaherpesviruses seem to be essentially transmitted through re-excretion in saliva, but can also be sexually transmitted and even be associated with genital pathologies. Although current knowledge seems to determine this second mechanism as a secondary route of transmission for gammaherpesviruses, its precise impact remains undetermined so far.

2.

Objectives

Objectives

The MHV-68 strain of MuHV-4 was isolated in 1980 from a bank vole (*Myodes glareolus*) (Blaskovic *et al.* 1980). This virus was rapidly considered of great interest by the scientific community. A lot of studies were subsequently performed in this model, allowing progress in understanding of gammaherpesvirus biology both *in vitro* and *in vivo*. However these three decades of studies have not allowed researchers to evidence the re-excretion or transmission of this virus among laboratory mice populations. To date, this remains the major problem associated with this model, as the capacity to test reexcretion and/or transmission is necessary for the development of new vaccination strategies. **The main objective of this work is therefore to analyze the *in vivo* cycle of MuHV-4 with modern techniques in order to establish an experimental transmission model of this virus.**

Firstly, we want to compare MuHV-4 infection in mice and bank voles. Indeed, given that gammaherpesviruses have co-evolved with their host, they are specifically adapted to their immune system. They may therefore behave very differently when infecting a species which differs from the natural host. Thus, even if the gnu (*Connochaetes taurinus*) and domestic cattle are phylogenetically separated by only 15 to 20 million years (Chaves *et al.* 2005), transmission of the Alcelaphine herpesvirus 1 (which is highly prevalent among populations of gnu), to cattle, leads to a fatal lymphoproliferative disease (Dewals *et al.* 2008). Likewise, bank voles and house mice present a similar phylogenetic distance, estimated at 12 to 19 million years (Kilpatrick 1996). We want therefore to know if MuHV-4 behaves differently in mice and in bank voles. **Secondly**, based on the results obtained in the first part of this work, we will try to develop a model of transmission of MuHV-4 in the experimental conditions that will be judged as the most suitable.

3.

Experimental section

3.1

Comparative study of murid gammaherpesvirus 4 infection in mice and in a natural host, bank voles

Journal of general virology (2010), 91, **2553-63**

S. François, S. Vidick, M. Sarlet, J. Michaux, P. Koteja, D. Desmecht, P.G. Stevenson, A. Vanderplasschen and L. Gillet

Preamble

The MHV-68 strain of MuHV-4, isolated from a bank vole in 1980 (Blaskovic *et al.* 1980), has been especially used as a model of gammaherpesvirus infection for a long time. However, it can be hypothesized that the use of a mouse model of infection is potentially inadvisable given the particularly narrow host range that gammaherpesviruses usually have and the lack of consensus on the natural host(s) of MuHV-4. Subsequently, the determination of potential differences during the MuHV-4 infection of mice (the experimental model), and bank voles (the species from which the virus was isolated), should be particularly informative.

To answer this question, we have designed a comparative study of the infection of *Mus musculus* and of *Myodes glareolus*, using various methods of classical virology but also *in vivo* imaging. This study aimed to evaluate the quality of mice as a model for gammaherpesvirus infection but also to determine the potential of voles as an alternative model for vaccinal and viral transmission studies.

Notably, this has been rendered possible through a collaboration established with Professor Pawel Koteja of the University of Krakow, who brought to us knowledge concerning voles and individuals from his animal husbandry to enable us carry out this study. In the same way, Johan Michaux, of the University of Liège has brought to us his large knowledge about rodent phylogeny but also about vole lifestyle.

The results obtained have been published in the *Journal of General Virology* in 2010 (Francois *et al.* 2010) and are presented in this section.

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Comparative study of murid gammaherpesvirus 4 infection in mice and in a natural host, bank voles

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Gammaherpesviruses are archetypal pathogenic persistent viruses. The known human gammaherpesviruses (Epstein–Barr virus and Kaposi's sarcoma-associated herpesvirus) are host-specific and therefore lack a convenient *in vivo* infection model. This makes related animal gammaherpesviruses an important source of information. Infection by murid herpesvirus 4 (MuHV-4), a virus originally isolated from bank voles (*Myodes glareolus*), was studied here. MuHV-4 infection of inbred laboratory mouse strains (*Mus musculus*) is commonly used as a general model of gammaherpesvirus pathogenesis. However, MuHV-4 has not been isolated from house mice, and no systematic comparison has been made between experimental MuHV-4 infections of mice and bank voles. This study therefore characterized MuHV-4 (strain MHV-68) infection of bank voles through global luciferase imaging and classical virological methods. As in mice, intranasal virus inoculation led to productive replication in bank vole lungs, accompanied by massive cellular infiltrates. However, the extent of lytic virus replication was approximately 1000-fold lower in bank voles than in mice. Peak latency titres in lymphoid tissue were also lower, although latency was still established. Finally, virus transmission was tested between animals maintained in captivity. However, as observed in mice, MuHV-4 was not transmitted between voles under these conditions. In conclusion, this study revealed that, despite quantitative differences, replication and the latency sites of MuHV-4 are comparable in bank voles and mice. Therefore, it appears that, so far, *Mus musculus* represents a suitable host for studying gammaherpesvirus pathogenesis with MuHV-4. Establishing transmission conditions in captivity will be a vital step for further research in this field.

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INTRODUCTION

Gammaherpesviruses have been identified in a range of animals from mice to man (Davison *et al.*, 2009). They establish persistent, productive infections, with virus carriers making antiviral immune responses that protect against disease and continuing to secrete infectious virions. Most gammaherpesviruses establish a long-term latent infection of circulating lymphocytes. They drive lymphocyte proliferation as part of normal host colonization, and this feature of the virus life cycle predisposes the host to neoplastic disease. Such disease can be particularly marked when cross-species transmission occurs, as observed for saimiriine herpesvirus 2, ovine herpesvirus 2 and alcelaphine herpesvirus 1 (Dewals *et al.*, 2006; Fickenscher & Fleckenstein, 2001; Hart *et al.*, 2007).

The best studied gammaherpesviruses are human herpesvirus 4 (Epstein–Barr virus) and human herpesvirus 8 (Kaposi's sarcoma-associated herpesvirus), which are associated with a range of cancers. As these viruses have no well-established *in vivo* infection model, related animal gammaherpesviruses are an important source of information. We have been studying murid herpesvirus 4 (MuHV-4). The archetypal MHV-68 strain was originally isolated from bank voles (*Myodes glareolus*) in Slovakia (Blaskovic *et al.*, 1980) together with four other MuHV-4 strains,

A supplementary figure showing IVIS Spectrum sensitivity in *Myodes glareolus* is available with the online version of this paper.

MHV-60 and -72 also isolated from bank voles and MHV-76 and -78 isolated from yellow-necked mice (*Apodemus flavicollis*). More recently, closely related viruses have been isolated from shrew (*Crocidura russula*) (Chastel *et al.*, 1994) and wood mouse (*Apodemus sylvaticus*) (Blasdell *et al.*, 2003; Hughes *et al.*, 2010b).

Although MuHV-4 has not been isolated from house mice (*Mus musculus*), infection of inbred laboratory mouse strains is commonly accepted as a viable model for studying gammaherpesvirus pathogenesis *in vivo*. Experimental MuHV-4 infection typically employs intranasal virus inoculation under general anaesthesia. This leads to a lytic infection of lung alveolar epithelial cells that is controlled within 2 weeks (Sunil-Chandra *et al.*, 1992). Meanwhile, the virus seeds to lymphoid tissue, mainly draining lymph nodes and spleen (Milho *et al.*, 2009), and drives the proliferation of latently infected B cells. This peaks at 2 weeks post-infection (p.i.) and is controlled by 4 weeks. A predominantly latent infection of memory B cells then persists (Flano *et al.*, 2002). Some inbred mouse strains infected with MuHV-4 tend to develop lymphomas (Sunil-Chandra *et al.*, 1994).

An unresolved feature of the MuHV-4/*Mus musculus* infection model is that virus re-excretion and transmission have not been observed. Whilst this could mainly reflect the restrictions on normal murine social behaviour imposed by conventional housing, it is also possible that the lack of transmission reflects the fact that one or more virus functions necessary for efficient host exit work(s) poorly in carrier mice. The different host species infectable by MuHV-4-like viruses are indeed separated by several millions of years (Fig. 1). In this study, we therefore characterized MuHV-4 infection in bank voles, the species from which it was first isolated, in order to reveal any major defects of the *Mus musculus* infection model.

RESULTS

Luciferase imaging sensitivity in bank voles

The purpose of this study was to compare MuHV-4 infection in mice, either inbred or outbred, and in one reported natural host, the bank vole. We first monitored infection by bioluminescence imaging of animals infected with luciferase-expressing MuHV-4 (Milho *et al.*, 2009). As bank voles have darker fur pigmentation than the BALB/c or CD1 mice used for comparison, we first established the sensitivity of bioluminescence imaging for each host. Different numbers of baby hamster kidney (BHK-21) cells infected with a MuHV-4 strain expressing luciferase under the control of the M3 promoter were therefore injected subcutaneously on the ventral part of animals before imaging. Removing the fur from animals prior to imaging had little effect on the signal obtained (data not shown), so all animals were imaged with fur present. After bioluminescent imaging, the total flux of photons for each injection site was reported on a graph (see Supplementary Fig. S1, available in JGV Online). Each group showed a similar sensitivity and linearity of signal with injected BHK-21 cell number. Therefore, bioluminescence imaging was considered a viable means of comparing infections in the different hosts.

Luciferase imaging of MuHV-4 infection in bank voles and BALB/c and CD1 mice

We then infected anaesthetized animals intranasally with 10^4 p.f.u. luciferase$^+$ MuHV-4 and tracked infection by D-luciferin injection and charge-coupled-device (CCD) camera scanning. Based on previous analysis (Milho *et al.*, 2009), we considered thoracic signals to come from the lungs, abdominal signals from the spleen and neck signals from the superficial cervical lymph nodes (SCLNs). The signal intensities in the nose (Fig. 2a), lungs (Fig. 2b), SCLNs (Fig. 2c) and spleens (Fig. 2d) were monitored over time. A strong signal was visible in the lungs and noses of BALB/c mice at the peak of lytic replication (5–7 days p.i.). CD1 mice were very similar. In contrast, bank voles showed no signal in the nose and only sporadic weak signals in the lungs. At the peak of latency amplification (12–15 days p.i.), luciferase signals were weak or undetectable in the lungs and noses of most of BALB/c and CD1 mice, but strong in the SCLNs and spleen. Again, the corresponding bank vole signals were weak or undetectable.

These results were confirmed by *ex vivo* imaging of dissected organs (Fig. 3). In the noses of all groups, we observed sporadic signals at day 7 p.i., which had

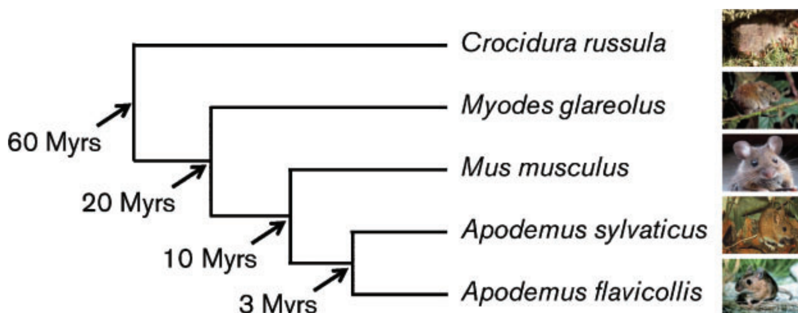

Fig. 1. Evolutionary relationships among the studied species. Synthetic phylogenetic tree summarizing the evolutionary relationships and an estimation of the divergence times among the studied species (Michaux *et al.*, 2001, 2002; Murphy *et al.*, 2001). Myrs, Million years.

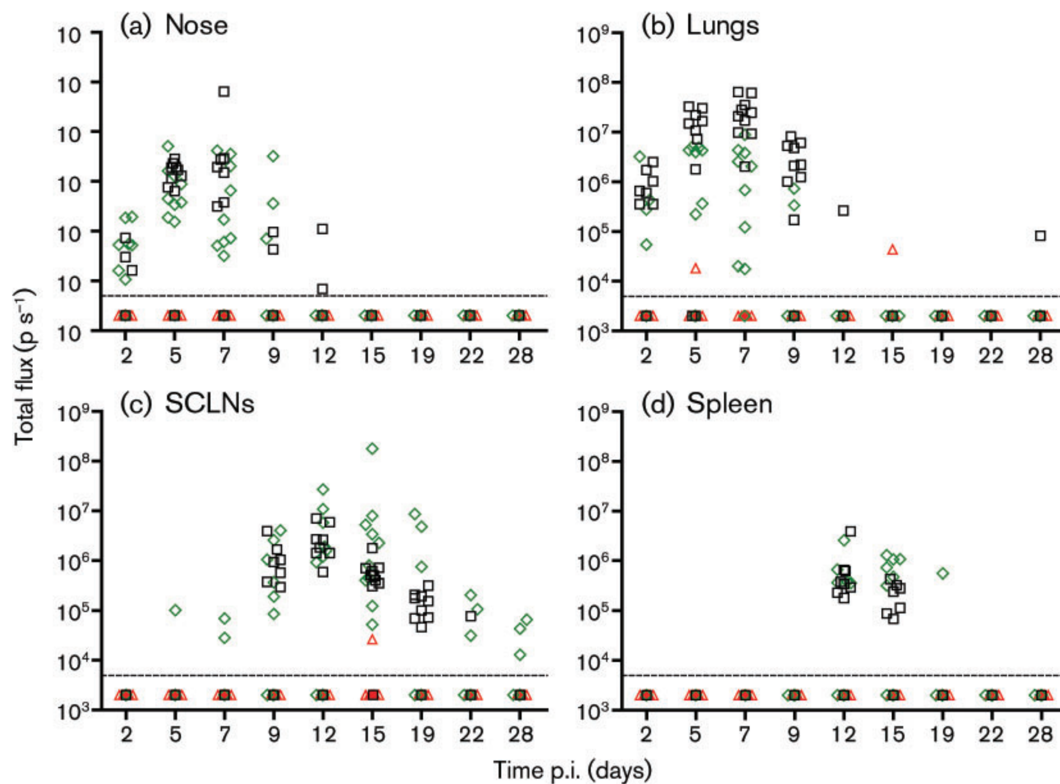

Fig. 2. Luciferase imaging of MuHV-4 infection in *Myodes glareolus* (△) and BALB/c (□) and CD1 (◇) mice. Animals were infected intranasally (10^4 p.f.u. in 30 μl) under general anaesthesia with wild-type luciferase+ MuHV-4 strain MHV-68. The maximum luciferase signal intensities in the nose (a), lungs (b), SCLNs (c) and spleen (d) were monitored in the different animals (ten per group: five males and five females) over time. Each point shows the signal of one animal. The dashed lines indicate the lower limits of detectable signal intensity.

disappeared by days 14 and 21 p.i. Signals from lungs were maximal at day 7 p.i. and again much weaker in bank voles than in mice, consistent with the analysis of living animals. Only sporadic lung signals were observed at days 14 and 21. Signals from SCLNs and spleens were maximal in mice at day 14 and only rarely observed in dissected bank voles. Based on the images, the signal in CD1 mice spleen appeared to be larger than in BALB/c mice spleen. Fig. 2 also suggested that there was more replication around day 14 in CD1 mice spleens and SCLNs than in similar organs from BALB/c mice. This probably reflects differences between mice strains and requires more experiments for clarification. No signals were observed in other organs. Therefore, it appeared that MuHV-4 follows a similar *in vivo* cycle in bank voles as in mice, but replicates much less well.

Classical analysis of MuHV-4 infection in bank voles and BALB/c and CD1 mice

Luciferase expression by luciferase+ MuHV-4 reflects predominantly lytic gene expression (Milho *et al.*, 2009). Therefore, to explore further the establishment of MuHV-4 latency in bank voles, we infected bank voles or mice intranasally with 10^4 p.f.u. wild-type MuHV-4 (strain MHV-68). Consistent with the bioluminescence imaging results, lytic replication, as measured by plaque assay, was greatly reduced in bank voles compared with mice (Fig. 4). At day 7 p.i., infectious virus was recovered only sporadically from noses (Fig. 4a) but was recovered from all lungs (Fig. 4b). However, plaque assay titres were approximately 1000 times lower in bank voles than in mice. Although the lung virus titres of bank voles were significantly lower than those of BALB/c mice ($P<0.001$), they were not significantly lower than those of outbred CD1 mice ($P>0.05$).

The colonization of SCLNs and spleens by latent virus was then determined by infectious centre assay. The recovery of replication-competent virus from SCLNs and spleens of mice was maximal at 14 days p.i. (Fig. 4c, d), consistent with published data (Milho *et al.*, 2009). Virus recovery from the SCLNs and spleens of bank voles was much less consistent. However, it was possible to observe a peak latent load in spleens at 14 days p.i., suggesting that latency amplification occurs in bank voles much as it does in mice.

Lower virus recovery in infection centre assays could have reflected a lower capacity of MuHV-4 to reactivate productively from latently infected bank vole spleen cells

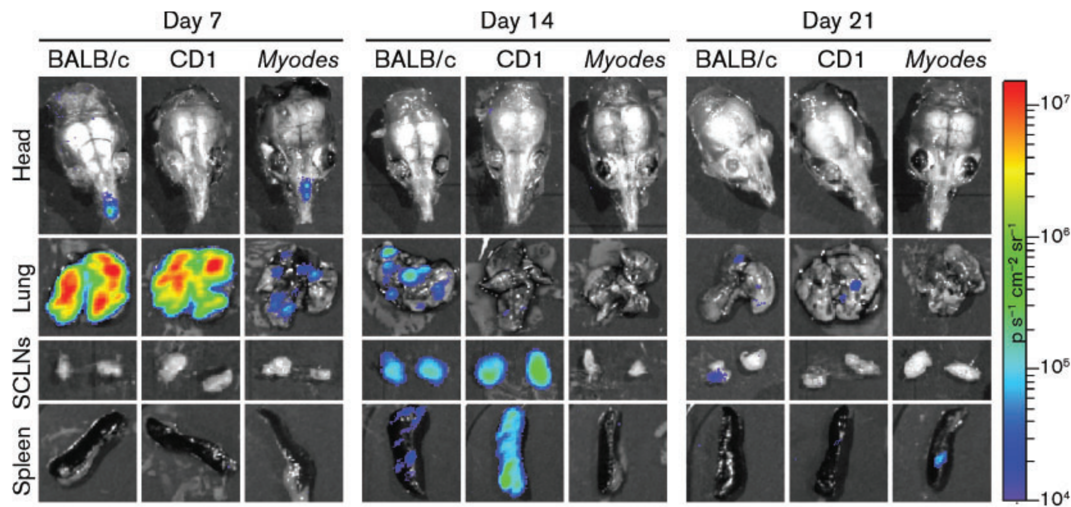

Fig. 3. Luciferase signals from isolated organs after intranasal MuHV-4 infection. Mice equivalent to those in Fig. 2 were dissected and their organs imaged *ex vivo*. The images are representative of data from at least five mice and show photographs overlaid with luciferase signals. The scale bar [photons (p) s^{-1} cm^{-2} steradian $(sr)^{-1}$] shows the colour scheme for signal intensity.

explanted onto BHK-21 monolayers. In order to evaluate latent loads in SCLNs and spleens further, we quantified viral genomes by real-time PCR. In spleens, the viral DNA loads of infected bank voles were approximately ten times lower than those of BALB/c mice (Fig. 5a). However, the difference was less when compared with CD1 mice. Moreover, viral DNA was recovered from the spleens of all infected bank voles, implying that MuHV-4 robustly establishes latency in this site. The SCLN latent loads were similar between bank voles and mice (Fig. 5b). Together, these results showed that MuHV-4 can efficiently establish latency in bank voles, despite lower levels of replication than in mice.

Tissue histology and immunohistochemistry

Dramatic peribronchiolar, perivascular and interstitial lymphocytic infiltrates were observed in mouse lungs at day 7 p.i. (Fig. 6a). These lesions were associated with virus replication as identified by immunohistochemical staining for viral antigens (Fig. 6a). A very similar interstitial pneumonia occurred in MuHV-4-infected bank voles. Viral antigens were detectable, but with a much more limited distribution than observed in mice. This was consistent with the limited luciferase expression and low virus titres of infected bank voles. Therefore, infection of bank vole lungs appeared to be qualitatively similar to that of mice, but quantitatively less.

At 14 days p.i., MuHV-4-infected mice show marked splenomegaly and lymphadenopathy (Nash *et al.*, 2001). The same was observed here. *Myodes* spleens are naturally much smaller than those of mice, but overall similar changes could be observed. Histological examination of mouse SCLNs at day 21 p.i. showed enlargement with increased numbers of germinal centres [Fig. 6b(ii, iv)]. Although bank vole SCLNs were luciferase-negative and yielded few observable infectious centres, viral antigens were more readily detectable than in mice [Fig. 6b(xii); 18.0 ± 9.8 positive cells per field in *Myodes* vs 3.8 ± 1.6 and 3.2 ± 1.2 positive cells per field in BALB/c and CD1 mice, respectively; $P < 0.05$ by one-way ANOVA and Bonferroni's multiple comparison test].

Antibody response analysis

Lower viral loads in bank voles might be expected to induce weaker antibody responses. Sera taken at various times p.i. were analysed by a plaque reduction assay to determine the titres of neutralizing antibodies (Fig. 7). As viral neutralization assays can be influenced by the cell types on which they are performed, we repeated the experiment on three cell types from different origins: BHK-21 cells, derived from hamsters, and mouse NAMRU mammary gland (NMuMG) cells and 3T3 cells representing epithelial and fibroblastic murine cells, respectively. In each cell type, MuHV-4-specific neutralizing titres were low at day 7 p.i. in all animals, but increased dramatically by day 14. For some time points, the titres measured in bank voles were significantly lower than in mice (Fig. 7a–c).

MuHV-4 is not transmitted between mice or voles in captivity

Finally, we evaluated the ability of MuHV-4 to undergo transmission between female mice or voles maintained in captivity. In each group, three 6–8-week-old females were inoculated intranasally (10^4 p.f.u. in 30 μl). At 2 days p.i., three naïve females were placed in the same cage as the inoculated animals. Luciferase signals were monitored once a week (data not shown) and sera were taken at various

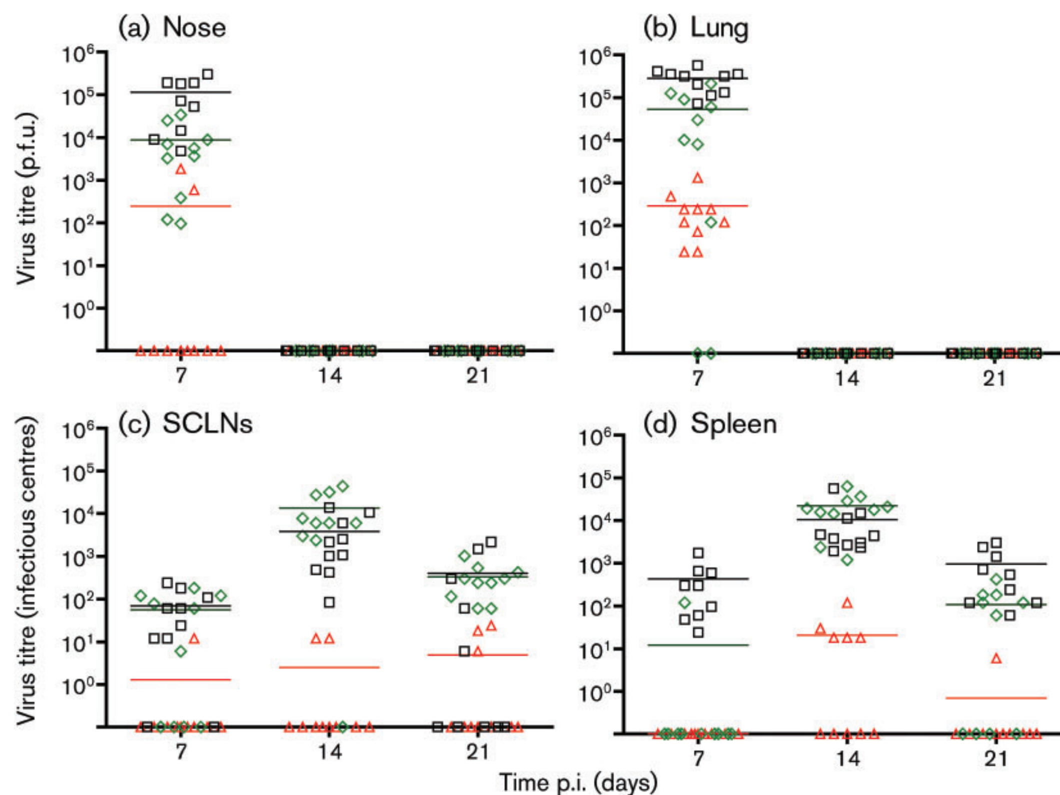

Fig. 4. Classical analysis of MuHV-4 strain MHV-68 infection in *Myodes glareolus* (△) and BALB/c (□) and CD1 (◇) mice. Ten animals (five males and five females) were infected intranasally (10^4 p.f.u. in 30 μl) under general anaesthesia with wild-type MuHV-4 strain MHV-68. Each horizontal line shows the mean for each group of ten (including negatives) and each point shows the signal for one entire organ. (a) At 7, 14 and 21 days p.i., the infectious virus titre in noses was determined by plaque assay. The titres at day 7 p.i. in *Myodes glareolus* noses were reduced significantly relative to those in BALB/c mice ($P<0.001$ by two-way ANOVA and Bonferroni post-test), but not relative to those in CD1 mice ($P>0.05$). (b) At 7, 14 and 21 days p.i., the infectious virus titre in lungs was determined by plaque assay. The titres in *Myodes glareolus* lungs were reduced significantly at day 7 p.i. relative to those in BALB/c mice ($P<0.001$), but not relative to those in CD1 mice ($P>0.05$). (c) Superficial cervical lymph nodes were removed at the indicated times and assayed individually for reactivatable MHV-68 by infectious centre assay. The titres in *Myodes glareolus* SCLNs were only reduced significantly at day 14 relative to those in CD1 mice ($P<0.001$). (d) Spleens from the same animals were analysed individually by infectious centre assay. The titres in *Myodes glareolus* spleens were reduced significantly at day 14 relative to those in BALB/c and CD1 mice ($P<0.05$ and $P<0.001$, respectively), but not at days 7 and 21.

times p.i. and analysed by a plaque reduction assay to determine the titres of neutralizing antibodies (Fig. 8a). Although all infected animals showed luciferase signals characteristic of the MuHV-4 *in vivo* cycle and developed neutralizing antibodies, none of the contact animals presented any luciferase signal or neutralizing antibody. Finally, all the animals were sacrificed at day 28 and latent virus loads in spleens were quantified by real-time PCR (Fig. 8b). Again, viral DNA was only recovered from infected individuals and not from any of the contact individuals.

DISCUSSION

Co-speciation has evidently been the prominent mode of evolution in the order *Herpesvirales* (Davison, 2002; McGeoch, 2001; McGeoch *et al.*, 2000, 2006). In nature, most herpesviruses are effectively closely associated with a single host species. They have evolved over long periods of time with their host and are extremely well-adapted to them. This view is supported not only by phylogenetic studies, but also by the modest pathogenicity of herpesviruses in their natural settings (Davison, 2002). In contrast, herpesvirus infections resulting from *trans*-species transmission are generally associated with severe diseases (Dewals *et al.*, 2006; Fickenscher & Fleckenstein, 2001; Hart *et al.*, 2007). We have been studying MuHV-4 in mice. The power of MuHV-4 to harness mouse genetic and immunological tools makes it an obvious choice for initial analysis. Indeed, we recently described several immune-evasion mechanisms that could explain why the antibody response to natural infection does not prevent its transmission (Gillet

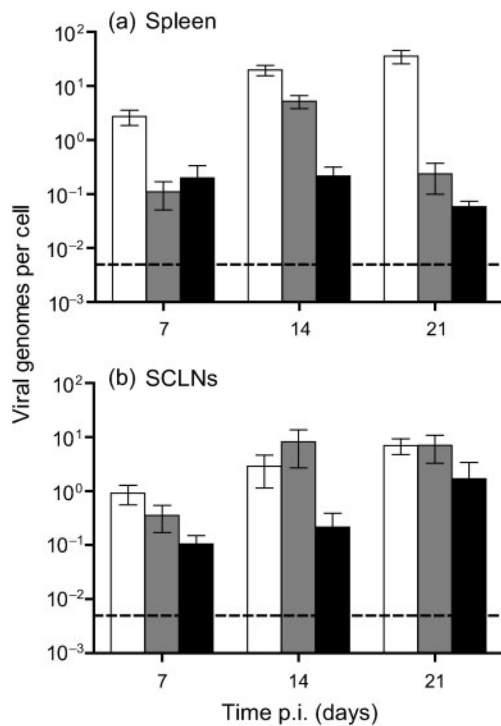

Fig. 5. Latent loads in spleens and SCLNs of *Myodes glareolus* (filled bars) and BALB/c (empty bars) and CD1 (grey-shaded bars) mice. The same samples as in Fig. 4(c, d) were analysed for viral genomes by real-time PCR of DNA from spleens or SCLNs. Each bar shows the mean viral genome copy number per host genome ± SEM for each group of ten. The dashed lines show the lower limits of assay sensitivity. (a) At day 7, viral genome loads in *Myodes glareolus* spleens were not significantly different from those measured in mice (two-way ANOVA and Bonferroni post-test). However, they were reduced significantly at days 14 and 21 relative to BALB/c but not CD1 mice ($P<0.01$ and $P<0.001$, respectively). (b) Viral genome loads in *Myodes glareolus* SCLNs were not significantly different from those measured in mice (two-way ANOVA and Bonferroni post-test).

& Stevenson, 2007; Gillet *et al.*, 2007a, 2008a, b). However, no transmission model has yet been defined in mice for MuHV-4. Thus, so far it has proved difficult to test the significance of viral antibody evasion and strategies of vaccination in the setting where they are likely to be most important.

All viruses must be selected primarily for efficient transmission. Failure of experimental transmission in the mouse could be due to several reasons, among which is the fact that the house mouse (*Mus musculus*) may not be a natural host for MuHV-4. Although there are serological indications that MuHV-4 (or a close relative) naturally infects house mice (Mistrikova *et al.*, 2000), the virus has never been isolated from this species. We therefore do not have any idea whether it is a relevant model. Comparisons of MuHV-4 infection in mice and natural hosts could provide answers to that question.

Recently, two gammaherpesvirus infection models were described in wood mouse (*A. sylvaticus*) (Hughes *et al.*, 2010a, b). In the first study, the authors characterized a novel wood mouse virus related to MuHV-4 (Hughes *et al.*, 2010b). However, wood mouse herpesvirus and MuHV-4 share only 85 % nucleotide sequence identity and therefore cannot be seen as strains of the same species. Although wood mouse herpesvirus seems to be a very interesting model, it will be very difficult to transpose all of the knowledge accumulated about MuHV-4 infection to this new model. In a second study, Hughes *et al.* (2010a) described MuHV-4 infection in the wood mouse. Based on PCR results, Blasdell *et al.* (2003) proposed MHV-68 as a wood mouse virus (Blasdell *et al.*, 2003). However, they did not provide any sequence analysis. Moreover, subsequent data from the same group strongly suggested that they were amplifying a related virus rather than MHV-68 (Ehlers *et al.*, 2008). This was reinforced by their recent paper describing wood mouse herpesvirus (Hughes *et al.*, 2010b). Therefore, although their characterization of wood mouse infection by MuHV-4 is interesting, there is major evidence suggesting that wood mice cannot be considered a natural host of MHV-68, only of the wood mouse virus. As MHV-68 has been isolated from bank voles (Blaskovic *et al.*, 1980), analysis of the MuHV-4 *in vivo* cycle in this species was therefore needed.

Intranasal virus inoculation led to productive replication in bank vole lungs, as is observed in mice; however the extent of lytic replication was approximately 1000-fold lower in bank voles than in mice. This observation could be related to the fact that the nose – but not the lung – is the most likely point of normal host entry for MuHV-4 (Milho *et al.*, 2009). Indeed, intranasal inoculation of mice without anaesthesia gave luciferase expression in just the nose and not in the lung, with a normal latent colonization of draining lymph nodes. In this study, despite lower replication levels in the lung, we observed similar latent loads in voles and mice (Fig. 5). MuHV-4 host colonization is relatively independent of the extent of primary lytic infection (Coleman *et al.*, 2003; Stevenson *et al.*, 1999). It depends much more on latency-associated lymphoproliferation (May *et al.*, 2004). As gammaherpesvirus epidemiology indicates that transmission correlates with the latent load, our results suggest that gammaherpesviruses may have evolved to infect their hosts without extensive lytic spread, which could provide a powerful immune stimulus. Besides a lower extent of lytic replication, Hughes *et al.* (2010a) reported focal granulomatous infiltrations in *Apodemus* lungs, rather than diffuse lymphocytic interstitial pneumonitis as observed in mice. Our experiments in *Myodes*, however, revealed lung lesions comparable to those observed in mice (Fig. 6a). Although lytic replication was limited in *Myodes* lungs, infection was accompanied by a characteristic diffuse interstitial pneumonitis. In the future, it would be interesting to see whether similar pathological changes are observed in mice after intranasal inoculation without anaesthesia. In comparison with

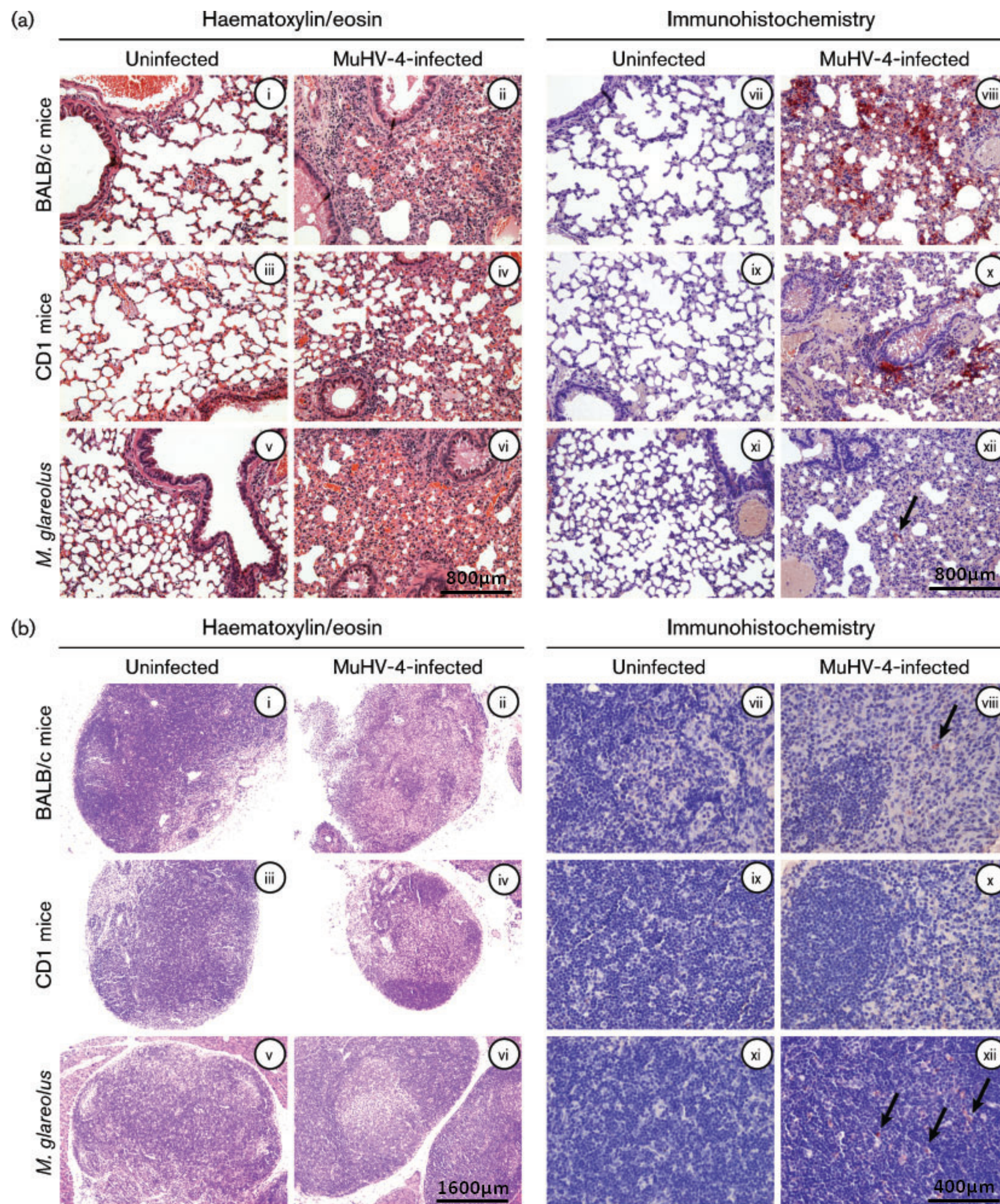

Fig. 6. Tissue histology and immunohistochemistry. (a) At 7 days after infection with wild-type MuHV-4 strain MHV-68, the lungs of *Myodes glareolus* and BALB/c and CD1 mice were removed and fixed in formaldehyde before haematoxylin/eosin staining. In the three species, lung infection evoked interstitial pneumonia with massive cellular infiltrates and oedema (i–vi). Samples from the same organs were then processed for immunohistochemistry and stained with anti-MHV-68 rabbit polyserum (vii–xii). The results confirmed that viral pulmonary replication was much lower in *Myodes glareolus* than in BALB/c and CD1 mice. The arrow indicates focal MuHV-4 antigen detection. The images are representative of data from at least five animals. (b) At 21 days after infection with wild-type MuHV-4 strain MHV-68, SCLNs of *Myodes glareolus* and BALB/c and CD1 mice were removed and fixed in formaldehyde before haematoxylin/eosin staining (i–vi). An increased number of germinal centres was observed mainly in BALB/c and CD1 mice. Samples from the same organs were then processed for immunohistochemistry and stained with anti-MHV-68 rabbit polyserum (vii–xii). The arrows indicate representative focal MuHV-4 antigen detection. The images are representative of data from at least five animals.

laboratory mice, the spleens of infected bank voles showed reduced splenomegaly. However, long-term latency was maintained similarly in voles and mice, suggesting that splenomegaly is not required for long-term latency. Finally, an intriguing observation was that, although bank vole SCLNs were luciferase-negative and yielded few observable

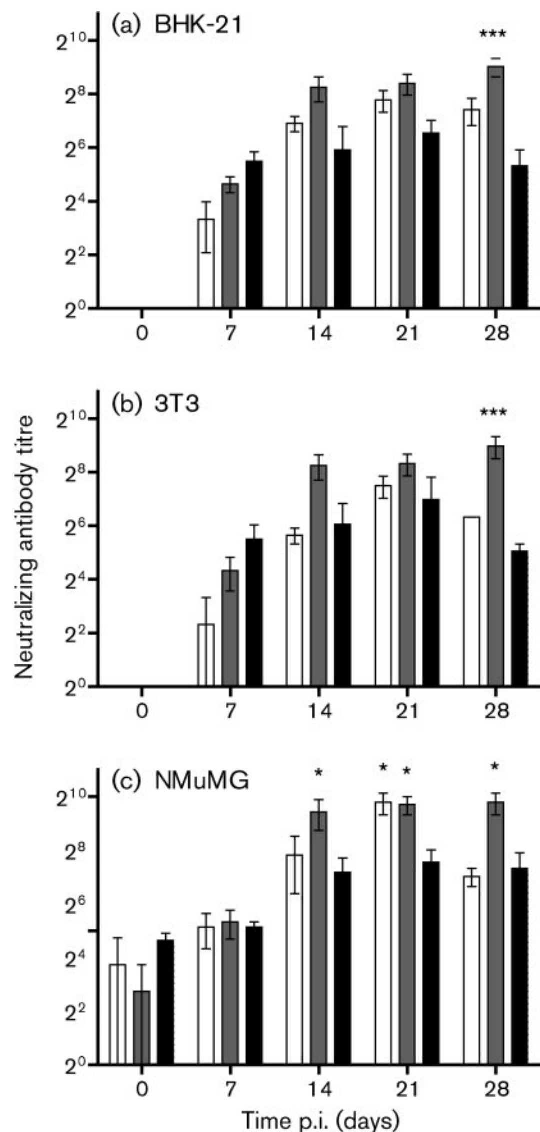

Fig. 7. Kinetics of the virus-specific neutralizing humoral response following infection with MuHV-4. Five females per group were infected intranasally (10^4 p.f.u. in 30 μl) under general anaesthesia with wild-type MuHV-4 strain MHV-68 and blood samples were taken at the indicated times p.i. The neutralizing antibody titre was determined by plaque inhibition on BHK-21 (a), 3T3 (b) or NMuMG (c) cells (see Methods). All measurements were made relative to a standard pool of immune sera and the reciprocal of the dilution that resulted in a >50 % reduction in the number of MuHV-4 plaques was plotted. Empty bars, BALB/c; grey-shaded bars, CD1; filled bars, *Myodes glareolus*. Each bar shows the mean ± SEM for each group of five. Asterisks indicate the values for each day that are significantly different from the *Myodes* values: *$P<0.05$, ***$P<0.001$ by two-way ANOVA and Bonferroni post-test.

infectious centres, viral antigens were more readily detectable than in mice. Several hypotheses can explain this observation, such as differences in tropism, differences in spreading and differences of antigenicity of some viral proteins. The significance of this phenomenon therefore remains unclear and will require further experiments in the future to be understood.

The main features of MuHV-4 infection in *Myodes glareolus* are very similar to those observed in *A. sylvaticus.* However, unlike what we found in *Myodes*, Hughes *et al.* (2010a) described titres of neutralizing antibody to MuHV-4 that were significantly higher in wood mice than in BALB/c mice. They related this observation to the histological changes that they found and that we did not observe in *Myodes glareolus.* Whilst this is possible, another explanation could be that their results mainly reflected poor neutralizing antibody titres in BALB/c mice, although numerous studies have investigated MuHV-4 neutralization in this model. This difference could be due to the cell type they used to perform their experiments, as every laboratory 3T3 cell line is potentially a bit different. For example, the mouse cells that they used could have provided Fc receptors that could have reduced BALB/c serum neutralizing activity (Rosa *et al.*, 2007). Other effects of wood mouse sera on 3T3 cells are also possible. We therefore performed our plaque reduction assays on three cell types from mouse and hamster origins. In each cell type, we observed neutralizing antibody titres in *Myodes* that were similar overall to those observed in mice, even if they tended to be lower at some time points. Further comparative studies on both *Myodes* and *Apodemus* sera in parallel are therefore needed to assess the differences between MuHV-4 neutralizing antibody responses in *Myodes glareolus* and *A. sylvaticus.*

The tendency towards lower antibody titres in *Myodes glareolus* suggests that transmission could be easier in bank voles than in mice. We therefore created epidemiological situations by mixing MuHV-4-infected and naïve animals. We monitored possible transmission for 1 month by serology, *in vivo* bioluminescence and genome quantification in spleens. However, it was impossible to establish experimental transmission in populations of either mice or voles. Several hypotheses could explain this lack of transmission. Firstly, these results could reflect the fact that gammaherpesvirus transmission is a rare event. However, the prevalence of these viruses in natural populations suggests the opposite. Secondly, luciferase expression could prevent virus transmission by altering the long-term behaviour of the virus, as recently shown by a MuHV-4 strain expressing the non-structural protein NS3 of hepatitis C virus under the control of the murine cytomegalovirus promoter (MHV-68-NS3; El-Gogo *et al.*, 2008). To avoid this problem, luciferase was placed under the control of the MuHV-4 M3 promoter and therefore was only expressed in lytically infected cells. In contrast to MHV-68-NS3, no decreased viral latent load was observed for this strain in comparison with the wild-type strain (Milho *et al.*, 2009). However, further studies with the wild-type MuHV-4 strain will be needed to determine whether luciferase expression during lytic replication could prevent virus

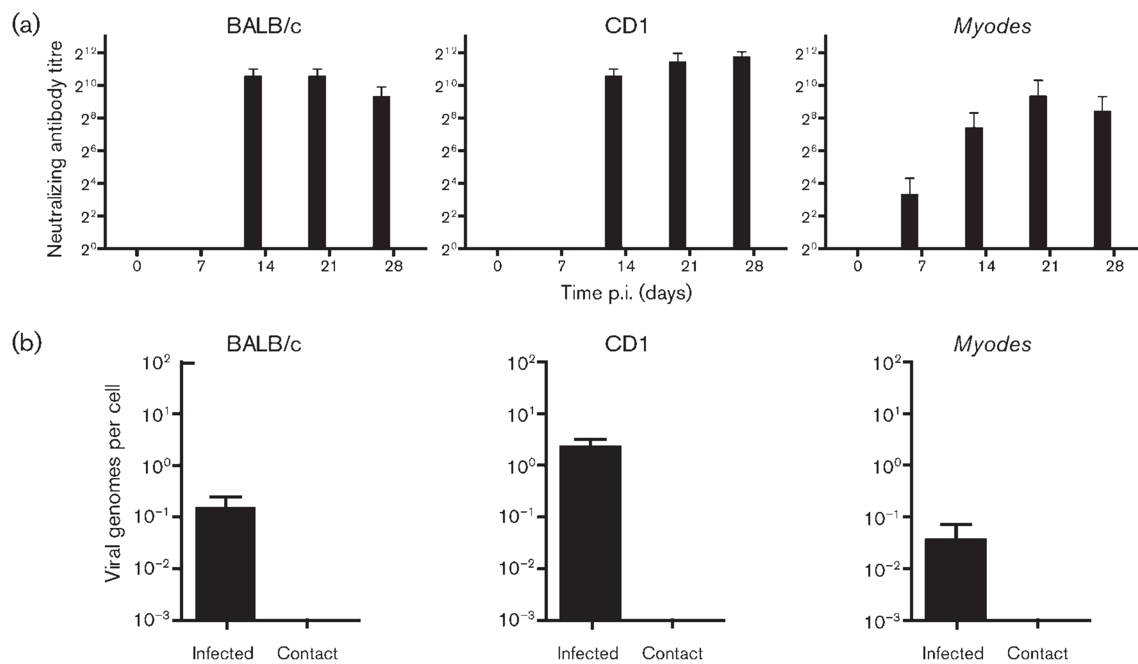

Fig. 8. Transmissibility of MuHV-4 in *Myodes glareolus* and BALB/c and CD1 mice. Three females per group were infected intranasally (10^4 p.f.u. in 30 µl) under general anaesthesia with wild-type MuHV-4 strain MHV-68. At 48 h p.i., three naïve females (contact) were placed in the same cage as the inoculated animals. (a) Blood samples were taken at the indicated times p.i. The neutralizing antibody titres were determined by plaque inhibition on BHK-21, 3T3 or NMuMG cells (see Methods). All measurements were made relative to a standard pool of immune sera and the reciprocal of the dilution that resulted in a >50 % reduction in the number of MuHV-4 plaques was plotted. Empty bars, contact animals; filled bars, infected animals. Each bar shows the mean ± SEM for each group of three. (b) The animals were sacrificed at day 28 p.i. and their spleens were analysed for viral genomes by real-time PCR. Each bar shows the mean viral genome copy number per host genome ± SEM for each group of three.

transmission in *Myodes glareolus* populations. Thirdly, failure of experimental transmission could be because the transmission conditions that we used were not appropriate.

MuHV-4 and its close relatives are, unusually, found in a wide variety of hosts (mouse, shrew and bank vole). We are used to thinking of herpesviruses as being highly host-restricted (McGeoch *et al*., 2006). However, recent phylogenetic analysis has shown that for some gammaherpesvirus species, including MuHV-4, distant interspecies transfer has been an important part of their evolutionary history (Ehlers *et al*., 2008). We can assume that there may be more horizontal transmission between small rodents than between large mammals, simply because more species share overlapping ecological niches. This diversity of hosts could explain why MuHV-4 genomes have been accumulating sequence changes atypically fast compared with other members of the *Gammaherpesvirinae* (McGeoch, 2001; McGeoch *et al*., 2005). Furthermore, nose infection has raised the possibility of transmission through scent marking. This behaviour is distorted in caged mice. Therefore, we need to test MuHV-4 transmission between *Mus musculus* in a more physiological context than conventional animal caging. Another way would be to test MuHV-4 transmission in another described host species. However, it is unclear whether the infections observed in field studies are always productive. MuHV-4 (or a related virus) has been isolated repeatedly from yellow-necked mice (*A. flavicollis*), so these could be the natural host. In the future, testing whether they transmit in captivity would be an interesting point.

In conclusion, MuHV-4 infection of bank voles follows the same route as in mice, but the virus replicates to a lesser degree. These differences in the extent of lytic replication could just mean that strain MHV-68 is better adapted to replication in mice. An MHV-68 isolate has actually been obtained from successive intracranial passages in newborn mice (Blaskovic *et al*., 1980; Nash *et al*., 2001). By contrast, limited lytic replication with normal long-term latent loads could also represent better adaptation of the virus to its host, as is also observed for wood mouse herpesvirus in the wood mouse (Hughes *et al*., 2010b). In the future, we hope to be able to choose between these hypotheses in light of virus transmission. Whilst MuHV-4 infection in yellow-necked mice – another species known to be naturally infected – may yet yield surprises, it appears so far that *Mus musculus* represents a suitable host for studying gammaherpesvirus pathogenesis with MuHV-4.

METHODS

Animals. Female and male BALB/c and CD1 mice were purchased from Charles River Laboratories. We used adult female and male bank voles from generation 2 of a colony established from voles maintained in a large experimental colony in Poland (Sadowska *et al.*, 2008). Their offspring (generation 1) were weaned at day 21 and maintained individually in standard polypropylene mouse cages (26 × 20 × 16 cm). Voles from generation 1 were paired at the age of 4–6 months to produce generation 2 (some individuals in generation 2 were cousins or paternal half-siblings). All the animals were housed in the Department of Infectious Diseases, University of Liège. The animals were infected with MuHV-4 at 6–12 weeks old. Intranasal infections with anaesthesia were in 30 µl aliquots. For luciferase imaging, animals were anaesthetized with isoflurane, injected intraperitoneally with D-luciferin (150 mg kg^{-1}), then scanned with an IVIS Spectrum (Caliper Life Sciences). Animals were routinely imaged after 10 min. For quantitative comparisons, we used Living Image software (Caliper Life Sciences) to obtain either the maximum radiance [photons (p) s^{-1} cm^{-2} per steradian (sr)] or total flux (p s^{-1}) over each region of interest. All experiments conformed to the rules of the local animal ethics committee of the University of Liège.

Cells and virus. BHK-21, NMuMG and 3T3 cells were propagated in Dulbecco's modified Eagle's medium (DMEM; Invitrogen), supplemented with 2 mM glutamine, 100 U penicillin ml^{-1}, 100 mg streptomycin ml^{-1} and 10 % FCS. We used the wild-type MHV-68 strain of MuHV-4 (Blaskovic *et al.*, 1980). The MuHV-4 strain expressing luciferase under the control of the M3 promoter has been described previously (Milho *et al.*, 2009).

Viral infectivity assays. Virus stocks were titrated by plaque assay on BHK-21 cells (Gillet *et al.*, 2007b). Cell monolayers were incubated with virus (2 h, 37 °C), overlaid with 0.3 % carboxymethylcellulose, and 4 days later were fixed and stained for plaque counting. Infectious virus in lungs was measured by homogenizing the lungs in 6 ml complete medium prior to plaque assay. To determine nasal titres, we removed a block of tissue bound (i.e. not including) anteriorly by the cartilaginous tip of the nose, posteriorly by the orbits, laterally by the zygomatic arches, ventrally by the palate and dorsally by the nasal bones. This region contained all the luciferase signal measurable by *ex vivo* CCD camera scanning. Bone fragments were discarded after homogenization in 3 ml complete medium and the lysate was plaque assayed. Latent virus in *ex vivo* tissues was measured by infectious centre assay (Gillet *et al.*, 2007b): spleen or SCLN suspensions were co-cultured with BHK-21 cells, fixed and stained for plaque counting after 5 days. Pre-formed infectious virus titres in lymphoid tissue, as measured by plaque assay of freeze–thawed cells, were always <1 % of infectious centre assay titres, so the latter essentially measured reactivatable latent virus.

Viral genome quantification. Viral genome loads were measured by real-time PCR (Gaspar *et al.*, 2008). DNA from organs (100 ng) was used to amplify MuHV-4 genomic co-ordinates 4166–4252 (iCycler; Bio-Rad) (gene M2: forward primer 5′-GTCAGTCGAGCCAGAGTCCAACA-3′, reverse primer 5′-ATCTATGAAACTGCTAACAGTGAAC-3′). The PCR products were quantified by hybridization with a TaqMan probe (genomic co-ordinates 4218–4189, 5′-6-FAM-TCCAGCCAATCTCTACGAGGTCCTTAATGA-BHQ1-3′) and converted to genome copies by comparison with a standard curve of cloned plasmid template serially diluted in control spleen DNA and amplified in parallel. Cellular DNA was quantified in parallel by amplifying part of the interstitial retinoid binding protein (IRBP) gene (forward primer 5′-ATCCCTATGTCATCTCCTACYTG-3′, reverse primer 5′-CCRCTGCCTTCCCATGTYTG-3′). The PCR products were quantified with SYBR Green (Invitrogen) and the copy number was calculated by comparison with standard curves of cloned IRBP templates from each species amplified in parallel. Amplified products were distinguished from paired primers by melting curve analysis, and the correct sizes of the amplified products were confirmed by electrophoresis and staining with ethidium bromide.

Lung histology and immunohistochemistry. Portions of lungs and SCLNs were fixed in buffered formol saline, processed routinely to 5 µm paraffin wax-embedded sections, stained with haematoxylin and eosin, and examined by light microscopy. Immunohistochemistry was performed using an EnVision Detection System (Dako) with anti-MHV-68 rabbit hyperimmune serum (Sunil-Chandra *et al.*, 1992) as the primary antibody.

Measuring neutralizing antibody. Duplicate twofold serum dilutions, starting from an initial concentration of 1 : 10 in DMEM containing 10 % FCS, were incubated with 40 p.f.u. MHV-68 at 37 °C for 1 h in 96-well plates. Freshly trypsinized cells (2×10^4) were added to each well and allowed to adhere for 2 h. The cells were then overlaid with 0.3 % carboxymethylcellulose, and 4 days later fixed and stained for plaque counting. A standard immune serum was included in each experiment to ensure uniformity of results. The neutralization titre was defined as the highest serum dilution giving a >50 % reduction in viral plaques. Naïve mouse and *Myodes* sera had no effect on plaque formation.

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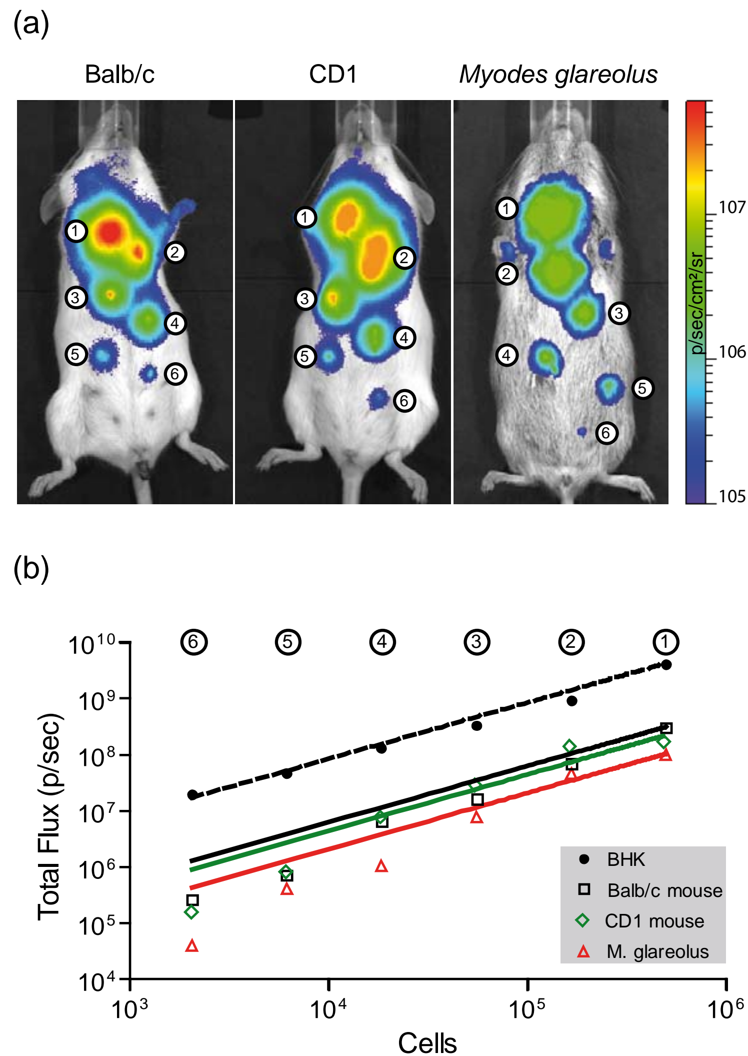
Supplemental figure 1



Supplementary Fig. S1. IVIS spectrum sensitivity in *Myodes glareolus. (a)* MuHV-4-infected cells (5×10^5) (dot 1) and serial threefold dilutions (dots 2–6) were injected subcutaneously into *Myodes glareolus and* BALB/c and CD1 mice before D-luciferin injection and imaging. All points could be detected in the different species used in this study. (b) The maximum luciferase signal intensities for each dot were monitored and plotted on a graph. These were compared with signals measured *in vitro* (BHK-21 cells). Linear regression curves were calculated for each condition.

3.2

Illumination of Murid Herpesvirus 4 cycle reveals a sexual transmission route in laboratory mice.

Submitted

S. François, S. Vidick, M. Sarlet, Desmecht, P-V. Drion, P.G. Stevenson, A. Vanderplasschen and L. Gillet

Preamble

As previously mentioned, herpesviruses establish lifelong latency within their host. In the particular case of gammaherpesviruses such as EBV or KSHV, temporary reactivation episodes with low levels of infectious virions produced are observed. This phenomenon of re-excretion is an epidemiological necessity for these latent viruses, ensuring their spread amongst host populations. Understanding and knowledge of this re-excretion is therefore crucial in order to consider the containment of the epidemiological cycle of a virus through vaccination.

For EBV, re-excretion occurs principally in the saliva and transmission often occurs by direct contacts with the saliva of an infected host undergoing a reactivation phase. Moreover, several studies demonstrated that EBV can be transitorily detected in the cervix of infected women. Several epidemiological studies have also shown a relationship between the age at the onset of sexual activity and the age of infection by EBV.

KSHV is clearly associated with a sexually transmitted disease as the majority of the associated pathologies are described in patients infected by HIV. The literature describes, as for EBV, re-excretion in the saliva, but also the presence of the virus in the genital tract of men and women.

For MuHV-4, the literature does not describe, as of yet, any observation of viral re-excretion. However, thanks to *in vivo* imaging, we have detected MHV-68 infectious virus in the genital area of female mice infected intranasally by MHV-68 and this, after the establishment of latency. Moreover the implication of sexual steroids in the observed phenomenon of genital re-excretion was demonstrated *in vivo*.

The study presented in this section is displayed as an article currently in preparation, and consists of the description of the phenomenon of MuHV-4 re-excretion as it is observed in laboratory mice. We also present the in-depth analysis of the consequences of this observation concerning the potential mechanisms of transmission. Lastly, we demonstrate the existence of sexual transmission of the virus from re-excreting female mice to naïve males.

Abstract

Transmission is a matter of life or death for pathogen lineages and can therefore be considered as the main motor of their evolution. Gammaherpesviruses are archetypal pathogenic persistent viruses which have evolved to be transmitted in presence of specific immune response. Identifying their mode of transmission and their mechanisms of immune evasion is therefore essential to develop prophylactic and therapeutic strategies against these infections. As the known human gammaherpesviruses, Epstein-Barr virus and Kaposi's Sarcoma-associated Herpesvirus, are host-specific and lack a convenient *in vivo* infection model, related animal gammaherpesviruses, such as Murid herpesvirus 4 (MuHV-4), are commonly used as general models of gammaherpesvirus infections *in vivo*. To date, it has however never been possible to monitor viral re-excretion or virus transmission of MuHV-4 in laboratory mice population. In this study, we have used the MHV-68 strain of MuHV-4 associated with global luciferase imaging to investigate potential re-excretion sites of this virus in laboratory mice. This allowed us to identify a genital re-excretion site of MuHV-4 following intranasal infection and latency establishment in female mice. This re-excretion occurred at the external border of the vagina and was dependent on the presence of oestrogens. However, MuHV-4 vaginal re-excretion was not associated with vertical transmission to the litter or with horizontal transmission to female mice. In contrast, we observed efficient virus transmission to naïve males after sexual contact. *In vivo* imaging allowed us to show that MuHV-4 firstly replicated in penis epithelium and *corpus cavernosum* before spread to draining lymph nodes and spleen. All together, those results revealed the first experimental transmission model for MuHV-4 in laboratory mice. In the future, this model could help us to better understand the biology of gammaherpesviruses and could also allow the development of strategies that could prevent the spread of these viruses in natural populations.

Introduction

Gammaherpesviruses are important pathogens which are both ubiquitous in human and animal populations. They establish persistent, productive infections, with virus carriers both making anti-viral immune responses that protect against disease and excreting infectious virions. Most gammaherpesviruses establish a long-term latent infection of circulating lymphocytes. They drive lymphocyte proliferation as part of normal host colonization and consequently they can induce some lymphoproliferative disorders. In humans, Epstein-Barr virus (EBV) and the Kaposi's Sarcoma-associated Herpesvirus (KSHV) are associated with several human malignancies such as Burkitt's and Hodgkin's lymphomas, nasopharyngeal carcinoma, Kaposi's sarcoma and post-transplant lymphoproliferative disease (Verma and Robertson 2003; Thorley-Lawson and Gross 2004). Human cancers associated with these two viruses are particularly prevalent in Africa where they are linked to malaria (Young and Rickinson 2004) and human immunodeficiency virus-1 (HIV-1) infection (Mesri *et al.* 2010). More generally, individuals with inherited or acquired immunodeficiency have an increased risk of developing a malignancy caused by one of these two viruses (Cesarman 2011). Efficient control of these infections is therefore of major interest, particularly in some epidemiological circumstances.

Experimental studies are difficult to perform directly with human gammaherpesviruses because they show limited lytic growth *in vitro* and have no well-established *in vivo* infection model. However, the identification of a closely related virus, Murid Herpesvirus 4 (MuHV-4), in wild rodents offered the possibility of developing a mouse model of gammaherpesvirus pathogenesis (Blaskovic *et al.* 1980). MuHV-4 readily infects laboratory mice (*Mus musculus*) in which it establishes a chronic infection that is harboured for life (Nash *et al.* 2001; Barton *et al.* 2011). We have recently confirmed that mouse is a valuable model for *in vivo* studies of MuHV-4 (Francois *et al.* 2010).

Experimental MuHV-4 infection of laboratory mice typically employs intranasal virus inoculation. When it is performed under general anaesthesia, this leads to a lytic infection of nose and of lung alveolar epithelial cells that is controlled within 2 weeks (Sunil-Chandra *et al.* 1992). It has to be noted that, after intranasal infection of non-anaesthetized mice, which seems most likely to mimic natural conditions, the nose is the only site of non-lymphoid luciferase expression (Milho *et al.* 2009). Virus meanwhile seeds to lymphoid tissue, mainly draining lymph nodes and spleen (Milho *et al.* 2009), and drives the proliferation of latently infected B cells. This peaks at 2 weeks post-infection (p.i.) and is controlled by 4 weeks. A predominantly latent infection of memory B cells then persists (Flano *et al.* 2002). Although differences exist, the other inoculation routes also lead to B-cell

infection, and latently infected B cells can transport MuHV-4 all over the organism. After latency establishment, B cells, along with macrophages and dendritic cells (DCs), harbour latent MuHV-4 infection (Barton *et al.* 2011).

Although MuHV-4 has been studied for more than 30 years (Blaskovic *et al.* 1980), attempts to demonstrate horizontal transmission in laboratory mice have been almost entirely unsuccessful, leaving unresolved how MuHV-4 is spread in wild rodent hosts (Nash *et al.* 2001; Barton *et al.* 2011). Different hypotheses can be mounted to explain these poor results. Firstly, conventional animal caging could not allow physiological behaviours observed in the wild such as scent-marking or male fighting. Secondly, although the MuHV-4 life cycle in mice following experimental infection is considered as well-known, unexplored inoculation routes could lead to important differences.

Methods available to follow viral infections are constantly evolving, becoming more sensitive and efficient. Recently, a bioluminescence imaging technique has been developed to measure the activity of luciferase reporters in living mice noninvasively and repetitively (Contag *et al.* 1998). This technique has been successfully applied to MuHV-4 (Hwang *et al.* 2008; Milho *et al.* 2009; Francois *et al.* 2010). In this study, we pursued this work. This allowed us to detect infectious virus in the genital tract of female mice after the time of latency establishment. This presence of infectious virus in the genital tract of latently infected females was transient and under the dependance of sexual steroid hormones. Strikingly, presence of infectious virus in female genital tract allowed us to observe sexual transmission of MuHV-4 to naïve males.

Material and methods

Ethics Statement. The experiments, maintenance and care of mice complied with the guidelines of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (CETS n° 123). The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Liège, Belgium (Permit Number: 1051). All efforts were made to minimize suffering.

Animals. Females and males BALB/c mice were purchased from Charles River Laboratories. All the animals were housed in the University of Liège, Department of infectious diseases. The animals were infected with MuHV-4 when 6–12 weeks old. Intranasal infections with anaesthesia were in 30 µl aliquots. For luciferase imaging, animals were anaesthetized with isoflurane, injected intraperitoneally with luciferin (150 mg/kg), then scanned with an IVIS Spectrum (Caliper Life Sciences). Animals were routinely imaged after 10 min. For quantitative comparisons, we used Living Image software (Caliper Life Sciences) to obtain the maximum radiance (photons per s per cm^2 per steradian, i.e. photons s^{-1} cm^{-2} sr^{-1}) over each region of interest.

Cells and virus. We used a MuHV-4 strain expressing luciferase under control of the M3 promoter that was described previously (Milho *et al.* 2009). Virus was propagated on BHK-21cells cultured in Dulbecco's modified Eagle's medium (Invitrogen) and supplemented with 2 mM glutamine, 100 U penicillin ml^{-1}, 100 mg streptomycin ml^{-1} and 10% fetal calf serum. Virions were concentrated as described previously (Gillet *et al.* 2007b).

Viral infectivity assays. Virus stocks were titrated by plaque assay on BHK-21 cells (Gillet *et al.* 2007a). Cell monolayers were incubated with virus (2 h, 37 °C), overlaid with 0.3% carboxymethylcellulose (CMC, medium viscosity, Sigma), and 4 days later fixed and stained for plaque counting (Gillet and Stevenson 2007). Infectious virus in organs was measured by homogenizing them after freezing (-80°C) in 6 ml complete medium prior to plaque assay.

Virus detection by infectious centre assay. Virus detection in genital organs cell suspension was assayed by infectious centre assay (ICA) as follows. 5.10^5 BHK-21 cells grown in 6 well cluster dishes (Becton Dickinson) were co-cultured for 5 days at 37°C with ex vivo cell suspension in MEM containing 10% FCS, 2% PS, 0.3% CMC and 5.10^{-5}M of β-mercaptoethanol (Merck). Cells were then fixed and stained for plaque counting.

Viral genome quantification. Viral genome loads were measured by real-time PCR (Francois *et al.* 2010). DNA from organs (100 ng) was used to amplify MuHV-4 genomic co-ordinates 4166–4252 (iCycler, Biorad) (gene M2, forward primer 5’- GTCAGTCGAGCCAGAGTCCAACA-3’, reverse primer 5’-ATCTATGAAACTGCTAACAGTGAAC-3’). The PCR products were quantified by hybridization with a TaqMan probe (genomic co-ordinates 4218–4189, 5’ 6-FAM-TCCAGCCAATCTCTACGAGGTCCTTAATGA-BHQ1 3’) and converted to genome copies by comparison with a standard curve of cloned plasmid template serially diluted in control spleen DNA and amplified in parallel. Cellular DNA was quantified in parallel by amplifying part of the interstitial retinoid binding protein (IRBP) gene (forward primer 5’-ATCCCTATGTCATCTCCTACYTG-3’, reverse primer 5’-CCRCTGCCTTCCCATGTYTG-3’). The PCR products were quantified with Sybr green (Invitrogen), the copy number was calculated by comparison with standard curves of cloned mouse IRBP template amplified in parallel. Amplified products were distinguished from paired primers by melting curve analysis and the correct sizes of the amplified products confirmed by electrophoresis and staining with ethidium bromide.

Detection of infectious virus in vaginal fluids. Vaginal lavage fluids were obtained by gentle flushing of the mouse vagina with 100µl of sterile PBS. Lavage fluids were then centrifuged and the supernatant was titrated as described above.

Ovariectomy and hormonal supplementation. Ovariectomy were performed at 3 weeks of age under isoflurane anaesthesia. Hormonal treatment was started 3 weeks after ovariectomy. 60 days slow-release pellets (Innovative Research of America, Sarasota, FL, USA) containing 0.05 mg 17β-estradiol (SE-121), or 25 mg progesterone (SP-131) per pellet were implanted subcutaneously, giving a release of ~0.8 µg 17β-estradiol or ~400 µg progesterone per 24 hours. Groups of mice were the following: 10 non ovariectomized mice, 7 ovariectomized mice and 5 ovariectomized mice complemented per hormonal condition.

***In vitro* and *ex vivo* hormonal stimulation.** 17-β-estradiol (Sigma) stock solution was prepared in DMSO (1 mg/ml). For *in vitro* stimulation, A20-Syndecan-1 cells (Bennett *et al.* 2005) were persistently infected with a MuHV-4 strain expressing eGFP under an EF1a promoter, between the 3' ends of ORFs 57 and 58 (Dr P.G. Stevenson, unpublished data). For *ex vivo* stimulation, spleen of WT MuHV-4 intranasally infected mice were harvested 14 days post-infection, cells were dissociated and erythrocytes were lysed by using red blood cells lysis buffer. Cells were cultivated in RPMI medium without phenol red, to avoid the presence of steroids, supplemented with 2 mM glutamine, 100 U penicillin ml^{-1}, 100 mg streptomycin ml^{-1}, $5*10^{-5}$M of β-mercaptoethanol (Merck) and 10% Charcoal Stripped Fetal Bovine Serum (CSFBS, Sigma). Stimulation of virus reactivation by

17-β-estradiol was performed as follows. Briefly, $3*10^5$ BHK-21 cells grown in 6 well cluster dishes were co-cultured for 5 days at 37°C with $5*10^3$ MuHV-4 infected A20 cells or $5*10^5$ infected spleen cells (from 5 mice) in RPMI containing 10% CSFBS, 2% PS, 0.3% CMC, 5.10^{-5}M of β-mercaptoethanol (Merck) and complemented with increasing doses of 17-β-estradiol. After 5 days, cells were fixed and stained for plaque counting.

Organ histology and immunohistochemistry. Portions of genital organs were fixed in buffered formol saline, processed routinely to 5-mm paraffin wax-embedded sections, stained with hematoxylin and eosin, and examined by light microscopy. Immunohistochemistry was performed using EnVision™ Detection Systems (DAKO) with anti-MuHV-4 rabbit hyperimmune serum against MuHV-4 as primary antibody (Francois *et al.* 2010).

Quantification of anti-MuHV-4 specific antibodies by ELISA. Nunc Maxisorp ELISA plates (Nalgene Nunc) were coated for 18 h at 37°C with 0.1% Triton X-100-disrupted MuHV-4 virions (2.10^6 PFU/well), blocked in PBS/ 0.1% Tween-20/ 3% BSA, and incubated with mouse sera (diluted 1/200 in PBS/ 0.1% Tween-20/ 3% BSA). Bound antibodies were detected with Alkaline Phosphatase conjugated goat anti-mouse Ig polyclonal antibody (Sigma). Washing were performed with PBS/ 0.1% Tween-20/ 3% BSA. p-Nitrophenylphosphate (Sigma) was used as substrate and absorbance was read at 405nm using a Benchmark ELISA plate reader (Thermo).

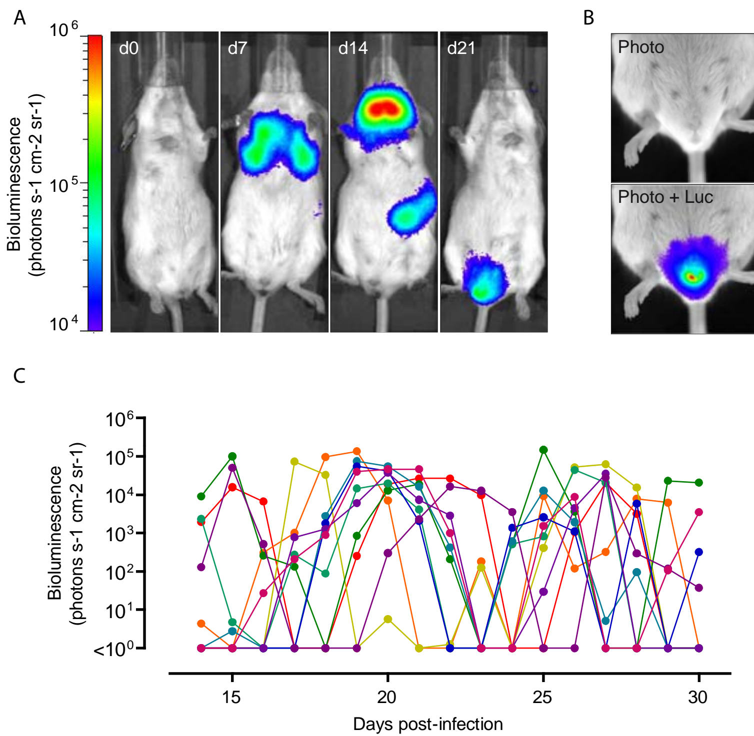


Figure 1. *In vivo* infection by luciferase-expressing MuHV-4. Female mice were infected intranasally (10^4 PFU) with WT luciferase$^+$ MuHV-4 under general anaesthesia, and then injected with luciferin and imaged every days. The results presented are representative of at least 5 experiments, each including 10 mice. **A.** Images show a representative mouse at days 0, 7, 14 and 21 post-infection (p.i.). **B.** Specific signal from the genital region was highlighted in an equivalent mouse. The scale bar (photons sec^{-1} cm^{-2} steradian^{-1}) shows the color scheme for signal intensity. The same scale bar is used in A and B. **C.** Temporal progression of the genital signal in different mice (each curve represents one mouse). For the reliable comparison of signal intensities, the signal intensities were measured from equivalent regions of interest after subtraction of individual backgrounds measured in the right abdominal region.

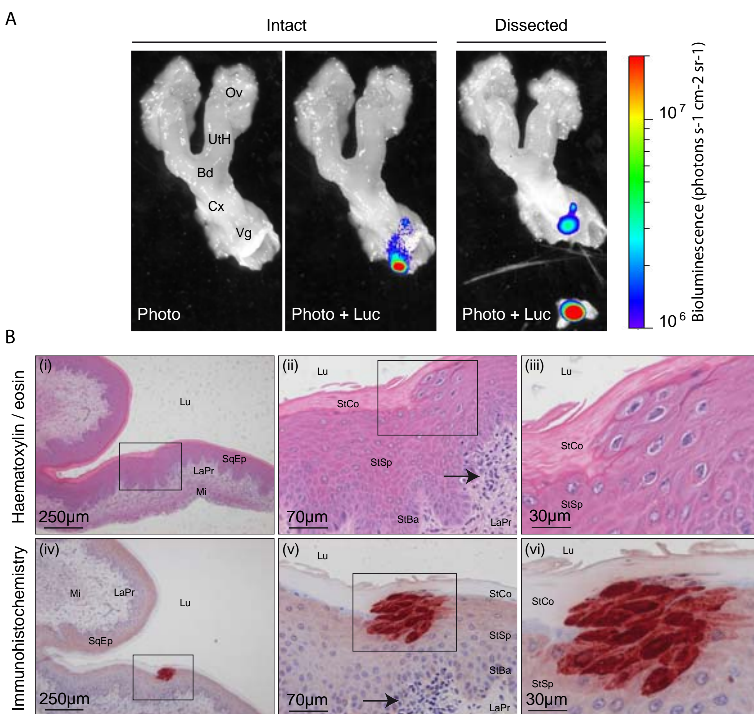


Figure 2. Luciferase signal and MuHV-4 antigen detection in isolated genital tract after intranasal infection. A. A mouse equivalent to those in Figure 1 was dissected and its genital tract imaged *ex vivo*. The images are representative of data from at least 10 mice, and show either a standard photograph (Photo) or that photograph overlaid with the luciferase signal (Photo + Luc). The region with the highest signal was isolated and processed for histological analysis. The scale bar (photons sec^{-1} cm^{-2} steradian^{-1}) shows the color scheme for signal intensity. Ov, ovary; UtH, uterine horn; Bd, body of the uterus; Cx, cervix; Vg, Vagina. **B.** The piece of vagina isolated in A. was fixed in formaldehyde and organ slices were either stained with hematoxylin-eosin (panels i to iii) or processed for immunohistochemistry with anti-MuHV-4 rabbit polyserum (panels iv to vi) as described in the Materials and Methods section. Rectangles indicate regions highlighted in the following panels. Arrows indicate focal recruitment of leukocytes at the periphery of MuHV-4 antigen detection. Lu, lumen; SqEp, stratified squamous epithelium; LaPr, lamina propria; Mi, Muscularis; StCo, stratum corneum; StSp, stratum spinosum; Stba, stratum basale.

Results

MuHV-4 reaches female genital tract after intranasal infection. The main advantage of whole body imaging of luciferase-expressing MuHV-4 cycle in living mice is the revelation of novel sites of viral replication. Therefore, we infected 6 weeks female BALB/c mice intranasally under general anaesthesia with 10^4 PFU of luciferase$^+$ MuHV-4 and tracked infection daily by luciferin injection and charge-coupled-device camera scanning. Representative images are shown in Figure 1A. As previously described (Hwang *et al.* 2008; Milho *et al.* 2009; Francois *et al.* 2010), we observed signals coming from the nose (d4 p.i.), the thoracic region (d7 p.i.), the neck (d14 p.i.) and the left abdominal region (d14 p.i.). Based on former descriptions (Hwang *et al.* 2008; Milho *et al.* 2009; Gaspar *et al.* 2011), we considered the nose signals to come from the nasal turbinates (Gaspar *et al.* 2011); thoracic signals from the lungs; neck signals from the superficial cervical lymph nodes (SCLNs); and the abdominal signals from the spleen. As previously described (Hwang *et al.* 2008; Milho *et al.* 2009; Francois *et al.* 2010), the nose and lung signals peaked at 5-7 days after infection and were undetectable after day 14. On the opposite, signal appeared around day 7 in SCLNs and was maximal at day 14, the peak of latency amplification. SCLNs signal then disappeared over the two following weeks. Signals appeared in the spleens around day 10 but were more transient and randomly observed than in the SCLNs. Anyway, the observation of spleen signal revealed virus dissemination over the body, likely through B-cells transport.

Surprisingly, we randomly observed appearance of luciferase signal in the genital region of infected female mice (Figure 1A-B). This signal appeared after the initial clearance of acute lytic replication in nose and lungs. Moreover, the signal in the genital region was concomitant or appeared after disappearance of the SCLNs and spleen signals (Figure 1A-B and Figure S1). To further investigate MuHV-4 replication in the female genital region, we followed it over time among different mice (Figure 1C). Interestingly, ~80% of the mice displayed luciferase signal in the genital region during this period. This signal was transient (no more than 4 consecutive days) and recurrent. To confirm the sites of infection and to further investigate the origin of the signal, *ex vivo* imaging of individual organs was performed after euthanasia of luciferase$^+$ MuHV-4 infected mice. This approach revealed that the luciferase signal observed in the genital region was coming from small regions of the vagina (Figure 2A). Fragments of vagina identified as positive for light emission were dissociated from the rest of the organs (Figure 2A) and processed for histological analysis. Immunohistochemical staining for viral antigens identified focal sites of MuHV-4 antigen expression in the superior layers of the vaginal epithelium (Figure 2B). This was associated with morphological changes of infected cells

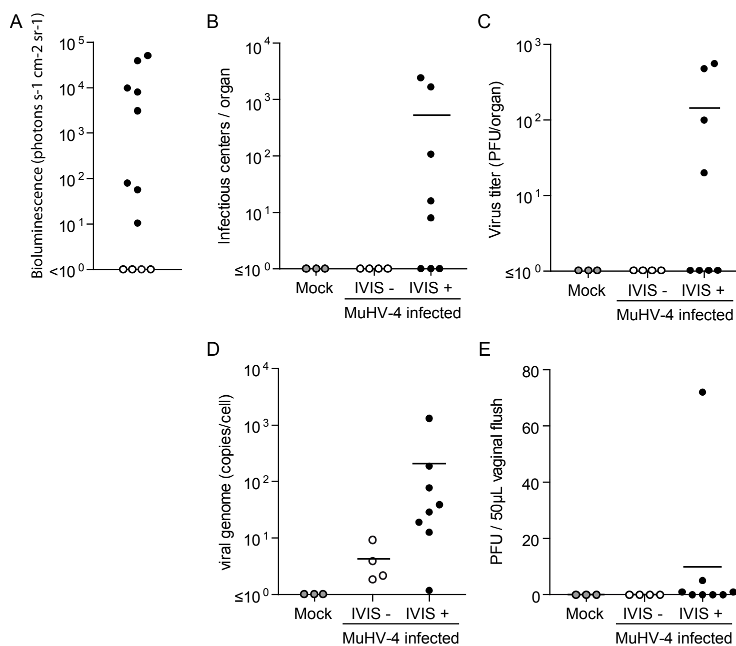


Figure 3. Quantification of MuHV-4 infection in female genital tract after intranasal infection. A. 12 female mice were infected intranasally (10^4 PFU) with WT luciferase$^+$ MuHV-4 under general anaesthesia. 23 days post-infection, luciferase signal in the genital region was assessed and mice were categorized as IVIS- (white dots) and IVIS+ (black dots). For the reliable comparison of signal intensities, the signal intensities were measured from equivalent regions of interest after subtraction of individual backgrounds measured in the right thoracic region. **B-D.** Individual genital tracts were removed at day 23 p.i. and assayed individually for the presence of MuHV-4 by infectious center assay (B), infections virus titration (C) and viral genome quantification (D). Groups were compared by student t-test (*****P* < 0.0001). **E.** Vaginal flush samples collected before euthanasia were tested for the presence of infectious virus. Samples from mock infected mice (grey dots) were used as controls.

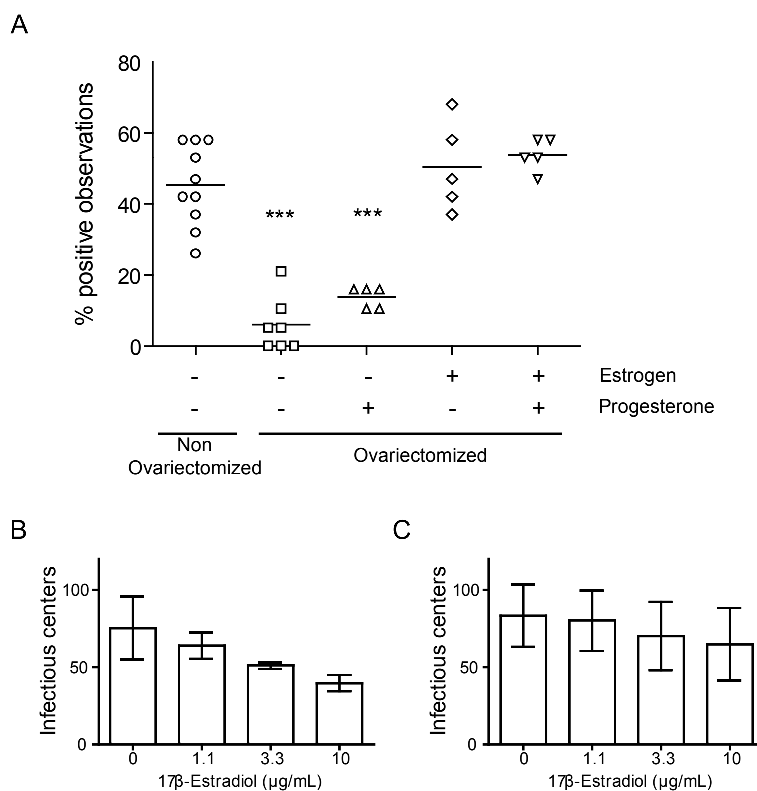


Figure 4. Influence of oestrus cycle on genital MuHV-4 reexcretion after intranasal infection. A. Control female mice (n=10) and ovariectomized mice, implanted (n=5 per condition) or not (n=7) with slow-realease hormonal pellets (progesterone and/or oestrogen), were infected intranasally (10^4 PFU) with WT luciferase$^+$ MuHV-4 under general anaesthesia. Presence of genital signal was monitored between days 14 and 32 post-infection and percentages of positive observations were recorded individually. For the reliable comparison of signal intensities, the signal intensities were measured from equivalent regions of interest after subtraction of individual backgrounds measured daily in the right thoracic region. Each point shows percentage of positive observation for one animal. Groups were compared by ANOVA1 and Bonferroni post-test (****P* < 0.001). **B-C**. Stimulation of MuHV-4 reactivation from persistently infected cells with 17β-Estradiol was tested. MuHV-4 persistently infected A20 cells (B) or bulk splenocytes (C), obtained from 5 mice 14 days following MuHV-4 intranasal inoculation (10^4 PFU), were analyzed for the frequency of cells reactivating virus with and without increasing concentrations of 17β-Estradiol as described in the Materials and Methods section. The data presented are the average for triplicate measurements +/- standard error of the mean and were analyzed by 1way ANOVA and Bonferroni posttests, no statistically significant difference was observed upon treatment.

(Figure 2B, panel iii) and with the presence of leukocytic infiltrate in the lamina propria (Figure 2B, panels ii and v). These lesions were not observed every time, likely because of their restricted size.

MuHV-4 presence in vagina is associated with release of infectious virions. In order to further investigate this observation, 12 mice were infected intranasally and light emission from the genital region was measured 23 days p.i. (Figure 3A). This allowed us to categorize mice into two groups: the first in which genital signal was observed was called IVIS+ and the other IVIS-, three uninfected mice were used as mock infected controls. Genital tracts of these mice were isolated as shown in Figure 2A and light emitting regions of the vagina were isolated. Equivalent regions were isolated in mock and IVIS- groups. These different samples were then analyzed by infectious center assays, infectious virus titration and viral genome quantification (Figure 3B-D). These experiments identified the presence of reactivable virus (Figure 3B) and infectious virions (Figure 3C) only in the IVIS+ group. Moreover, there were statistically more copies of MuHV-4 genome in the IVIS+ samples than in the IVIS-. Finally, titration of vaginal lavage fluids, collected before euthanasia, revealed the presence of infectious virions in half of the IVIS+ samples (Figure 3E). The latter experiment was repeated on a higher number of mice between days 21 and 30 post-infection (Figure S2). This revealed that excretion of infectious MuHV-4 virions in female genital tracts occurred randomly and was limited in terms of number of PFUs. All together, these experiments showed that MuHV-4 luciferase signal in female genital tract is associated with the presence of infectious virus in the vaginal epithelium and in the vaginal fluids. This could therefore represent a potential portal of transmission of this virus.

Oestrogens indirectly influence MuHV-4 excretion in female genital tract. Random and recurrent observations of MuHV-4 associated luciferase signal in female genital tract suggest an association of this phenomenon with the oestrus cycle. To investigate this possibility, we compared occurrence of MuHV-4 associated luciferase signal in genital tract among groups of control and ovariectomized female mice between days 14 and 32 post-infection (Figure 4A and S3). This revealed that ovariectomy greatly diminished observation of MuHV-4 associated luciferase expression in the genital tract (Figure 4A and S3) although the global levels of MuHV-4 latency were not affected by the treatment (data not shown). In order to indentify if it was associated with specific hormonal deprivation, we implanted ovariectomized mice with slow-release progesterone and/or oestrogen pellets (Figure 4A and S3). Oestrogens alone or in combination with progesterone were sufficient to restore occurrence of genital luciferase signal to levels similar to the ones observed in the non ovariectomized group.

To determine whether oestrogen treatment can trigger MuHV-4 reactivation from latently infected cells, we used murine A20 B cells latently infected with MuHV-4 (Figure 4B) or explanted

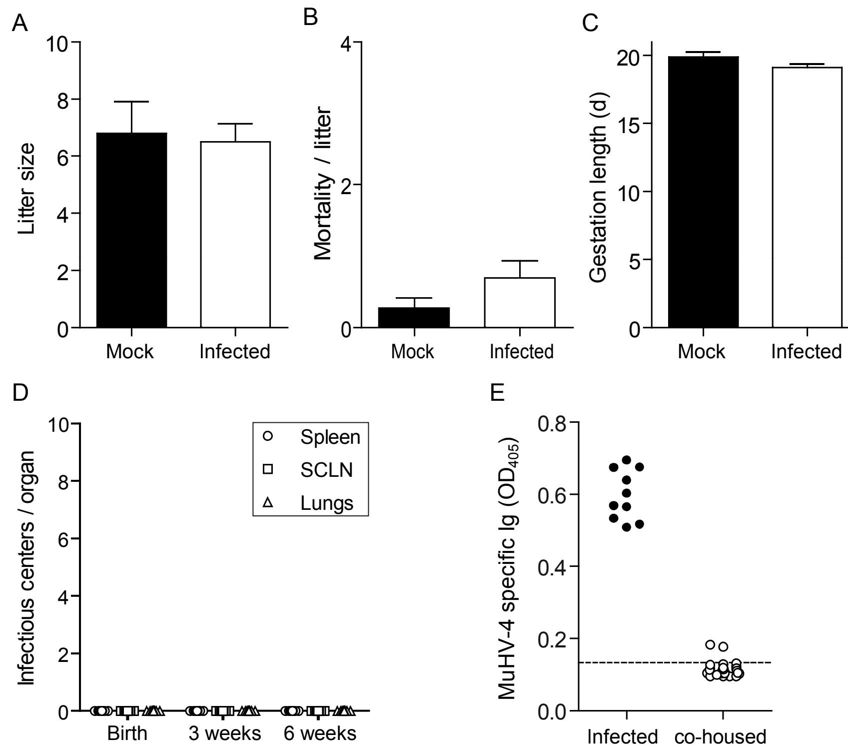


Figure 5. Vertical and non sexual transmissibility of MuHV-4 from virus-excreting mice. A-D. Female mice were infected intranasally (10^4 PFU) with WT luciferase$^+$ MuHV-4 under general anaesthesia, and then injected with luciferin and imaged every day. At the time of the first observation of genital signal, infected females were mated with uninfected males. Mock infected female mice were used as controls. Effect of MuHV-4 infection on litter size (A), mortality/litter (B) and gestation length (C) was then monitored. The data presented are the average for 20 (infected) and 11 (mock) pregnancies +/- standard error of the mean and were analyzed by 1way ANOVA and Bonferroni posttests, no statistically significant difference was observed. Transmission to the progeny (n≥20 per group) was assessed by infectious center assays performed on isolated organs taken from newborn or at 3 or 6 weeks after birth (C). Data are plotted individually. **E.** Female mice (n=10) were infected intranasally (10^4 PFU) with WT luciferase$^+$ MuHV-4 under general anaesthesia, and then injected with luciferin and imaged every day. At the time of the first observation of genital signal, infected females were co-housed with 3 uninfected females. Potential MuHV-4 transmission was monitored 45 days later by detection of anti-MuHV-4 specific antibodies. The dashed line indicates the mean value obtained with sera from 3 uninfected mice taken as controls.

splenocytes from MuHV-4 infected mice, 14 days p.i. (Figure 4C). These cells were treated with increasing amounts of 17β-Estradiol and MuHV-4 reactivation was analyzed by infectious center assays. The results obtained did not show that oestrogen stimulation of latently infected cells induces MuHV-4 reactivation. The observed effect of oestrogens on occurrence of MuHV-4 associated luciferase signal (Figure 4A and S3) is therefore likely indirect.

Genital excretion of MuHV-4 is not associated with vertical transmission to the litter or horizontal transmission between female mice. The presence of MuHV-4 replication in latently infected females could affect gestation. To investigate this hypothesis, luciferase+ MuHV-4 infected female mice were mated with uninfected males at the time of the first observation of genital signal. Mock infected female mice were used as controls. Effect of MuHV-4 infection on litter size (Figure 5A), mortality/litter (Figure 5B) and gestation length (Figure 5C) was then monitored. We did not observe any effect of MuHV-4 infection on any of these parameters (Figure 5A-C). Moreover, we also did not observe transmission to the progeny either at birth or after 3 or 6 weeks (Figure 5D). Similarly, we also did not observe seroconversion (Figure 5E) or detectable levels of MuHV-4 DNA in the spleen of co-housed naïve female mice (data not shown).

Genital excretion of MuHV-4 is associated with sexual transmission. To determine whether the presence of infectious virus in the vaginal epithelium and in the vaginal fluids can result in sexual transmission of MuHV-4, we mated luciferase+ MuHV-4 infected female mice with uninfected males at the time of the first observation of genital signal. We then tested transmission to males by serology at day 10 post-contact and more than 20 days post-contact. Interestingly, we observed seroconversion of 10 individuals among the 30 males that were tested (Figure 6A). As this seroconversion was moderate in comparison to the one observed after intranasal infection (Figure 6A), presence of MuHV-4 DNA in spleens was tested. At least 9 out of the 10 previously identified males displayed detectable levels of MuHV-4 DNA in the spleen (Figure 6B). All together, these results therefore show that MuHV-4 can be transmitted from infected female mice to naïve males.

Transmission to males is associated with penis infection. To determine the route of MuHV-4 transmission to naïve males, we repeated the previous experiment and tracked MuHV-4 infection of males daily by luciferin injection and charge-coupled-device camera scanning (Figure 7). We observed that light emission appeared in the genital region around 4 days post-contact. This signal peaked around 10 days post-contact but was maintained for at least 3 weeks. To confirm the site of infection and to further investigate the origin of the signal, *ex vivo* imaging of individual organs was performed after euthanasia of luciferase^{+} MuHV-4 infected males at different time points. This approach revealed that the luciferase signal observed in the genital region was coming from small regions of the penis (Figure 8A). Fragments of the penis identified as positive for light emission were dissociated from the

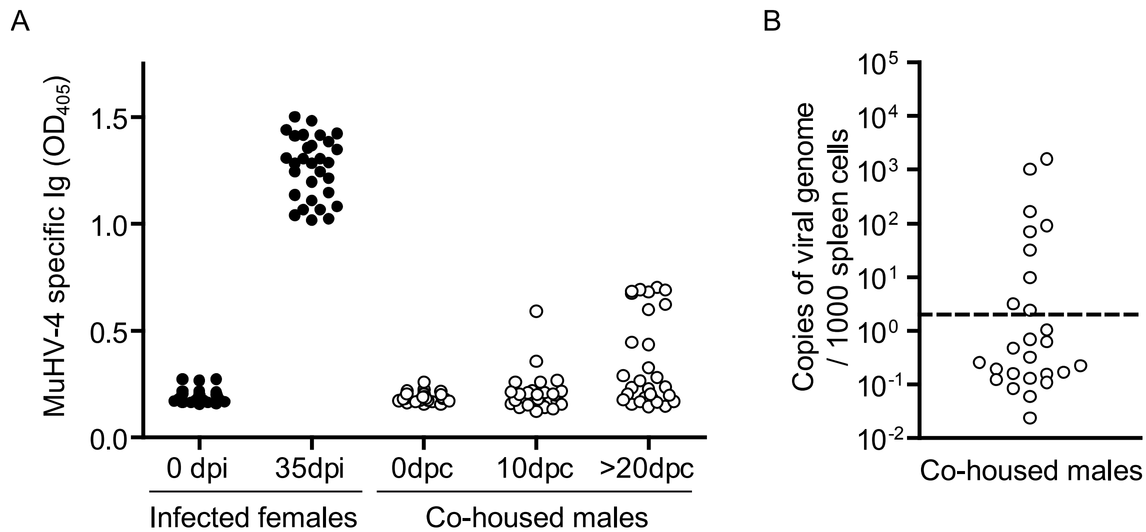


Figure 6. Sexual transmission of MuHV-4 from virus-excreting female mice. A-B. Female mice (n=30) were infected intranasally (10^4 PFU) with WT luciferase$^+$ MuHV-4 under general anaesthesia, and then injected with luciferin and imaged every day. At the time of the first observation of genital signal, infected females (3 per cages, total n=30) were mated with uninfected males (3 per cages, total n=30). MuHV-4 infection was monitored at the indicated times by detection of anti-MuHV-4 specific antibodies (A) or quantification of viral genomes in male spleens performed after at least 20 days post-contact (B). The dashed line shows the lower limit of the assay sensitivity. Dpi, days post-infection; dpc, days post-contact.

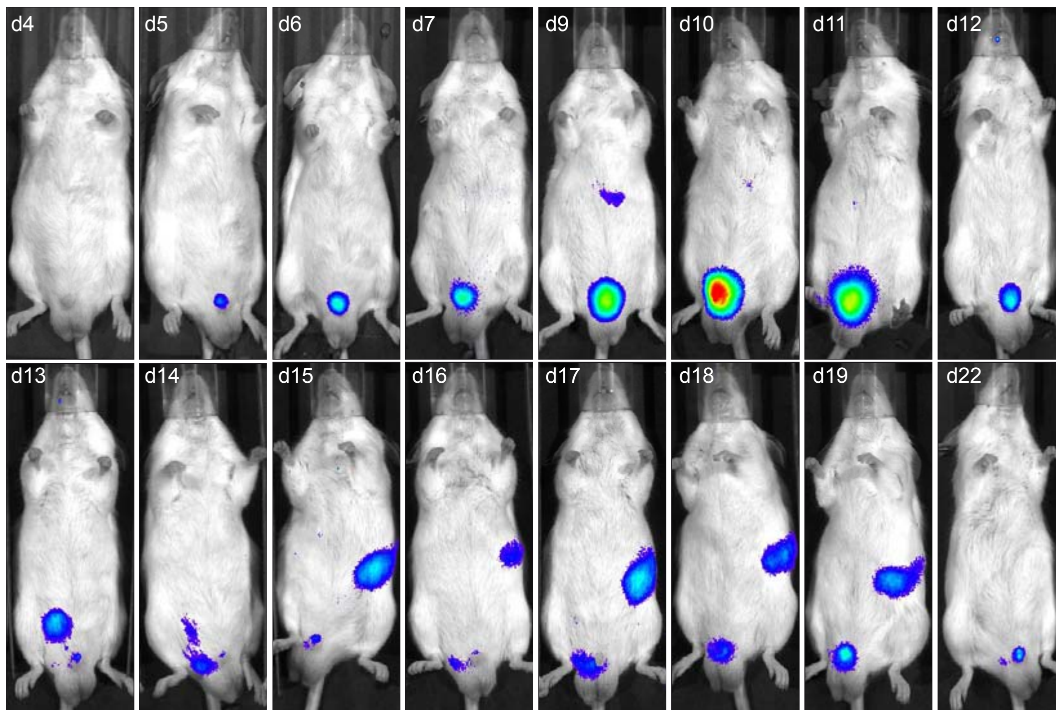


Figure 7. Spatial and temporal progression of MuHV-4 infection after sexual transmission to male mice. Female mice were infected intranasally (10^4 PFU) with WT luciferase$^+$ MuHV-4 under general anaesthesia, and then injected with luciferin and imaged every day. At the time of the first observation of genital signal, infected females were mated with uninfected males. The males were then injected with luciferin and imaged every day. Images show a representative mouse over time. The day post-contact with the infected female (e.g., d4 is day 4 post-contact) is shown at the top of each image. Pictures are representative of at least 15 males from 3 independent experiments.

rest of the organ (Figure 8A) and processed for histological analysis. Immunohistochemical staining for viral antigens identified focal sites of MuHV-4 antigen expression in the superior layers of the penis epithelium and of the corpus cavernosum (Figure 8B). Viral antigens were also detected in deeper regions of the Corpus cavernosum (Figure 8B, panel iii). Penis infection was associated with propagation of the infection to draining lymph nodes. *Ex vivo* imaging revealed that they were mainly lumbar aortic medial iliac lymph nodes (Figure 9). Light emitted by these lymph nodes had already been observed during imaging of living animals (Figure 7, days 13 to 17). Finally, colonization of the spleen was observed (Figure 7, days 15 to 19) as already showed by viral genome detection (Figure 6B). All together, these results show that MuHV-4 can be sexually transmitted between laboratory mice.

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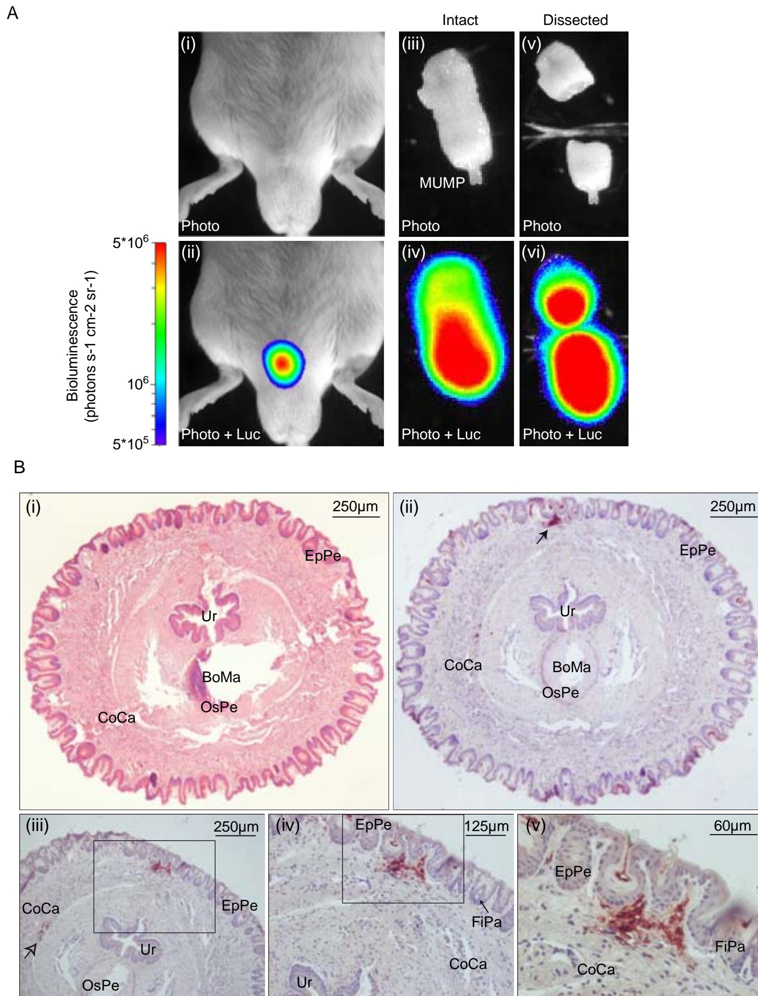

Figure 8. Luciferase signal and MuHV-4 antigen detection in isolated male genital tract after sexual transmission. A. A mouse equivalent to that in Figure 7 was dissected and its genital tract imaged *ex vivo* at day 10 post-contact with the infected female. The images are representative of data from at least 5 mice, and show either a standard photograph (Photo) or that photograph overlaid with the luciferase signal (Photo + Luc). The region with the highest signal was isolated and processed for histological analysis. The scale bar (photons sec^{-1} cm^{-2} steradian^{-1}) shows the color scheme for signal intensity. MUMP, male urogenital mating protuberance. **B.** The piece of penis isolated in A. was fixed in formaldehyde and organ slices were either stained with hematoxylin-eosin (panel i) or processed for immunohistochemistry with anti-MuHV-4 rabbit polyserum (panels ii to v) as described in the Materials and Methods section. Rectangles indicate regions highlighted in the following panels. Filled and open arrows indicate detection of MuHV-4 antigens in superficial regions of the penis and in deeper region of the Corpus cavernosum, respectively. Ur, urethra; EpGl, Epithelium of the glans; BoMa, Bone marrow; OsPe, Os penis; CoCa, Corpus cavernosum; FiPa, Filiform papilla.

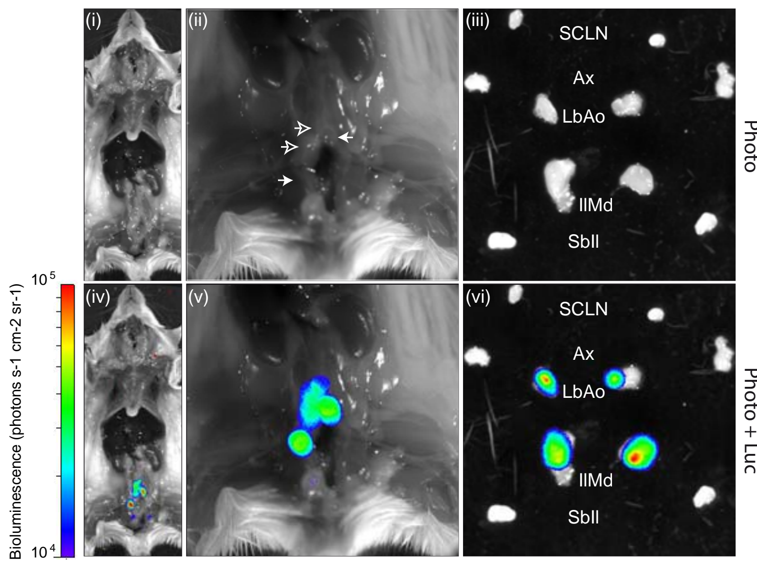

Figure 9. Direct visualization of lymph node colonization after sexual transmission of MuHV-4 infection. A mouse equivalent to those in Figure 8 and 9 was dissected 2 weeks post-contact with the infected female. The images are representative of data from at least 5 mice, and show either a standard photograph (Photo) or that photograph overlaid with the luciferase signal (Photo + Luc). The entire body (panels i and iv) and the pelvis region (panels ii and v) are shown after displacement of the genital tract. Panels iii and vi show isolated lymph nodes. The scale bar (photons sec^{-1} cm^{-2} steradian^{-1}) shows the color scheme for signal intensity. SCLN, superficial cervical lymph nodes; Ax, axillary lymph nodes; LbAo, lumbar aortic lymph nodes; IlMd, medial iliac lymph nodes; SbIl, subiliac lymph nodes.

Discussion

Transmission in host population is the main motor of viral evolution (Derdeyn *et al.* 2004; Keele and Derdeyn 2009; Alizon *et al.* 2011). Herpesviruses have co-evolved with their host for millions of years and have therefore developed sophisticated mechanisms to persist and transmit in presence of protective immune response (Griffin *et al.* 2010; Lee *et al.* 2010). This is particularly well illustrated by the fact that infection by multiple herpesvirus strains in a single individual is not a rare event. Indeed, recent studies on Herpes Simplex viruses (HSV) have found multiple-strains genital infection in 15% of healthy adults infected with genital HSV-1 and HSV-2 (Roest *et al.* 2004; Roest *et al.* 2006). Similarly, multiple strains of human cytomegalovirus (HCMV) are detected in more than 90% of HCMV-infected women and these can even be congenitally transmitted together to infants (Novak *et al.* 2008; Ross *et al.* 2011). Understanding how they do this and what countermeasure can be taken is therefore of major importance for the development of new prophylactic or therapeutic strategies. Until now, most of the immune evasion strategies of gammaherpesviruses have been studied *in vitro* or in animal models (Barton *et al.* 2011). However, none has been investigated in the light of transmission mainly due to the lack of experimental transmission model.

EBV and KSHV, the two human gammaherpesviruses, are host-specific and cannot be experimentally studied *in vivo*. The identification of a closely related viral species, MuHV-4, in wild rodents (Blaskovic *et al.* 1980) opened therefore new horizons to the study of gammaherpesviruses. However, despite several attempts, MuHV-4 transmission had never been observed. In this study, using *in vivo* imaging, we observed that MuHV-4 is genitally reexcreted after latency establishment in intranasally infected female mice (Figures 1-3, S1 and S2). This allowed us to observe, for the first time, experimental transmission to naive males after sexual contact (Figures 6-9).

The first question is why genital reexcretion of MuHV-4 has never been documented before. Firstly, in comparison with initial investigations, we used a very powerful approach able to identify very few infected cells (Costes *et al.* 2009; Francois *et al.* 2010). As MuHV-4 genital reexcretion is weak and transient, similar reexcretion could have happened in other studies but could have been below their detection threshold. Interestingly, the two previous studies using *in vivo* luciferase imaging of MuHV-4 cycle identified potential genital infection. Milho *et al.* showed that the female genital tract is a site of virus replication after intraperitoneal infection (Milho *et al.* 2009) and one of the mice used by Hwang *et al.* displayed light emission in the genital region (Figure 2A, day 18 p.i. (Hwang *et al.* 2008)). Interestingly, similarly to our observations, this mouse had also been infected intranasally

and the signal appeared after latency establishment (Hwang *et al.* 2008). The latter observation strengthens the results presented in our study. Secondly, the frequency of genital signal observation in our study (~80% of the infected mice) could reflect particular experimental conditions. For example, co-infection with other pathogens could favour MuHV-4 genital excretion as observed for HSV-2 and human immunodeficiency virus-1 which enhance reciprocal virus replication (Van de Perre *et al.* 2008). However, despite several attempts, we did not manage to identify another specific infection that could explain our observations.

The observation of genital signal in females was transient and recurrent (Figure 1C). Moreover, this was dependent on the oestrus cycle as ovariectomy nearly abolished the phenomenon and as oestrogens supplementation restored it (Figure 4). However, we did not observe any direct effect of oestrogens on latently infected B-cells either *in vitro* or *ex vivo* (Figure 4 B and C). Similar observations of a role of the oestrus cycle on virus reactivation have been made for HSV-1 (Vicetti Miguel *et al.* 2010). In this case, HSV-1 reactivation through 17β-Estradiol was leukocyte independent but depended on oestrogen receptor activation (Vicetti Miguel *et al.* 2010). Further experiments will be required to identify the mechanism involved in MuHV-4 reactivation. The fact that the genital signal lasted for at most 3-4 days (Figure 1C) could be linked to the cyclic remodelling of the epithelium observed during the oestrus cycle. Indeed, infected cells are located in the superior layers of the vaginal epithelium (Figure 2B) and could therefore be removed at each cycle. How MuHV-4 reaches this location is still unknown. In the future, we could investigate this hypothesis by using cell-type specific Cre/lox genetic labelling of MuHV-4 to track the route of viral reexcretion *in vivo* as it has recently been done to explore the *in vivo* entry pathway (Gaspar *et al.* 2011).

In contrast to what was reported by Stiglincova *et al.* (Stiglincova *et al.* 2011), we did not observe premature termination of pregnancy, reduced number of newborns, vertical transmission or transmission through milk of MuHV-4 in mice (Figure 5). We have no explanation for this discrepancy. However, mother to child transmission of human gammaherpesviruses, both transplacental or perinatal, seems also to be very limited (Meyohas *et al.* 1996; Pica and Volpi 2007). On the opposite, sexual transmission has been proposed for both viruses (Crawford *et al.* 2002; Thomas *et al.* 2006; Pica and Volpi 2007). The observation of MuHV-4 sexual transmission from infected females to naive males is therefore particularly interesting in that context.

In males, initial infection was localized in the superior layers of the penis epithelium and of the corpus cavernosum (Figure 8B). Infection then spreads to draining lymph nodes and spleen (Figure 7 and 9). Again, cell-type specific Cre/lox genetic labelling of MuHV-4 (Gaspar *et al.* 2011) will be helpful to track the route of viral infection after sexual transmission. On the opposite, horizontal

transmission of the infection was not observed. Moreover, infectious virions were rarely detected in vaginal lavages although MuHV-4 induced luciferase signal was frequent. Therefore, we hypothesize that close contacts between genital organs of males and females were necessary to transmit infection. Indeed, the penis of the male mice is recovered of spines called filiform papilla. These structures could therefore induce abrasion of the vaginal epithelium and promote virus transmission. Interestingly, cells that were initially infected on penis were located around these filiform papilla (Figure 8B). Infection persisted at this site for at least three weeks (Figure 7). The importance of this observation for MuHV-4 epidemiology and transmission to females will therefore have to be tested. Interestingly, Telfer *et al.* showed that gammaherpesvirus (identified serologically as MuHV-4, though likely Wood Mouse Herpesvirus) infection in wood mice was more prevalent in heaviest males than in any other category of animal (Telfer *et al.* 2007). Finally, it has recently been shown that male circumcision significantly reduces the incidence of HSV-2 and HIV-1 infection and the prevalence of HPV infection (Golden and Wasserheit 2009; Tobian *et al.* 2009). Our results suggest that it could also be the same for some gammaherpesviruses.

The identification of a route of transmission for MuHV-4 in mice will allow testing the importance of various immune evasion strategies in the context of infection epidemiology. Thus, we have shown that the gp150 glycoprotein acts as an immunogenic decoy, distorting the MuHV-4-specific antibody response to promote Fc receptor-dependent infection and so compromise virion neutralization (Gillet *et al.* 2007b). In the future, it will therefore be interesting to test this hypothesis at the scale of a population. Similarly, it will be possible to test prophylactic vaccinal strategies in physiological conditions as well as antiviral and therapeutic vaccines, not only used to improve the clinical course in individual patients but also to potentially decrease virus shedding and hence transmission.

All together, in this study we identified for the first time a genital reexcretion site of MuHV-4 after latency establishment in intranasally infected female mice. This has allowed us to observe sexual transmission of the virus from infected females to naïve males. These results open new perspectives for the study of gammaherpesvirus in particular but also for the study of sexually transmitted infections in general.

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Supplemental figures

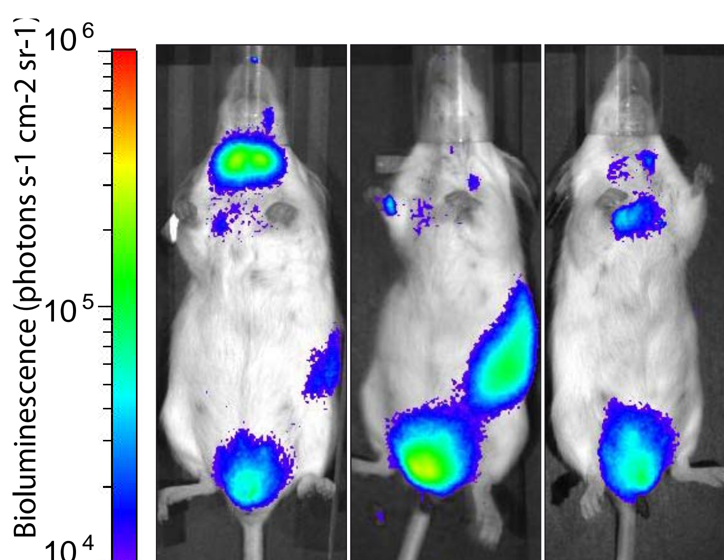

Figure S1. *In vivo* infection by luciferase-expressing MuHV-4. Female mice were infected intranasally (10^4 PFU) with WT luciferase$^+$ MuHV-4 under general anaesthesia, and then injected with luciferin and imaged every days. Images show a representative mouse around 2 weeks p.i. The scale bar (photons sec^{-1} cm^{-2} steradian^{-1}) shows the color scheme for signal intensity.

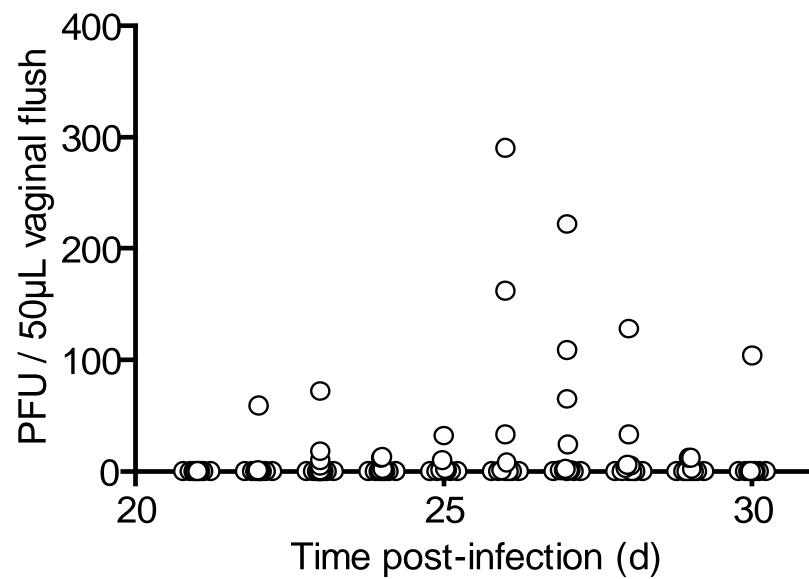

Figure S2 Quantification of infectious MuHV-4 virions in vaginal flushes after intranasal infection. Female mice were infected intranasally (10^4 PFU) with WT luciferase$^+$ MuHV-4 under general anaesthesia. Individual vaginal flush samples (at least 10 per time point) were collected between day 21 and 30 p.i. and were tested for the presence of infectious virions as described in the Material and Methods.

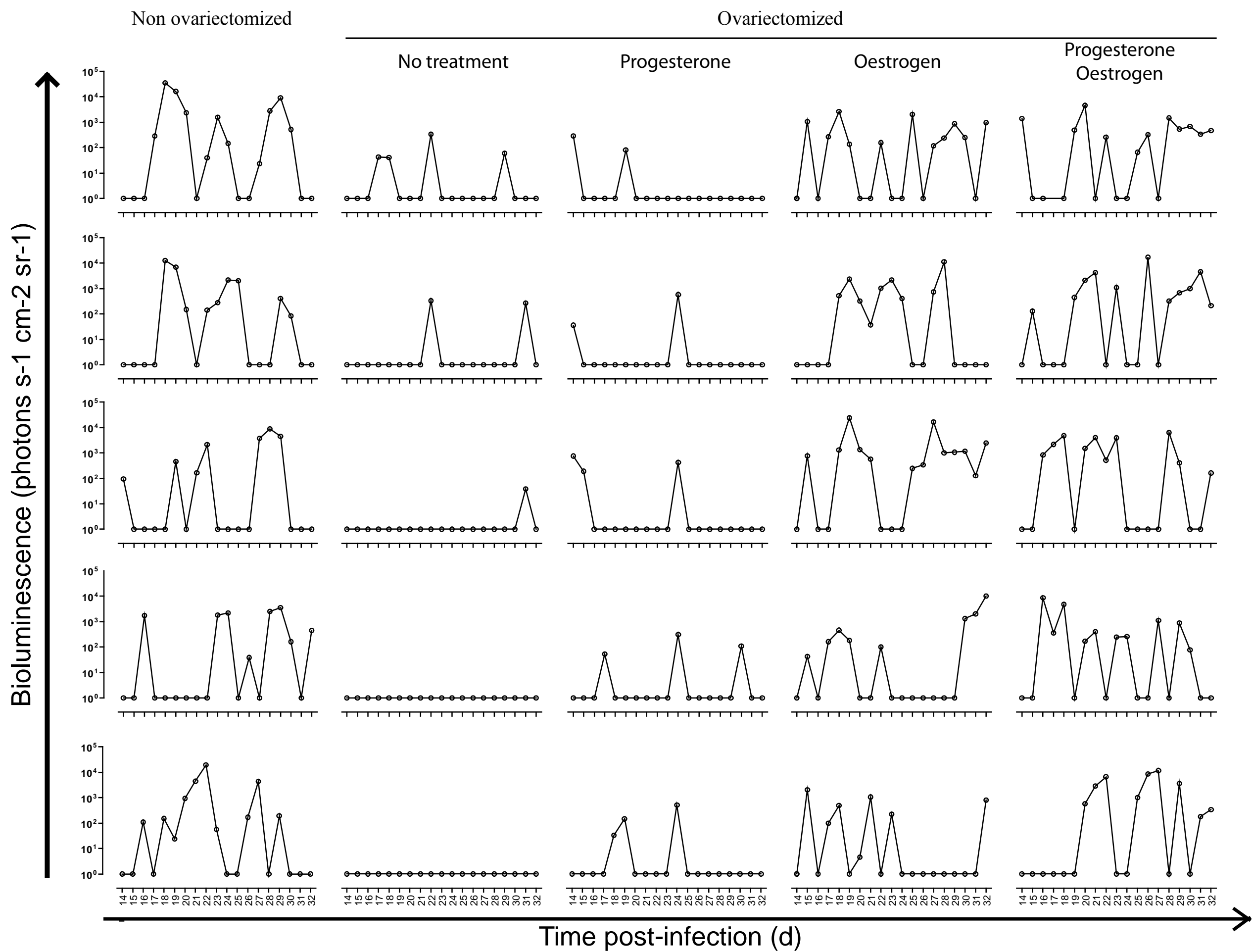

Figure S3. Influence of oestrus cycle on genital MuHV-4 reexcretion after intranasal infection. Control female mice and ovariectomized mice, implanted or not with slow-realease hormonal pellets (progesterone and/or oestrogen), were infected intranasally (10^4 PFU) with WT luciferase$^+$ MuHV-4 under general anaesthesia. Individual genital signals were monitored between days 14 and 32 post-infection. For the reliable comparison of signal intensities, the signal intensities were measured from equivalent regions of interest (ROI) after subtraction of individual backgrounds measured daily in the right thoracic region. Each point shows one measurement. 5 individual mice per group are shown.

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4.

Discussion - Perspectives

Discussion – perspectives

Herpesviruses are archetypal persistent viruses identified in a wide variety of animals, ranging from mice to man (Davison *et al.* 2009). They are able to establish persistent and productive infections in immunocompetent hosts, provoking an efficient immune response. Despite this defense of the host, infectious viruses are sporadically re-excreted from the infected individual, ensuring viral spread in the host population.

Herpesviruses are classified according to their cellular reservoir for the long-term latent infection. Alphaherpesviruses such as VZV and HSV are neurotropic viruses while betaherpesviruses and gammaherpesviruses are lymphotropic (Roizman and Pellet 2007). All of the human herpesviruses are of great interest in terms of human health. Indeed, alphaherpesviruses mostly cause ulcerative skin lesions during both primary infection and reactivation. The acquisition of infection by VZV, HSV-1 or HSV-2 during pregnancy is a risk of congenital infection that can lead to severe neurological sequels (Brown *et al.* 1997; Corey and Wald 2009; Smith and Arvin 2009). In the same way, CMV, a betaherpesvirus, is the major cause of deafness and neurological disorders in neonates especially when the primo-infection is contracted during pregnancy (Dollard *et al.* 2007; Grosse *et al.* 2008). Finally primo-infection by gammaherpesviruses such as EBV and KSHV is often mild and can even be asymptomatic. However, these viruses establish long-term latency in circulating lymphocytes and induce lymphoproliferation as part of normal host-colonization. This characteristic predisposes the infected host to the development of neoplasic diseases including certain cancers, particularly in high risk populations such as immunocompromised individuals (for instance in cases of HIV co-infection, grafts,…). Therefore the scientific community has made great efforts for more than 50 years to oppose the epidemiological cycle of herpesviruses. However, studying these human viruses is very difficult because of their limited *in vitro* growth and because of the impossibility to study biological properties *in vivo* in humans. Also, even if a given virus grows well *in vitro*, the observed infectious system is never completely similar to the one observed *in vivo* as herpesviruses require the cooperation of different cell types to complete their life cycle. *In vitro* studies are for that reason only partially informative. Consequently, animal models were considered to answer some important questions about the biology of herpesviruses. Research soon faced limitations due to the very host-restricted capacity of infection or the dramatic difference in the pathology induced by infection in non-natural hosts. Indeed, herpesviruses are generally moderately pathogenic in their natural host but cross-species infection often leads to severe disease, which is potentially fatal (Fickenscher and Fleckenstein 2001; Dewals *et al.* 2006b; Hart *et al.* 2007). Much attention has been drawn to research models that could provide effective answers about the biology of these viruses in order to consider halting their

epidemiological cycle by treatment or vaccination. Consequently some alternative methods were developed.

HSV is frequently studied in a mouse model as this small animal can readily reproduce the infection. However, HSV-1 is often studied following ocular inoculation which is proved not to be the major route of infection. A vaginal model of infection in mice is used for HSV-2 studies, but the mouse mucosa and the remodeling of vaginal tissue is so different in mice compared to that of women that this method, although informative, creates a bias in the observations made. The *in vivo* investigation of VZV infection was hampered by a strict restriction for human tissues and so no small animal model can recapitulate the human disease. To bypass this, a SCID (Severe Combined Immunodeficiency) mouse/human xenograft model was developed and proved to be a valuable experimental tool for the studies of VZV infection and pathogenesis in the human skin, the dorsal root ganglia and T cells (reviewed in Arvin *et al.* 2010; Zerboni *et al.* 2010). In this model, SCID mice are used as “incubators” for human tissues and the infection is restricted to xenografts due to the host cell restriction of the infection. To date this model is the most sophisticated, developed for the assessment of VZV “*in vivo*” pathogenesis. Human CMV is also very host-restricted, but closely related viruses naturally infecting mice (MCMV) (Cheung and Lang 1977), rats (RCMV) (Rabson *et al.* 1969) and guinea pigs (GPCMV) (Patrizi *et al.* 1967) can easily be studied in their natural hosts in laboratory conditions. The most studied amongst them is certainly MCMV because of the large panel of molecular tools available for this species. This virus/host homolog model is probably one of the most informative ones available and we can expect that the major part of the data obtained with this model should be valuable for HCMV as the genome of MCMV is closely related to that of HCMV (Rawlinson *et al.* 1996). Finally, gammaherpesviruses, like betaherpesviruses, are lymphotropic and they need several different cells to complete their life-cycle. Therefore, the development of a modified mouse model implied the use of a “humanized” mouse from the point of view of the complete immune system. This was performed for some studies of EBV (Kuwana *et al.* 2011; Sato *et al.* 2011). However, the epithelial system, that is to say the portal of entry of the virus, continues to be the one of the mouse in such systems. This probably leads to the acquisition of mouse specific proteins at the viral surface and consequently, creating non-realistic infections. In addition, a lot of gammaherpesviruses infecting a wide range of species were discovered, only rarely infecting accessible animal models. For example, BoHV-4, a gammaherpesvirus that naturally infects cows is able to infect rabbits, but only when using high quantities of intravenously injected viruses. This probably reflects the difficulty of the virus in the infection of the rabbit’s epithelial cells in this trans-species model of infection. Nevertheless, this virus replicates well *in vitro* and *in vivo*, and a BAC clone exists. This renders BoHV-4 a useful tool and using it led for example to the elucidation of some immunoevasion and entry mechanisms (Machiels *et al.* 2011a; Machiels *et al.* 2011b; Lete *et al.* 2012). Another useful model is the study of the herpesvirus saimiri in monkeys. Indeed, this model is

phylogenetically the closest to humans (Rosenwirth *et al.* 2011). However, the ethical trend is now to limit drastically the use of experimental animals and even more so when considering primates. Therefore we can expect that the use of such models will be restricted to the evaluation of data that we cannot obtain with small animal models.

Consequently, the isolation in 1980 of Murid herpesvirus 4 strains in various naturally infected rodents (Blaskovic *et al.* 1980) was of great interest. Although a lot of information was collected using MuHV-4 experimental infections of mice, no consensus has been reached concerning the natural host(s) of the virus. Indeed, MuHV-4 was isolated in different species such as field voles, wood mice and shrews (Blaskovic *et al.* 1980; Chastel *et al.* 1994), and field studies conducted in the UK determined that the prevalence of the infection was higher in wood mice than in bank voles (Blasdell *et al.* 2003; Telfer *et al.* 2007). In 2010, Hughes *et al.* compared the infection of wood mice and BALB/c laboratory mice by MuHV-4 (Hughes *et al.* 2010). They reported several differences including a lower replication rate in the lungs of wood mice when compared to laboratory mice and focal granulomatous infiltrations rather than the interstitial pneumonia observed in *Mus musculus*. However, the route followed by the infection was the same in the two species and latency was not significantly different.

As no consensus has been reached to define MuHV-4's natural host(s); we have evaluated the quality of another potential *in vivo* alternative model: the bank vole, which is the species in which the virus was isolated. For this purpose we have compared the infection by MuHV-4 of bank voles and of mice, the latter being the classical experimental model. The comparative characterization of the infection after intranasal inoculation demonstrated that the infection of mice and of bank voles follows the same route. The virus first replicates in the nasal mucosa and afterwards, in the lungs, causing pneumonia and leukocyte infiltration. However, replication is about 1000-fold less intense in bank vole tissues than in those of the mouse. This was demonstrated by different techniques. Following this acute phase, the virus establishes successful latency in the superficial cranial lymph nodes and in the spleen of the two species. This indicates that the virus follows the same cycle in mice and in bank voles. Moreover, MuHV-4 host-colonization is more related to latency-establishment than to viral replication, indicating that transmission should be dependent on a latent load (Stevenson *et al.* 1999b; Coleman *et al.* 2003; May *et al.* 2004). In parallel to our work, a recently published study was dedicated to the comparison of the MuHV-4 infection of bank voles and wood mice, another potential natural host (Hughes *et al.* 2012). The results of this study are very similar to ours except that they have compared the latency in lungs which was largely lower in bank voles than in wood mouse or laboratory mouse. In their study, the latency in spleen was also lower in bank voles, contrasting with our results. However, they concluded that bank voles is probably an occasional and inefficient host in the wild (Hughes *et al.* 2012). We are not so conclusive about this point. Indeed, the low level of replication could be a viral strategy dedicated to the limitation of the host's immunological response.

This hypothesis is reinforced by the observation of lower neutralizing antibodies titers in bank voles than in mice. On the whole, these results demonstrate no major differences between the infections of the two species and so mice could henceforth be considered as a good model for MuHV-4 *in vivo* studies. Another good model for the study of gammaherpesvirus biology should be the recently described Wood mouse herpesvirus (Hughes *et al.* 2009) in the wood mouse. This has to be considered very interesting as it is a real virus/host homologue model. This situation is rarely encountered and of great interest in concern with herpesvirus biology. However, molecular and analysis tools available for the study of wood mouse are largely less extended than those available for the mouse. This will probably leads to major limitations of the research on this model. Another very interesting perspective was recently opened with a screening by PCR which was designed to detect new herpesviruses in naturally infected rodents. For this purpose, more than 1100 samples were analyzed and 21 novel gammaherpesviruses were identified, including the first gammaherpesvirus naturally infecting house mice (Ehlers *et al.* 2007). Unfortunately, this virus was only identified by sequence analyses and was not isolated. The opportunity to study a gammaherpesvirus naturally infecting mice would be of great interest as on one hand it might be a real homologous host/virus model and on the other hand the scientific community has a lot of biological and molecular tools for this species. Therefore, when this virus will be isolated, it could potentially become the most sophisticated and suitable model for *in vivo* studies of gammaherpesviruses.

Up until now, the major argument to devaluate the MuHV-4/laboratory mouse model has been the absence of observed re-excretion and transmission. This information is not valuable any more as we have demonstrated that both re-excretion and viral transmission occur in laboratory mouse populations. The finding of re-excretion in laboratory mice was somewhat surprising as numerous people have been working on this model around the world for a long time without reporting such observations. However, several points can be mentioned. First, we have used a method of *in vivo* imaging that was recently developed and which is very sensitive, allowing the detection of low levels of replicative virus. Secondly, we have followed the infections daily and during a long period, generally between 14 and 32 days post infection. To our knowledge, such following of the infectious process has never been reported. Another hypothesis could come from a potential pre-existing infection of the mice by a pathogen helping viral reactivation. Indeed, such synergic relation has been demonstrated for others herpesviruses, notably HSV-2 and CMV, with HIV-1 (Rinaldo *et al.* 1992; Mostad *et al.* 1999; Van de Perre *et al.* 2008; Schoenfisch *et al.* 2011). If such a pre-existent infection exists, the causal agent remains to be identified. Finally, our housing facility homes both females and males and this can be an important element. Indeed, the re-excretion observed is on a genital level and we have demonstrated a link between the sexual steroid cycle and the re-excretion of the virus. The ovariectomy of mice leads to a drastic decrease of viral genital re-excretion, nearly abolishing it, and complementation by estrogens, but not by progesterone, restores the re-excretion to the level of non-

ovariectomized mice. However, estrogens do not act directly to reactivate MuHV-4 from latency as the effect is only seen *in vivo*. Such effects of sex hormones on herpesvirus reactivation have already been described. For HSV-1, it was shown that 17β-estradiol causes reactivation from latency (Vicetti Miguel *et al.* 2010). Moreover, Mostad *et al.* demonstrated that high levels of sexual hormones, resulting from contraception or pregnancy, increase the frequency of HSV-2 genital shedding (Mostad *et al.* 2000). Sex hormones are major actors of the cyclic cellular remodeling of the vaginal tissue. This could influence the re-excretion by two ways. First, the cyclic observation of shedding could reflect the renewal of epithelial cells at the surface of vaginal mucosa. Secondly, hormones could act to modify the expression of membrane proteins at the surface of cells, modifying their sensibility to the infection. However, the molecular and cellular pathways influenced by sex hormones and implied in the observed shedding remains to be elucidated.

It is well known that the immunity of the female genital tract is remodeled during the sexual cycle and that these changes are essentially controlled by steroid hormones. The recruitment of neutrophils, the antibodies in genital secretions, as well as T-cell mediated response and susceptibility are all dependent on the stage of the estrous cycle (Beagley and Gockel 2003). TLR are important mediators of the innate immunity and are known to be implied in gammaherpesviruses reactivation from latency (Gargano *et al.* 2009; Gregory *et al.* 2009). In a general fashion, TLR are expressed on phagocytic and epithelial cells (Akira *et al.* 2001; Takeda and Akira 2001) and some of them are expressed on female reproductive tract epithelial cells (Fichorova *et al.* 2002). Moreover, it is possible that the expression of TLR at the level of the genital tract varies upon the sexual cycle, modifying the sensibility of innate immunity at this anatomical site. The activation of TLR, by another infectious agent for example, in the genital tract could participate to the reactivation of MuHV-4 on a genital level. Moreover, it is possible that the expression of TLR at the level of the genital tract varies upon the sexual cycle, modifying the sensibility of innate immunity at this anatomical site. In addition, the humoral immunity is also greatly affected by the sexual cycle as the level of IgA in vaginal fluids reaches a maximum at the estrous stage and dramatically decreases during the diestrus while IgG levels show a reverse profile (Gallichan and Rosenthal 1996; Nardelli-Haefliger *et al.* 1999). This could be only a part of the phenomenon and the mechanisms underlying this phenomenon and the cell types implied remain however unclear. Moreover, the fact that the re-excretion occurs when the antibody level is maximum at the genital area could be an advantage in terms of transmission. Indeed, excreted virions could for example bind anti-gp150 antibodies present at the re-excretion sites and it was demonstrated that anti-gp150 antibodies were on one hand non neutralizing and on the other hand were promoters of the infection by favoring the infection of cells with Fc receptors (Gillet *et al.* 2007b; Rosa *et al.* 2007).

Elucidating the cellular origin of vaginally re-excreted virions will be very interesting to shed light on re-excretion mechanisms. For this purpose, we could use a newly-developed cell-type specific

virus genetic labeling based on Cre/lox recombination allowing to track viral infection *in vivo* (Barton and White 2008; Sacher *et al.* 2012). This system is based on the impairment of the viral survival after Cre/lox recombination because of the loose of an essential part of the viral genome. Another potential marking system is based on the insertion of two genes coding for different fluorescent proteins and switch from the expression of first transgene in absence of *Cre* to the expression of the second transgene after recombination by the *Cre* recombinase. Such *Cre/loxP* system has recently been applied to demonstrate that MuHV-4 uses DC's to colonize B cells after intranasal infection (Gaspar *et al.* 2011). We could use transgenic mice expressing *Cre* recombinase only in certain cell types, such as B cells, T cells subsets or dendritic cells, or only after specific induction. This, associated with infection by recombinant viruses specifically constructed for assaying exposure to *Cre* should allow revealing the route of re-excretion of MuHV-4 from latently infected female mice. In parallel, the determination of the cell-types implied in the entry of the virus in males during natural primary infection will be important in the context of the development of vaccinal strategies. Indeed, knowing which cell-types are firstly targeted could direct the choice of vaccinal antigen target. As an example of this, the gp350 of EBV is required for B-cell infection but is dispensable for epithelial infection and the induction of antibodies against gp350 failed to protect from infection as seroconversion rates do not decrease after immunization against gp350 (Sokal *et al.* 2007). Moreover, treatment by antibodies against gp350 enhances the ability of EBV to infect epithelial cells (Turk *et al.* 2006). This underlines the importance of deep understanding of the entry mechanisms in the context of treatment or vaccination.

The precedent results, indicative of potential routes of transmission, led us to address the question of vertical and horizontal transmission among laboratory mice populations. We have firstly addressed vertical transmission which is proven for some herpesviruses albeit it is a very rare event, especially for gammaherpesviruses. In 2011, Stiglincova *et al.* reported vertical transmission of MHV-68 in laboratory mouse population, *via* either transplacental infection or breast milk (Stiglincova *et al.* 2011). They also reported retardation of fetal development and premature termination of the pregnancy. This contrasts drastically with the results we have obtained. Indeed, in our experimental conditions, no transmission from mother to progeny occurred. They also reported the detection of infectious viruses in the newborns of all the litters tested by co-culture on Vero cells. This also contrasts with our results revealing no effect of the infection, either acute or latent, on litter size or gestational length. Moreover, regarding the very low frequency of transplacental transmission of the two human gammaherpesviruses (Pica and Volpi 2007), this type of transmission in mice should be expected at lower rates. Indeed, in women, the placental interface is of haemomonochorial type which means that only one layer of trophoblast separates the maternal blood from the fetus, but in mice, the placental interface is named heamotrichorial as three layers of trophoblast exist (Johnson 2007). This structural difference should render the transmission more difficult in mice than in humans. In our

experiments, no transmission *via* breast milk was observed as the progeny remains negative in terms of infectious viral particles after 6 weeks of life. However, breastfeeding transmission is not excluded for gammaherpesviruses as HHV-8's DNA was detected in some breast-milk samples (Dedicoat *et al.* 2004). On the contrary, another study failed to demonstrate the presence of KSHV in the breast milk of infected women (Brayfield *et al.* 2004). Finally, a link between the presence of viral DNA of CMV, EBV and HIV's RNA in breast milk was established in another field study (Gantt *et al.* 2008), indicating a potential link between these infections and their re-excretion site. Moreover, BoHV-4 was detected in the cellular fraction of milk of infected cows, suggesting a potential way of transmission (Donofrio *et al.* 2000). In conclusion, the mother-to-child transmission of gammaherpesviruses *in utero* or *via* breastfeeding remains a potential way of viral spread in natural populations but the impact of such transmission remains unclear and if it exists, it seems to be a very rare event.

Sexual horizontal transmission was also assessed as this route is more established although it is not considered as a major way of transmission for gammaherpesviruses. EBV and KSHV may be detected in the semen and cervical secretions of infected individuals (Sixbey *et al.* 1986; Israele *et al.* 1991; Naher *et al.* 1992; Diamond *et al.* 1997; Whitby *et al.* 1999; Ablashi *et al.* 2002). However, sero-epidemiological studies failed to definitively prove the existence of sexual transmission because these two viruses are also primarily re-excreted in the saliva of infected individuals (LaDuca *et al.* 1998; Rickinson and Kieff 2001). Consequently, even if it has been proved that the risk of acquisition of the infection is higher with sexual activity, it is impossible to link the transmission with sexual intercourse itself or with other associated behaviors such kissing. However, the possibility of genital acquisition of EBV was demonstrated with cases of ulcerative genital lesions in children from which infectious EBV was collected (Halvorsen *et al.* 2006).

Here, we have demonstrated the first case of female-to-male sexual transmission of MuHV-4. This transmission occurred from genitally re-excreting females to naïve males and sexual infection was clearly demonstrated because the first replication site in males was the penis. This transmission reached about 30% of exposed males. The co-housing conditions of both genders were chosen to rely as much as possible on the conditions encountered in nature. This implies the co-housing of three infected females with three naïve males, inducing the possibility of multiple sexual contacts, but also of some situations which provoke physiological stress in the cages. These elements can be part of the observed transmission phenomenon. Indeed, the presence of several males probably induces an increase in coupling attempts and contacts because of some non-fertilizing mating. Indeed, female mice are no more receptive to sexual attempts once fertilized. Moreover, it is possible that females re-excrete more viruses on a longer period in such stressful situations. However, the monitoring of re-excretion by imaging is not indicative of such a situation in females. When transmission takes place, it starts by an intense replicative signal at the level of the penis and the infection then spreads to surrounding lymph nodes and later to the spleen, demonstrating that primo-infection by MuHV-4 can

be acquired by the sexual route and that lymph nodes and spleen colonization is part of the natural infection. However, once transmitted to males, the virus seems to replicate at high levels during a long time (at least 20 days) in the urogenital tract of males. This shed new insights on the natural epidemiological cycle of MuHV-4 as viral replication in the urogenital tract of males could reveal several (and not mutually exclusive) ways of transmission: by sexual route, but also by territory-marking. This last point could only be relevant if infectious viruses are re-excreted in urine as it is frequently observed for CMV and sporadically for HHV-6 and HHV-7 (Gautheret-Dejean *et al.* 1997; Mocarski *et al.* 2007). Therefore, the systematic analysis of body fluids from infected mice on a long-term period should bring relevant information on that point. This could also be informative to determine if the infection could be transmitted between males by biting, fighting or any other means. Indeed, we have sporadically observed transmission to naïve males beginning by replication in the area of nose/mouth. Finally, the transmission from males undergoing viral replication at the level of the penis to naïve female should be investigated. Indeed, we have observed viral replication in sexual male tissue during a long time after primary infection. This is indicative of the great ability of the virus to replicate in these tissues. Thus it can also be hypothesized that virus will be maintained in the male genital tract, maybe at low levels, during long periods, favoring the transmission to females and thus spread in the whole population. In this case, the effect of such transmission on the issue of pregnancy and on progeny should be investigated.

Sexual transmission constitutes an easy way of spread for a virus in natural populations of wild animals. This is particularly the case for rodents. Indeed, rodents live generally in small groups of spread on a relatively large territory. Direct contact could be a relatively efficient route of transmission when considering urine (territory marking) or saliva (biting/fighting). However, the opportunity of transmission by these is probably considerably less than by sexual route. Taking this point in account, the fact that the viral shedding in the female genital tract is linked to sexual cycle and more precisely to the period of oestrus (high rates of estrogens) would be very beneficial for transmission as re-excretion would occur during the periods of female receptivity for mating.

Altogether, these results also question the natural routes of infection. To date, intranasal infection has been considered as the most probable way of natural infection. Indeed, several herpesviruses are spread by contact and the first replication site is often the respiratory tract. Moreover, several groups evaluated the intranasal, intravenous, and intraperitoneal routes of infection for MuHV-4 and in all cases, the viral inoculation led to spleen and lymph node colonization. However, among these tested routes, the most potent natural way of infection was the intranasal route and this was subsequently used experimentally. In 2009, Milho *et al.* compared the MuHV-4 infection following intranasal inoculation under general anesthesia or without anesthesia (Milho *et al.* 2009). In this study they revealed that mice infected without anesthesia do not undergo viral replication in the lung contrary to those infected under general anesthesia, which led to the conclusion that lungs are not

a natural site of replication. Other characteristics of the infection were otherwise very similar. Even if intranasal inoculation is considered for the moment as the best experimental route of infection to evaluate the natural biology of MuHV-4, we cannot exclude that sexual experimental infection could bring new important information about MuHV-4 biology. We have tried experimental intravaginal infection (data not shown). As the sexual intercourse in mice implies the deposit of a vaginal plug composed of male secretion, we have designed an experimental infection mimicking this physiological aspect of murine behavior. So we have infected mice under anesthesia and maintained them on their back during about half an hour after the infection. In this experiment only one mouse on the five used became infected, but the mice were not synchronized in terms of genital cycle and we can expect that the phase of the cycle and therefore the vaginal tissue structure can influence the acquisition of the infection. However, this has demonstrated the capacity of the virus to directly infect the vaginal epithelia or cells presents in this area. In the future, it could be interesting to test the vaginal route of infection also in bank voles. Indeed, sexual transmission could be seen as an easy way of natural viral transmission and the infection by this route could reveal some new interesting information in this alternative model of infection. Moreover, the suggested above systematic analysis of body fluids (blood, saliva, sperm, cervical secretions) during the time of the acute and latent infections should provide new insights into the mechanisms implied in viral shedding and transmission of MuHV-4 in mouse populations and maybe in bank vole populations.

In addition, several immune-evasion mechanisms, often implying glycoproteins, were described and offer perspectives in term of development of original vaccinal strategies as they could explain why the natural immune response fails to prevent transmission of gammaherpesviruses (Gillet *et al.* 2007b; Gillet and Stevenson 2007a; Gillet *et al.* 2008a; Gillet *et al.* 2008b; Gillet *et al.* 2009). However, to date, the absence of a transmission model has made these findings theoretical in terms of epidemiology. Now disposing of this new model of re-excretion/transmission will allow their evaluation. The first considered target for vaccination will be the gH/gL complex. Indeed, gH/gL is a part, along with gB and gp150, of a potential multiprotein entry complex (Gillet and Stevenson 2007b). Gp150 seems to act as an immunologic decoy, inducing massive production of non neutralizing antibodies (Gillet *et al.* 2007b). Those antibodies even promotes the infection of cells with Fc receptor (Rosa *et al.* 2007). Therefore, gp150 is supposed to protect the other proteins of the complex (essentially gH/gL) from neutralization (Gillet *et al.* 2007b). Thus, the induction of mucosal immunization against gH/gL should therefore be a key part in vaccinal strategies. In this context, immunization with adenoviruses expressing the gH/gL heterodimer should be tested in diverse epidemiological conditions experimentally created such as mixing of vaccinated naïve males and vaccinated infected females, unvaccinated naïve males and vaccinated infected females, vaccinated naïve males with unvaccinated infected females or unvaccinated naïve males with unvaccinated infected females.

This strategy could be applied to the evaluation of several potential targets for vaccination. The identification of novel candidates could be realized by random mutagenesis of the MuHV-4 BAC genome. Indeed, we could randomly mutate the genome in order to identify recombinant viruses less re-excreted. This could bring new information both on proteins implicated in transmission mechanisms and in reactivation and immune evasion. Then, the potential use of these mutated viruses for vaccination should be tested in our model.

Finally, as our model allows the evaluation of the level of the shedding as well as the transmission power of the virus by the sexual route, effects of enhancing the immunity at the genital mucosa should be evaluated. For this purpose, several strategies might be considered: direct genital immunization, antiviral vaginal treatments or nasal immunization. Indeed, nasal immunization not only stimulates an immune response on a respiratory tract level but also elicits a strong vaginal/genital mucosal immunization response (Holmgren and Czerkinsky 2005; Neutra and Kozlowski 2006). However, regardless to the chosen site of immunization, particular attention should be paid to hormonal conditions. Indeed, as previously mentioned, steroid hormones greatly influence the immune system. Many studies were designed to evaluate their effect in the context of vaccination, especially against HSV-2. Indeed, it was showed that intranasal and subcutaneous immunization by an attenuated HSV-2 strain led to better protection when performed under the effect of estradiol (Bhavanam *et al.* 2008). These results were confirmed by the enhancement of vaccination by estradiol in the context of a clinically relevant vaccine (Pennock *et al.* 2009). However, it could be different if an intra-vaginal treatment was proposed. Indeed, antigen presentation in the genital tract is also regulated by sex hormones. In the female reproductive tract, antigen presentation is carried out by specialized antigen presenting cells but also by non-specialized cells such as epithelial and stromal cells (Prabhala and Wira 1995; Wira and Rossoll 1995). It was demonstrated that antigen presentation is higher when estradiol levels in the blood are low, suggesting an inhibitory effect of estrogens on antigen presentation. This should be taken in account in the design of studies dedicated to the enhancement of mucosal immunity of the genital tract (Wira and Rossoll 1995).

Despites that gammaherpesvirus infections are associated with several important diseases in human and animal populations, recent studies on MuHV-4 have suggested that the relationship between these viruses and the host immune system could be more symbiotic than pathogenic (Barton *et al.* 2007; Larson *et al.* 2012). Even if these effects are limited in duration, the multiple herpesvirus infections acquired during early childhood may have significant beneficial consequences for human immunology (Barton *et al.* 2011).However, there is no evidence so far that this beneficial effect observed in mice has relevance for human gammaherpesvirus infections. Moreover, there are several epidemiological circumstances in which the need for a prophylactic and/or a therapeutic vaccination strategy remains an important goal (Yager *et al.* 2009). These include African populations where there is a high incidence of Burkitt's lymphoma, Southeast Asian populations displaying an increased

incidence of EBV-associated nasopharyngeal carcinoma and EBV-negative adolescents in whom late EBV infections can be associated with debilitating infectious mononucleosis and increased susceptibility to Hodgkin's disease (Klein *et al.* 2007). Moreover, the identification of a potential benefit of gammaherpesvirus infection does not argue against the development of prophylactic or therapeutic vaccines (Yager *et al.* 2009). The challenge will be to develop attenuated vaccines that could confer any potential benefits of latent gammaherpesvirus infection while simultaneously protecting against wild-type associated diseases.

In conclusion, we have hither confirmed that mouse is a valuable model for *in vivo* studies of MuHV-4. Moreover, we have reported the observation of genital viral re-excretion in female mice and of sexual transmission from re-excreting female to male. This has led to the development of the first relevant model of transmission for the MuHV-4 virus, the most studied model of gammaherpesvirus infection, in experimental conditions. In the future, this model should help to better understand the mechanisms underlying gammaherpesvirus reactivation and spread among host populations. The model should also be very helpful in the design and testing of novel vaccinal strategies.

5.

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