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~ Nulla tenaci invia est via ~

Unraveling herpesvirus mucosal invasion in an *ex vivo* organ culture

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Promotor

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" *Nulla tenaci invia est via*" - " For the tenacious, no road is impassable"

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TABLE OF CONTENTS

LIST OF ABBREVIATONS

aa	amino acid
AI	artificial insemination
AlHV-1	alcelaphine herpesvirus 1
Als	agglutinin-like sequence
ANOVA	analysis of variance
ANDV	Andes virus
APC	antigen presenting cells
ASL	airway surface liquid
BAC	bacterial artificial chromosome
BBB	blood-brain barrier
BM	basement membrane
BoHV-1, -2, -4, -5	bovine herpesvirus 1, 2, 4, 5
BRSV	bovine respiratory syncytial virus
bURT	bovine upper respiratory tract
BVD-1, -2	bovine viral diarrhea virus type 1
C3b	complement factor 3b
CCSP	Clara cell secretory protein
CD	cluster of differentiation
cDNA	complement DNA
CGRP	calcitonin gene related peptide
ChAT-IR	choline acetyltransferase immunoreactive
CNA	collagen-binding adhesion protein
CNS	central nervous system
CO_2	carbon dioxide
CPE	cytopathic effect
CT	cytoplasmic tail
C-terminal	carboxy terminal
CWP	cell wall-anchored surface protein
2D, 3D	two-dimensional, three-dimensional

DABCO	1,4-diazobicyclo-2.2.2-octane
DC(-Sign)	dendritic cell (-specific intercellular adhesion molecule-3-grabbing non-integrin)
DMEM	Dulbecco's modified eagle's medium
DNA	deoxyribonucleic acid
DPX	distyrene plasticizer xylene
drie V's	Verfijning, Vermindering en Vervanging
dUTP	2´-Deoxyuridine, 5´-Triphosphate
E	early
EBV	Epstein-Barr virus
EC(M)	extracellular (matrix)
E-cadherin	epithelial cadherin
EGF	epidermal growth factor
EHV-1, -2, -4, -5	equine herpesvirus 1, 2, 4, 5
ElHV-1	elephant endotheliotrophic herpesvirus
(S/T)EM	(scanning/transmission) electron microscopy
EMT	epithelial-to-mesenchymal transition
ET	extracellular tail
Fc region	fragment crystallizable region
FeHV-1	feline herpesvirus 1
FITC	fluorescein isothiocyanate
FGF-2	fibroblast growth factor 2
G1,-2,…	globular domain 1,-2,…
GAG	glycosaminoglycan
GaHV-1, -2, -3	gallid herpesvirus 1, 2, 3
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
gB, gC,…	glycoprotein B, glycoprotein C,…
Gly-X-Y	glycine, X and Y stand for any amino acid
GPI	glycosylphosphatidylinositol
GRP	gastrin releasing peptide
h	hour

HbX	hepatitis B virus x protein
(H)CMV	(human) cytomegalovirus
HE-staining	haematoxyline-eosine staining
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HHV-1, -2, -6, -7	human herpesvirus 1, 2, 6, 7
HMEC	human microvascular endothelial cells
HPV	human papillomavirus
HVEM	herpesvirus entry mediator
HIV	human immunodeficiency virus
H particle	heavy particle
HSV-1 and -2	herpes simplex virus 1 and 2
HTLV-1	human T-cell leukemia/lymphoma virus type I
IBR	infectious bovine rhinotracheitis
(B)ICP0	(bovine) infected cell polypeptide 0
IE	immediate early
IEL	intraepithelial lymphocytes
IF	immunofluorescence
Ig	immunoglobulin
ILC	innate lymphoid cells
ILK	integrin-linked kinase
INM	interkinetic nuclear migration
IPMA	immunoperoxidase monolayer assay
IPV/IPB	infectious pustular vulvovaginitis/balanoposthitis
JAM	junctional adhesion molecule
kbp	kilo base pairs
KIF(1A)	kinesin family protein (1 alpha)
KSHV	Kaposi’s sarcoma-associated herpesvirus
L	late
LC	Langerhans cell
Lmb	laminin-binding protein
Lmp-1	latent membrane protein 1

Ln-332	laminin-332
LPXTG motif	leucine-proline-any aa-threonine-glycine motif
Lsa	leptospiral surface adhesin
LVR	low viscosity resin
MALT	mucosa-associated lymphoid tissues
MAPK	mitogen-activated protein kinase
MARCO	macrophage receptor with collagenous structure
MCF	malignant catarrhal fever
McHV-1	macacine herpesvirus 1
MDBK	Madin-Darby bovine kidney cells
MHC	major histocompatibility complex
MLV	modified live vaccine
MMP	matrix metalloproteinase
mRNA	messenger RNA
MSCRAMMS	microbial surface components recognizing adhesive matrix molecules
MUC(5AC)	mucin(5AC)
MuHV-1, -4	murine herpesvirus 1, 4
MUPP-1	multi-PDZ domain protein 1
MV	measles virus
NC-1	non-collagenous 1
Nef	negative regulatory factor
NKA	neurokinin A
NK cell	natural killer cell
NSP	non-structural protein
N-terminal	amino terminal
OvHV-2	ovine herpesvirus 2
P4	progesterone
PAR	protease-activated receptor
PAS	periodic acid Schiff's
PBMC	peripheral blood mononuclear cell
PBS	phosphate-buffered saline

PCL	periciliary liquid
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
pH	-log10[H^+]
pi	post inoculation
PI-3	bovine parainfluenza virus type 3
PI3K	phosphatidylinositol 3-kinase
PilA	pilus-associated adhesin A
PMN	polymorphonuclear neutrophil granulocytes
PRRSV	porcine reproductive and respiratory syndrome virus
PRV	Pseudorabies virus
R_0	basic reproduction ratio
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
Roi	region of interest
SD	standard deviation
Ser/Thr/Asn	serine/threonine/asparagines
SgrA	serine glutamate repeat A
SH-2, -3	src homology 2, 3
SIV	simian immunodeficiency virus
Slr	streptococcus lactis R factor
SN	seroneutralization
SP-A, SP-D	surfactant protein A or D
SPARC	secretory protein acidic and rich in cysteine
SPLI	secretory leukocyte peptidase inhibitor
SPSS	statistical package for the social sciences
Src	sarcoma protein family
STI	sexually transmitted infection
TAP	transporter associated with antigen processing
Tat	trans-activator of transcription
$TCID_{50}$	tissue culture infectious dose with a 50% endpoint

TCR	T cell receptor
TF	tissue factor
TGN	trans Golgi network
Three R's	Refinement, Reduction and Replacement
TIMP-2	tissue inhibitor of metalloproteinase 2
TJ	tight junction
tPA	tissue-type plasminogen activator
TNF-α	tumor necrosis factor α
TR	threonine-rich
Tukey's HSD	Tukey's honestly significant difference
TUNEL	terminal deoxynucleotidyl transferase mediated dUTP nick end labeling
UL	unique long
uPA(R)	urokinase-type plasminogen activator (receptor)
US	unique short
Vhs	virion host shut-off
VEGF	vascular endothelial growth factor
VIP	vasoactive intestinal peptide
VP(16)	virion protein (16)
VZV	Varicella-zoster virus
WNV	West-Nile virus
WT	wild-type
ZO-1,-2,...	zonula occludens-1,-2,...

CHAPTER 1.

INTRODUCTION

1.1. Herpesviridae

Herpesviruses are widespread among both vertebrates and invertebrates, making them one of the most successful viruses. All existing herpesviruses are believed to be evolutionary derivates of one distant progenitor (divergent evolution) (299). Their extreme ability to co-adapt to and co-evolve with a single host species allowed them to persist over time. A seminal study of McGeoch and Cook showed that the phylogeny of the subfamily of the *Alphaherpesvirinae* is nearly an exact copy of that of their hosts (235). The evolutionary rate of herpesviruses is estimated to be 30 times faster than that of the hosts. Although their evolutionary rate is several orders less than RNA-viruses, herpesviruses have exploited many mechanisms for generating diversity including substitutions, deletions or insertions of nucleotides; capture of genes from the host genome via an RNA intermediate; duplication of genes via recombination and large scale gene rearrangement. The frequency of these events seems to be sufficient for efficient adaptation (73). Despite their differences at the level of gene content, host range and duration of the reproductive cycle; *Herpesviridae* share significant biological properties: (i) they encode for a variety of enzymes involved in processing of proteins and in DNA replication, (ii) synthesis of viral DNA and encapsidation occurs in the nucleus, (iii) productive infection results in cell destruction and (iv) a hallmark is the ability to establish latency to persist within the host (300).

1.1.1. Classification and virion structure

Classification

The recently revised order of the *Herpesvirales* contains three different families: *Alloherpesviridae*, *Malacoherpesviridae* and *Herpesviridae*. *Alloherpesviridae* contain fish and frog viruses, whereas *Malacoherpesviridae* consist of a bivalve virus. The family of the *Herpesviridae* incorporates pathogenic members that can cause disease in mammals, birds and reptiles (75). The *Herpesviridae* are subdivided according to biological properties in three distinct subfamilies, alpha-, beta- and gamma-herpesviruses. *Alphaherpesvirinae* and *Betaherpesvirinae* can be considered as ‘lytic’. Typically, most cells support lytic replication of these viruses whereas only a minority of cell types are able to harbor latent virus. On the contrary, *Gammaherpesvirinae* often remain latent/persistent in cells, while only a small subset of cells support lytic replication (6).

Alphaherpesvirinae

These viruses are present in many different species and are known to have a broad host cell range, a short replication cycle with a fast destruction of infected cells and a rapid spread among susceptible cells. They are able to establish latency primarily but not exclusively in sensory ganglionic neurons. Four distinct genera are present within this subfamily *Simplexvirus*, *Varicellovirus*, *Mardivirus* and *Iltovirus* (75, 300). Known species of the *Simplexvirus* genus are bovine herpesvirus 2 (BoHV-2), human herpes simplex virus 1 (HSV-1) and 2 (HSV-2). Animal herpesviruses bovine herpesvirus 1 (BoHV-1) and 5 (BoHV-5), equine herpesvirus 1 (EHV-1), pseudorabies virus (PRV), feline herpesvirus 1 (FeHV-1) and human herpesvirus varicella-zoster virus (VZV) are the best studied varicelloviruses. Gallid herpesviruses such as Marek's disease virus (GaHV-2 and GaHV-3) and infectious laryngotracheitis virus (GaHV-1) are the most important members of the genus *Mardivirus* and *Iltovirus* respectively (74, 75).

Betaherpesvirinae

The host cell range of the members of this family is much more restricted. Next to that, betaherpesviruses know a long productive cycle which is reflected by the slow infection progress in cultures. Typically, cells frequently become enlarged, known as cytomegalia. Latency can be established in different tissues including secretory glands, kidneys, lymphoreticular cells and others. *Cytomegalovirus*, *Muromegalovirus*, *Roseolovirus*, *Proboscivirus* are the different betaherpesvirus genera (75, 300). The prototype member of this subfamily belongs to the *Cytomegalovirus* genus and is human herpesvirus 5 also known as human cytomegalovirus (CMV). The best studied animal model for CMV is murine herpesvirus 1 (MuHV-1) and is a member of the *Muromegalovirus* genus (304). However, several studies exist about human roseolovirus human herpesvirus 6 and 7 (HHV-6/-7) (230, 338) and research on veterinary proboscivirus elephant endotheliotropic herpesvirus (ElHV-1) is on the increase (211).

Gammaherpesvirinae

Somewhat misleading is the host range of this subfamily. For these viruses a clear difference has to be made between infection and productive replication. Upon entry, the virus often prefers to enter the latency program instead of causing a lytic replication, which contributes to the fact that gammaherpesviruses are believed to infect a limited number of species. However,

for some veterinary gammaherpesviruses both the host range and the tissue tropism are quite broad. Latent virus is often demonstrated in lymphoid tissues. Gammaherpesvirinae encompasses the *Lymphocryptovirus*, the *Rhadinovirus*, the *Macavirus* and the *Percavirus* genus (6, 75, 300). Human herpesvirus 4 or Epstein-Barr virus (EBV) belongs to the lymphocryptoviruses. Bovine herpesvirus 4 (BoHV-4) together with human herpesvirus 8 (Kaposi's sarcoma-associated herpesvirus or KSHV) and murine herpesvirus 4 (MuHV-4) belong to the rhadinoviruses. Alcelaphine herpesvirus 1 (AlHV-1) and ovine herpesvirus 2 (OvHV-2), both causative agents of malignant catarrhal fever (MCF) belong to the macaviruses. Equine herpesvirus 2 (EHV-2) and 5 (EHV-5) belong to the percaviruses (75).

Data presented in the next sections mainly focus on the key players of this thesis namely BoHV-1 and HSV-1.

Virion structure

Mature fully assembled herpesvirus particles (referred to as H-particles) are on average 200-250 nm in size and consist of 4 indispensable structural elements: the genome, the capsid, the tegument and the envelope (27). The general architecture of a herpesvirus particle is given in Figure 1.

The DNA molecule of the herpesvirus genome is a linear double strand of about 150kbp. Two distinct regions interspersed by inverted repeats exist within the genome: the unique long region (UL) and the unique short (US). 162 capsomers form a rigid icosahedral capsid which protects the genetic information. Around the capsid more than 20 proteins make up for the tegument. Finally, a lipoprotein membrane, the envelope, surrounds the nucleocapsid (27, 245, 289). The envelope, which is from cellular origin, harbors different viral proteins with the majority of them being glycosylated transmembrane proteins, termed glycoproteins. The genome of BoHV-1 encodes at least 10 different glycoproteins whereas HSV encodes 11 (316). Glycoproteins are important in essential virus-cell interactions like adherence, penetration and spread (8, 357). They also evoke a strong immune response within the host (94).

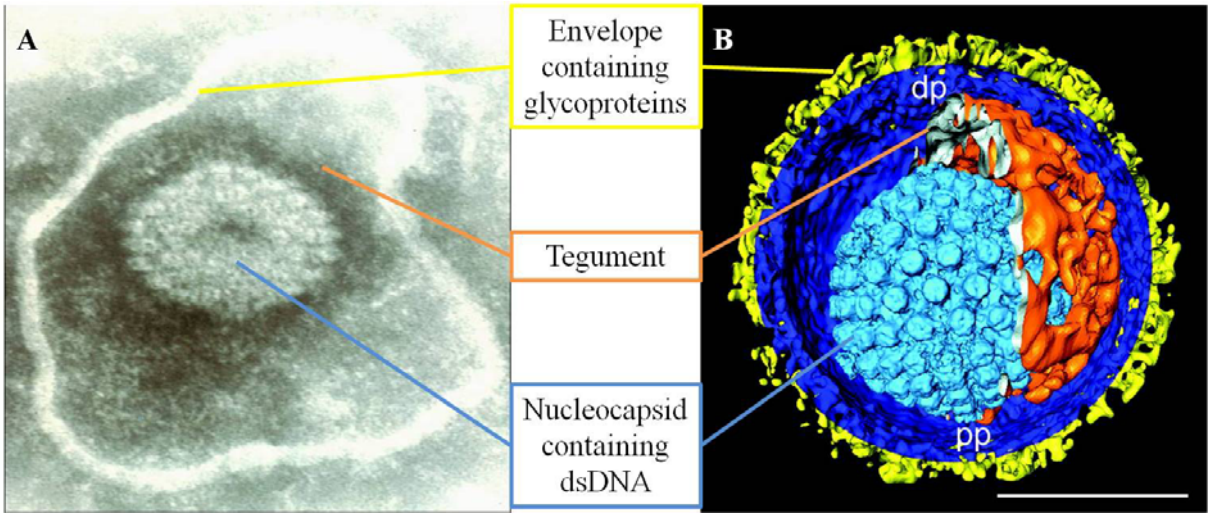

Figure 1. Structure of a herpesvirus virion. (A) Image taken by transmission electron microscopy of a herpesvirus particle. (B) Cutaway view of the virion interior by single virion tomography (pp, proximal pole; dp, distal pole). Scale bar: 100 nm. Adapted from (134).

Viral glycoproteins

gB (UL27)

Glycoprotein B is highly conserved among herpesviruses and the target of many neutralizing antibodies (92). BoHV-1 gB (932aa) and HSV-1 gB (904aa) share 45.9% homology at the amino acid level (360). gB is an essential part of the herpesvirus core fusion machinery. It initially binds to heparan sulfate moieties on the cell membrane and causes fusion of the viral envelope with the host cell (92, 343). The crystal structure provides the most convincing evidence that gB acts as a viral fusion protein and more specific, a Class III fusion protein, as the gB ectodomain was found to have an unexpected homology to glycoprotein G from vesicular stomatitis virus, which is a known Class III fusion protein (150). In the structure, Class III fusion proteins are trimeric with three long central α-helices (92). The gB ectodomain trimer forms a spike-like molecule and these spikes have been observed on HSV-1 virions using electron microscopy (333). However, the exact mechanism behind the conversion of the inactive pre-fusion to the active post-fusion form of gB is still unkown. Assumedly, gH/gL uses triggering mechanisms that are functionally analogous to known fusion triggering mechanisms like low pH or protease cleavage. In addition, pH was found to play a minor role in HSV fusion when it occurs in endosomes (92).

gC (UL44)

Glycoprotein C is composed of 508aa and 511aa for BoHV-1 and HSV-1 respectively (68, 114). Like gB, this glycoprotein mediates binding to heparan sulfate molecules on cells and is involved in cell entry of the virus (13). Moreover, gC of HSV-1 was found to bind to the macrophage receptor with collagenous structure (MARCO) for enhanced adsorption to epithelial cells and infection (220). gC is also known to bind complement factor C3b. This allows the virus to mediate inhibition of complement-mediated neutralization of cell-free virus and complement-mediated lysis of infected cells (13). Recently, gC was found to play a role in the exploitation of the host coagulation system by HSV-1. Once attached to the cell surface, HSV1 assembles a proteolytic complex, consisting of both cellular co-factors of coagulation such as Tissue Factor (TF) and viral gC, in proximity to protease activated receptor 2 (PAR2). These low levels of virus-bound proteases enhance host cell infection by activating PAR (334).

gD (US6)

Glycoprotein D determines cell tropism by binding to cellular receptors (92). Several entry mediators have been described for many herpesviruses, including nectins (nectin-1 and nectin-2), herpesvirus entry mediator (HVEM) and 3-O-sulfated heparan sulfates (339). The structure reveals that the binding site for nectin-1 on gD differs from the binding site of the HVEM receptor (81). Stable binding of gD to one of the above mentioned receptors triggers membrane fusion (92). HSV-1 gD is composed of 394aa and gD of BoHV-1 consists of 417aa (68, 237). Viruses, including BoHV-1 and HSV, lacking a functional gD protein are severely impaired in cell entry and spread within the host (260, 358).

gE (US8)

Glycoprotein E forms a non-covalently bound heterodimer with glycoprotein I. This nonessential protein is 575aa long in BoHV-1 and 552aa in HSV-1 (68, 270). The gE is a multifunctional virulence protein. It plays a role in immunomodulation by acting, together with gI, as an Fc-receptor but is also crucial for efficient antibody-induced viral glycoprotein capping (105). Moreover and most importantly gE is necessary for efficient spread of the virus between neurons and from cell-to-cell (105). Indeed, alphaherpesviruses mimic intracellular sorting pathways to transport virus particles towards the plasma membrane and gE/gI facilitates the envelopment into cytoplasmic vesicles (f.e. TGN) that are sorted to

epithelial junctions (173). Deletion of gE causes attenuation of the virus and current marker vaccines for BoHV-1 are based on this deletion (178).

gG (US4)

Glycoprotein G of HSV-1 and BoHV-1 is 238aa and 444aa long respectively (68, 346). Except for HSV-1 gG, secreted forms of gG have been identified for many alphaherpesviruses (261). It is the least conserved glycoprotein between HSV-1 and HSV-2 and is antigenically distinct in HSV-1 versus HSV-2. Thus, the antibody response to gG is type specific and therefore widely used as a discriminating tool for serological identification (280). gG is known for its immunomodulatory activities. The secretory part of HSV-2 gG has been shown to cause activation of phagocytes and downregulation of NK activity (21). Also, gG might postpone apoptosis in infected cells and contribute to efficient cell-to-cell spread (261). Finally, glycoprotein G functions as a host chemokine binding protein, a mimicry which allows modulation of the host immune response (38).

gH (UL22)

Glycoprotein H is essential and forms a heterodimer with glycoprotein L. The gH/gL complex is, together with gB, part of the fusion machinery of many herpesviruses (92). The protein has a quite large ectodomain and reaches 838aa for HSV-1 and 842aa for BoHV-1 (68, 92). gH/gL can bind cellular integrins as a regulator for fusion. At present, based on the unique structure of gH/gL, resolved in whole or in part for several herpesviruses, the complex is proposed to not function as a fusogenic protein in itself but instead regulates the fusogenic activity of gB (92).

gI (US7)

Glycoprotein I, together with gE, is present within the gE/gI complex. HSV gI (411aa) and BoHV-1 gI (382aa) associate soon after synthesis with gE to form a stable complex (68, 270). Knock-out of gI results in an export and modification defect of gE. However, this defect is kinetic in nature and not absolute as gE can be detected on the surface of BoHV-1 gI null infected cells (361). Functions of the gE/gI complex are described above.

gK (UL53)

For both HSV-1 and BoHV-1, glycoprotein K consists of 338aa (68, 270). gK is important for cell-to-cell spread, syncytia formation, neurovirulence and immunomodulation (188). HSV-1

gK physically interacts with UL20, which is mandatory for their intracellular transport and subsequent membrane expression, and functions in virion egress, virus-induced cell fusion and virus entry. Moreover, the amino terminus of gK interacts with gB and gH to regulate cell fusion (53).

gL (UL1)

Glycoprotein L is a small glycoprotein of 224aa in HSV-1 and 158aa in BoHV-1 (68, 92). The protein lacks a transmembrane region but is maintained within the stable heterodimeric complex gH/gL. In viruses lacking gH, gL is either secreted or retained in cells in its immature form. On the other hand, gL is necessary for correct folding and trafficking of gH (92). Functions of the complex gH/gL are mentioned above.

gM (UL10)

Glycoprotein M is a type III integral membrane protein with multiple transmembrane domains that form a complex with pUL49.5 (gN). The functions of gM, which is 438aa long in BoHV-1 and 473aa long in HSV-1, are promoting spread (neurovirulence) and retaining viral glycoproteins in the TGN or retrieving them from the plasma membrane to the TGN (68, 191). The heterodimer gM/gN complex is thought to modulate membrane fusion (191).

gN (UL49.5)

Glycoprotein N is a 91aa long glycoprotein in HSV-1 (236). However, in BoHV-1, this protein is not glycosylated and therefore, considered as a false glycoprotein (96aa) (260). Next to the functions that it exerts within the complex gM/gN mentioned above, UL49.5 plays a major immunomodulatory role. Herpesviruses evade elimination by cytotoxic T lymphocytes through specific interfering with the transporter associated with antigen processing (TAP) and subsequent antigen-presenting function of major histocompatibility complex class I (MHC I). For several herpesviruses, UL49.5 has been shown to act as a TAP-inhibiting molecule (352).

1.1.2. Replication cycle

A detailed overview of the alphaherpesvirus productive replication cycle, valid for BoHV-1 and HSV, is depicted in Figure 2.

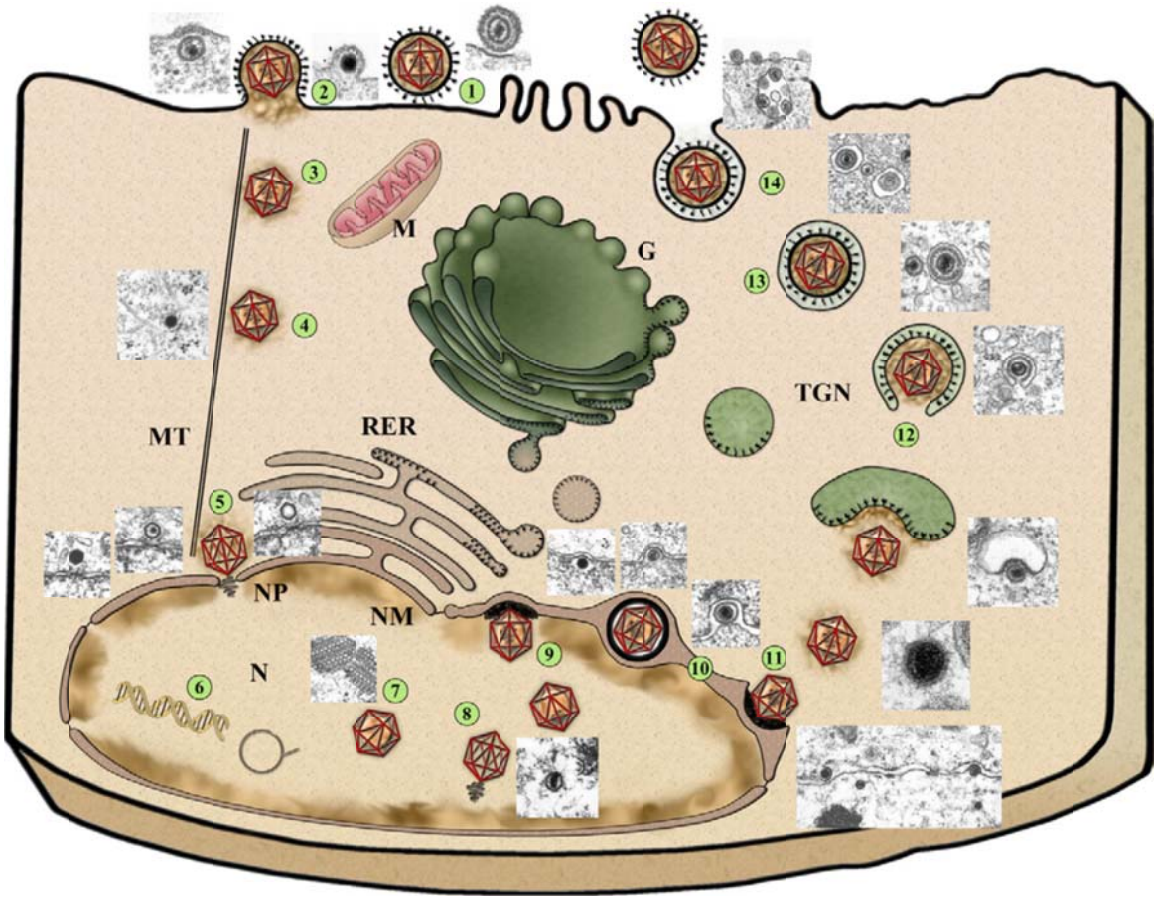

Figure 2. Productive replication cycle of alphaherpesviruses (243). (1) Initial non-stable binding to heparan sulfate moieties is followed by stable binding to cellular receptors. (2) The viral fusion apparatus is activated allowing nucleocapsid and tegument release into the cytoplasm. Next, nucleocapsids are transported (3) towards the nucleus (N) via interaction with microtubules (MT) (4) and dock at the nuclear pore (NP) (5). In the nucleus, DNA replication and transcription occur (6) which will finally result in a large amount of new viral genomes. Subsequent packaging of these genomes into newly formed capsids (7) is known as the encapsidation process (8). Hereafter, nucleocapsids leave the nucleus by budding into the inner nuclear membrane (INM) (9). Once in the perinuclear space (10) these primary enveloped particles fuse with the outer nuclear membrane (ONM) (11) resulting in cytoplasmic release of unenveloped particles. Secondary envelopment happens through budding of these particles into vesicles of the trans-Golgi network (TGN) (12). This secondary envelope contains all viral glycoproteins. Finally, after transport to the plasma membrane (13), fusion of the vesicle and the plasma membrane takes place which releases mature virions in the extracellular space (14).

Virion entry and transport to the nucleus

As described above, attachment to susceptible cells happens trough interaction of gC (and to a lesser extent gB) with heparan sulfate proteoglycans. Next, gD will form a stable binding with one of the possible cellular entry receptors (see above). This binding will trigger membrane fusion governed by the fusion apparatus gB and gH/gL (92). Although a non-endocytotic pathway via direct fusion with the cell membrane is still considered as the major entry mode for herpesviruses, there is growing evidence that, at least in some cell types, entry might also happen through low-pH-dependent endocytosis (110, 286). Once inside, capsids misuse the cellular motor protein dynein for direct microtubule-based transport towards the nuclear pore complexes. At the nuclear pores, docking complexes are formed and the viral DNA is released within the nucleus (323, 371).

Gene expression and replication

Upon arrival in the nucleus, the genome circularizes. Next, transcription is initiated and this happens in a tightly regulated cascade manner. Viral genes can be subdivided into three categories and are sequentially transcribed: immediate-early (IE), early (E) and late (L) genes (264). The latter are currently being subdivided in leaky-late genes and true-late genes (271). First, IE genes are transcribed which results in the production of regulatory proteins that are crucial for activating transcription of E and L genes. This IE transcription is orchestrated by a transactivator protein, brought into the cell as a tegument protein, namely VP16 (203). Vhs (virion host shut-off), another tegument protein, is part of the viral arsenal to express viral transcripts at the expense of cellular protein synthesis by affecting the stability of cellular mRNAs (99). HSV-1 encodes 5 IE genes: ICP0, ICP4, ICP22, ICP27 and ICP47 (140); whereas BoHV-1 only encodes four different IE genes: BICP0, BICP4, BICP22 and circ protein (310). Second, the E gene products are formed and mainly play a role in nucleotide metabolism (e.g.UL23 and UL50) and DNA synthesis (e.g. UL5 and UL42) (210). Third, expression of L genes is triggered by DNA synthesis, which typically encode structural proteins, such as glycoproteins, required for virus assembly and egress (210).

Assembly and egress

Rolling circle replication generates tandem head to tail concatemers consisting of multiple unit-length genomes that accumulate in the nucleus. These concatemers are cleaved to monomeric units and packaged in individual, pre-formed capsids (251). Since all viral proteins are synthesized in the cytoplasm and viral DNA replication takes place inside the nucleus, a translocation of capsid proteins to the nucleus is necessary for nucleocapsid formation (241, 242). The encapsidation process is tightly regulated and requires for HSV-1 the action of at least 7 HSV-1 gene products (157, 160). Fully assembled nucleocapsids are too large to pass through the nuclear pores, hence they utilize an envelopment/development mechanism for transport to the cytoplasm (242). Although the virus was thought to hijack a membrane disassembly mechanism, recent data suggest that it may instead be subverting an endogenous pathway. Indeed, the mechanism by which large ribonucleoprotein particles exit the nucleus is quite reminiscent of that used by herpesviruses during the release of viral capsids (175, 315). First, nucleocapsids bud through the inner nuclear membrane thereby gaining both tegument proteins and a primary envelope. For all herpesviruses, 2 viral proteins UL31 and UL34 are required in this process (242). Primary enveloped nucleocapsids, located in the perinuclear space, will undergo a subsequent fusion with the outher nuclear membrane. Thus de-enveloped nucleocapsids will be released in the cytosol. Once here, the majority of the tegument is added to the nucleocapsids, which obtain their final envelope by budding into glycoprotein-containing Golgi derived vesicles (242, 244).

Intercellular spread

Once fully assembled and enveloped, virions use two different pathways to infect naïve susceptible cells. Either progeny virus is released via exocytosis in the extracellular space. These then bind to new cells via the traditional entry pathway. Or, virus can spread directly from one cell to another. A quite ingenious mechanism as this allows the virus to escape from antibodies, complement and phagocytes. Different mechanisms of cell-to-cell spread have been described (262). This process is mediated by many proteins, including gB, gD, gE/gI and US3 (106, 262).

1.1.3. Pathogenesis of bovine herpesviruses

Bovines host both alpha- and gammaherpesviruses. Table 1 provides an overview of the different herpesviruses that are associated with diseases in ruminants (93, 260). Transmission of herpesviruses in ruminants happens mainly via direct contact between infected animals, via aerosols or via semen but also intrauterine (93). We will focus on bovine herpesvirus 1 and bovine herpesvirus 4 as they are the bovine viruses used in this thesis.

Table 1. Herpesviruses isolated from naturally infected cattle

Virus species	Acronym	Natural host	Subfamily	Clinical signs
Bovine herpesvirus 1	BoHV-1	Cow	α	Respiratory disease (IBR), genital disease (IPV/IPB), abortion, high fever, milk drop, diarrhea (260)
Bovine herpesvirus 2	BoHV-2	Cow	α	Mammilitis (341)
Bovine herpesvirus 4	BoHV-4	Cow	γ	Associated with post-partum metritis, mastitis, infertility (137)
Bovine herpesvirus 5	BoHV-5	Cow	α	Meningo-encephalitis in calves (169)
Bovine herpesvirus 6	BoHV-6	Cow	γ	Lymphotrophic herpesvirus – disease image unknown (56)
Alcelaphine herpesvirus 1	AlHV-1	Wildebeest	γ	Malignant catarrhal fever (MCF) (303)
Ovine herpesvirus 2	OvHV-2	Sheep	γ	Malignant catarrhal fever (MCF) (303)
Pseudorabies virus	PRV	Pig	α	Aujeszky's disease (262)

Bovine herpesvirus 1

Bovine herpesvirus 1 (BoHV-1) is the known etiological agent of infectious bovine rhinotracheitis (IBR) and infectious pustular vulvovaginitis/balanoposthitis (IPV/IPB) in cattle and may replicate in both respiratory and genital mucosa. However, respiratory and genital infections have been assigned to different BoHV-1 strains in the past (260).

Although there is proof of the existence of the virus as early as 1941, BoHV-1 was firstly isolated in 1955 (125, 221). In the late 1950's both the respiratory disease prototype strain Cooper and the genital disease prototype strain K22 were isolated from field cases of respectively IBR and IPV in the United States (186, 367). Importantly, before 1977, a continental discrepancy was present. Genital infections were predominant throughout Europe whereas respiratory infections were mainly prevalent at feedlots in the United States and Canada. Some authors believe that the virulent respiratory strains emerged out of the less virulent genital strains. They state that the enhanced virulence of the virus for the respiratory epithelium is a consequence of rapid passages of the virus in crowded susceptible populations present in "feedlots", typically for the United States at that time. Since 1977, severe "North American like" IBR emerged on the European continent (162, 238, 322).

An attempt was made to see if BoHV-1 could be subdivided into distinct types with different tropisms i.e. whether a correlation could be found between IBR and IPV on the one hand and distinct virus subtypes on the other hand. Using restriction endonuclease digestion and reactivity tests to a panel of monoclonal antibodies, a classification was made of different BoHV-1 subtypes. BoHV-1.1 was associated with respiratory disease and abortion whereas BoHV-1.2 was regarded as a genital type (232, 246, 249). Subtype BoHV-1.3 was renamed into BoHV-5 as new findings showed significant differences in genomic and antigenic properties between BoHV-1.3 and other BoHV-1 strains (59, 247). Furthermore, a distinction was made between different BoHV-1.2 subtypes. BoHV-1.2b causes local genital lesions and possibly mild respiratory illness; BoHV-1.2a seems to have both tropism for the genital and respiratory mucosa and is associated with abortion (232, 246, 329). However, this postulation about several subtypes possessing diverse mucosa tropisms has been contested since several studies showed no correlation between the different genotypes and their clinical manifestations (54, 66, 162, 222, 238, 257, 329).

Respiratory and genital infection

Upon entering the host at the respiratory or genital tract, BoHV-1 will initiate replication in epithelial cells. The pathogenesis of BoHV-1 is shown in Figure 3. BoHV-1 sets up a lytic replication cycle which will result in cell ballooning and eventually in cell death (260). Primary encounter with the virus will result in extensive nasal shedding of progeny virus which will last 10 to 16 days post infection. Maximum virus titers within nasal and genital secretions can reach up to 10^8-10^{11} Tissue Culture Infectious Dose ($TCID_{50}$)/ gram mucus.

These high titers are substantial considering the fact that 10^2 $TCID_{50}$/gram mucus is enough to cause infection in cattle and will allow the virus to quickly disseminate within a herd (82).

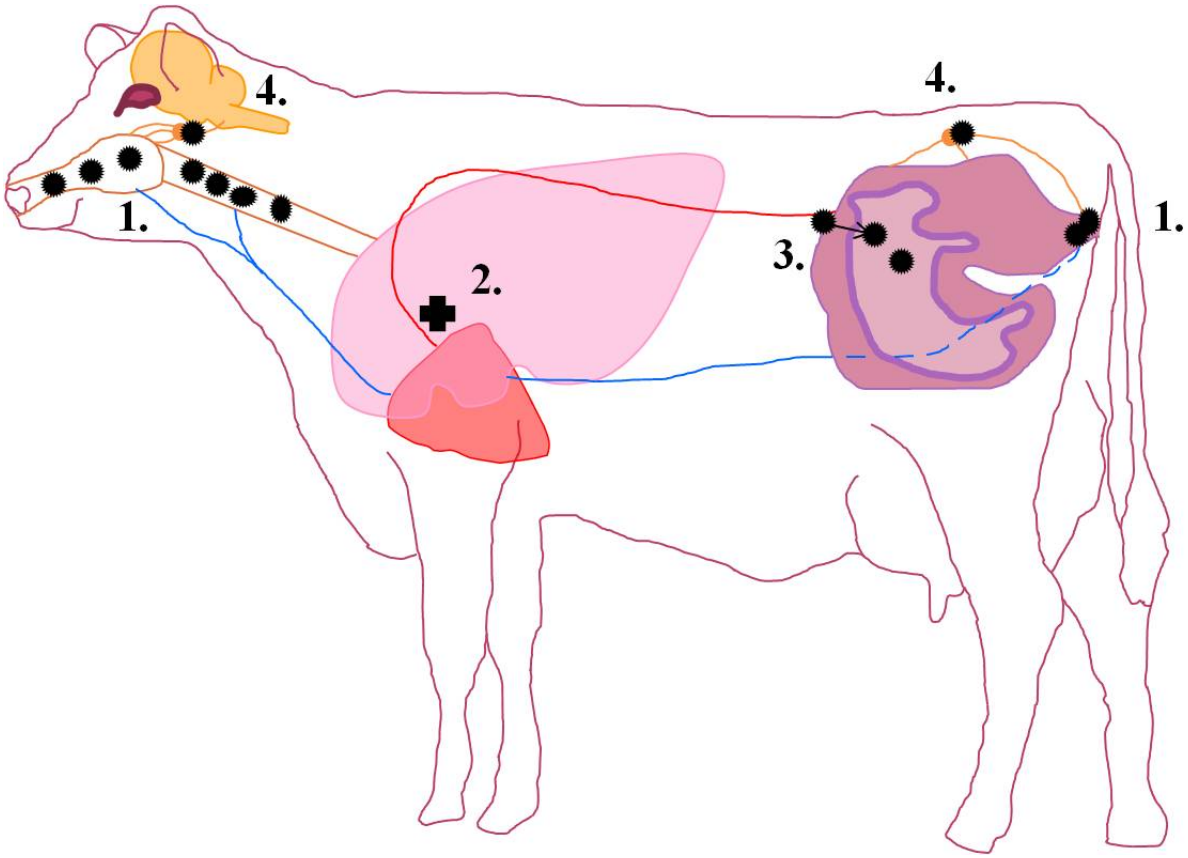

Figure 3. Pathogenesis of BoHV-1. The primary replication sites of BoHV-1 are either the respiratory or genital tract (1.) After local dissemination, the virus gains access to the blood vessels and spreads via viremia to internal organs (2.) In pregnant cows, the virus might reach the uterus and causes extensive replication within the fetus, resulting in abortion (3.). Moreover, the virus will invade nerve endings at primary replication sites and establishes life-long latency within the host (4.)

The basic reproduction ratio (R_0) is a parameter for the average amount of secondary cases generated by one primary case and describes the infection dynamics within a herd. For BoHV-1, R_0 is estimated to be at least 7 (260). Clinical symptoms of respiratory infection are mucopurulent discharge from the nose and eyes, high fever (>41°C), anorexia, hypersalivation and milk drop. Secondary bacterial colonization is a common complication and might aggravate the clinical image. These animals are in severe dyspnee, show open mouth breathing and often succumb. The clinical entity is called infectious bovine

rhinotracheitis (IBR) and causes significant economical losses worldwide if not controlled (7). Initial genital manifestations of BoHV-1 are pustules in the caudal vagina and/or vulva for cows and pustules on the penis for bulls, termed infectious pustular vulvovaginitis (IPV) and infectious pustular balanoposthitis (IPB) respectively. Additionally, hyperemia and edema can be observed. In general, genital infections are less severe than respiratory infections and also less common, as the use of artificial insemination (AI) and negative bulls repressed this clinical image (82, 260). Importantly, subclinical BoHV-1 infections are common. Indeed, the severity of disease is influenced by multiple factors such as virus strain, age and host resistance factors (260). After local dissemination the virus can go up two ways: (i) reaching internal organs via leukocytes (viremia) and (ii) invade nerve endings and hide in the infected host (latency).

Systemic spread via viremia

Very little information is known regarding the mechanisms by which this virus gains access to blood vessels and spreads to internal organs. Although a cell-associated viremia is readily described for equine herpesvirus 1 (EHV-1) and pseudorabies virus (PRV), it is less clear for BoHV-1. Only one report describes the isolation of BoHV-1 from peripheral blood monocytic cells (PBMC) after nasal infection (46). If the virus reaches the uterus in seronegative pregnant cows, it can induce abortion. Under field circumstances, BoHV-1 induced abortion is mainly seen at 4 to 8 months of gestation. The mechanism by which the virus spreads through the placenta to the fetus is unknown but happens most likely via the umbilical vein. The incubation period between inoculation with BoHV-1 and abortion is 15 to 64 days (254, 260). Fatal systemic infections might occur in young seronegative calves following congenital or early post-natal exposure (179, 260).

Neuroinvasion and latency

Within the mucosa, herpesviruses in general can gain access to local sensory nerve endings and ascend towards the central nervous system. BoHV-1 uses either the trigeminal nerve in the nasal mucosa or sacral nerves in the genital mucosa. Unlike, BoHV-5 and PRV, BoHV-1 rarely passes beyond the first order neurons in the trigeminal and sacral ganglia respectively. Therefore, BoHV-1 induced central nervous disorders are only seen sporadically in the field (69, 296).

A hallmark of herpesviruses is to establish latency within its natural host. Thus, after primary BoHV-1 infection, animals become latent carriers of the virus within neuronal cell bodies located in the previously mentioned nervous ganglia. Unlike in epithelial cells, only the BoHV-1 region containing the latency associated-transcripts is expressed in neurons which will inhibit a lytic viral cycle and induce an anti-apoptotic state. Consequently, no production of progeny viruses occurs. Latency in this particular cell type is a strategy of the virus for life-long survival in the host and enables the virus to avoid clearance by the host immune system. Upon specific natural stimuli (such as stress, parturition,…) or corticosteroid treatment, reactivation from this quiescent state might occur and give rise to recurrent viral spread. It has become clear that latency of alphaherpesviruses is a multi-factorial process, dependent on the interplay between neuron, virus and immune system (176, 177, 260).

Bovine herpesvirus 4

Bovine herpesvirus 4 (BoHV-4) is a gammaherpesvirus belonging to the *Rhadinovirus* genus and is associated with postpartum metritis and chronic infertility in cows (65). Nevertheless, this association is not absolute as BoHV-4 has been isolated from different pathologies including conjunctivitis, dermatitis and respiratory disease; and even from healthy animals (84, 111). At present, there is increasing evidence that BoHV-4 more likely plays a secondary pathogenic role in postpartum metritis (111). The first reported isolation of BoHV-4 originates from a case of bovine metritis in 1973 (272). Although the virus seems to have a tropism for endometrial stromal cells and epithelial cells, the exact pathogenesis remains to be elucidated (84).

1.1.4. Pathogenesis of human herpesviruses

Nine different herpesviruses are known to use humans as hosts (Table 2) (75). Human herpesvirus 1 and 2 will be discussed in this section as they are the human viruses used in this thesis.

Table 2. Overview of herpesviruses known to infect humans

Virus species	Acronym	Natural host	Subfamily	Clinical signs
Human herpesvirus 1 Herpes simplex virus 1	HHV-1 HSV-1	Humans	α	Gingivostomatitis, mucosal blisters, genital herpes, encephalitis, keratitis (12)
Human herpesvirus 2 Herpes simplex virus 2	HHV-2 HSV-2	Humans	α	Gingivostomatitis, mucosal blisters, genital herpes, encephalitis, keratitis (12)
Human herpesvirus 3 Varicella-zoster virus	HHV-3 VZV	Humans	α	Chickenpox, shingles, chronic pain, rash, vasculopathy, zoster paresis, ocular disorders (124)
Human herpesvirus 4 Epstein-Barr virus	HHV-4 EBV	Humans	γ	Associated with lymphoid and epithelial malignancies e.g. Burkitt's lymphoma and nasopharyngeal carcinoma, infectious mononucleosis (24, 166)
Human herpesvirus 5 Human cytomegalovirus	HHV-5 HCMV	Humans	β	Congenital infections – neurodevelopmental delay, infectious mononucleosis (226, 295)
Human herpesvirus 6	HHV-6	Humans	β	Linked with neurological disease, multiple sclerosis, exanthema subitum (290, 359)
Human herpesvirus 7	HHV-7	Humans	β	Linked with exanthema subitum, largely unknown (117, 230)
Human herpesvirus 8 Kaposi's sarcoma-associated herpesvirus	HHV-8 KSHV	Humans	γ	Low-grade vascular tumor induction, often muco-cutaneous (104)
Macacine herpesvirus 1 B-virus	McHV-1	Monkeys	α	Encephalitis, encephalomyelitis, death (100)

Human herpesvirus 1 and 2

Human herpesvirus 1 (HHV-1), also known as herpes simplex virus 1 (HSV-1) and human herpesvirus 2 (HHV-2), termed herpes simplex virus 2 (HSV-2) are both members of the *Simplex* genus within the alphaherpesviruses. HSV infections are prevalent worldwide and have no seasonal variation (12). Transmission of HSV happens through direct contact with virus-containing body fluids (aerosol, genital secretions, saliva, exsudates of active lesions,…). Importantly, viral shedding of HSV occurs often during the prodromal phase of primary or recurrent infections and results in nearly 60% of the cases without clear symptoms (12, 307). In Europe, the seroprevalence of HSV-2 within the adult population varies from 4% to 24% which is comparable to that in the United States (2% to >25%). For HSV-1, a seroprevalence of 52-84% in Europe and of 90% in the United States among adolescents was registered (275). At present, no efficacious HSV-vaccine is present on the market, and the likelihood of one coming to market soon is low: a relatively recent publication in the journal Science describes the failure of a potential HSV-vaccine in a clinical trial (57). When treating herpes virus infections with antiviral compounds, resistance occurs frequently which limits the application of these products (87, 181).

In general, HSV-1 is accepted to cause infections 'above the waist' and HSV-2 'below the waist'. Nevertheless, the frequency of for example primary genital herpes infections caused by herpes simplex virus type 1 (HSV-1) is on the increase (50). Therefore, both HSV-1 and HSV-2 are nowadays considered as causative agents of similar clinical manifestations at multiple sites of the body (12).

Primary respiratory/oral infection

HSV can infect the oral mucosa and cause primary gingivostomatitis in children and young adults (12). Recently, HSV-1 was found to also severely infect human nasal mucosa (127) and possibly the neuroepithelium (320). However, most primary herpes simplex infections in children are asymptomatic. The incubation period is on average 2-20 days after which non-specific symptoms may arise like myalgia and malaise. 1-3 days after the onset of disease, the typical muco-cutaneous vesicular eruptions might arise. Lesions are usually accompanied by pyrexia, lethargy, loss of appetite,… Blisters heal gradually within 14 days, if not complicated by secondary bacterial infections (12, 197). If during HSV invasion nerve endings or blood vessels are reached, these viruses can spread in the host, resulting in establishment of latency and potentially nervous disorders and viremia respectively (174, 250).

Primary genital infection

Human genital herpes is worldwide one of the most prevalent causes of genital ulcer disease and with a prevalence reaching up to 80% (developing countries), one of the most important sexually transmitted infections (STI). Genital manifestations of initial infection display an array of symptoms going from asymptomatic to severe bilateral vesicular lesions which arise typically 4-7 days after initial infection. Because of extensive replication of the virus these lesions can be purulent, and in some cases lead to necrotic trauma. Systemic consequences consist of flu-like symptoms (fever, headache, myalgia) and dysuria (12). Moreover, genital herpes lesions promote the transmission of HIV (50, 287). Within the genital tract, similar as for BoHV-1, sacral ganglia may become latently infected by HSV after primary herpes genitalis (347). Importantly, in women experiencing primary genital herpes infections, the majority show signs of HSV-induced viremia (174).

Recurrent herpetic infections (herpes labialis and herpes genitalis)

Different stimuli can trigger the virus to reactivate from sensory ganglia. Typically, symptoms in the respiratory/oral region and genital region are less severe during a recurrent infection compared to a primary infection. Often, intervals of asymptomatic shedding periods exist and are key for HSV transmission (306, 307). The vast majority of lesions is located on muco-cutaneous junctions such as the lips and the *labiae vulva* (12).

Other manifestations of HSV infection

In Table 3, an overview of other manifestations of HSV infection is given. Congenital and perinatal herpes infections, and brainstem encephalitis in neonates, are well known complications of maternal genital herpes (250). Neonates often get exposed to the virus during vaginal delivery or a seldom intrauterine transmission. A clinical episode of genital maternal herpes is an important reason to perform a caesarian section (145, 216, 292). Primary or recurrent ocular infection can give rise to blepharitis, conjunctivitis, epithelial and stromal keratitis and more. Moreover, around 20% of HSV-induced keratitis can lead to a blinding stromal keratitis. Although the exact pathogenesis of herpetic stromal keratitis remains poorly understood, repeated bouts of recurrent HSV stromal keratitis initiate and sustain a chronic inflammatory reaction in the eye, which will lead to progressive irreversible cornea scarring and blindness (12, 301).

Table 3. Additional disease images caused by HSV infection

Name	Clinical signs
Herpetic whitlow	Pain, burning eruption of distal phalanx/fingers
Herpetic rash	Atopic dermatitis, eczema herpeticum
Herpes gladiatorum	Trigeminal, cervical or lumbo-sacral dermatomes
Ocular herpetic infections	Blepharitis, conjunctivitis, epithelial keratitis, stromal keratitis, iridocyclitis or retinitis
Neonatal herpetic infections	Meningo-encephalitis, death
Erythema multiforme	Cutaneous and oral lesions
Behcet's disease	Oculo-urogenital syndrome, immune-mediated disorder
Bell's palsy	Neuropathy, facial nerve paralysis
Meniere's disease	Hearing and balance disorders
Herpes lymphadenitis	Haematological malignancies
Herpes encephalitis	Neurological disorders

1.2. Characteristics of respiratory and genital mucosae

Upon entering the host, viruses face different barriers that hamper their invasion (Figure 4). At the level of the mucosa; mucus, epithelium, basement membrane (BM) and lamina propria are the principal barriers these viruses have to overcome. These structures will be considered briefly below. In addition, mucosa innervation and resident immune cells are summarized.

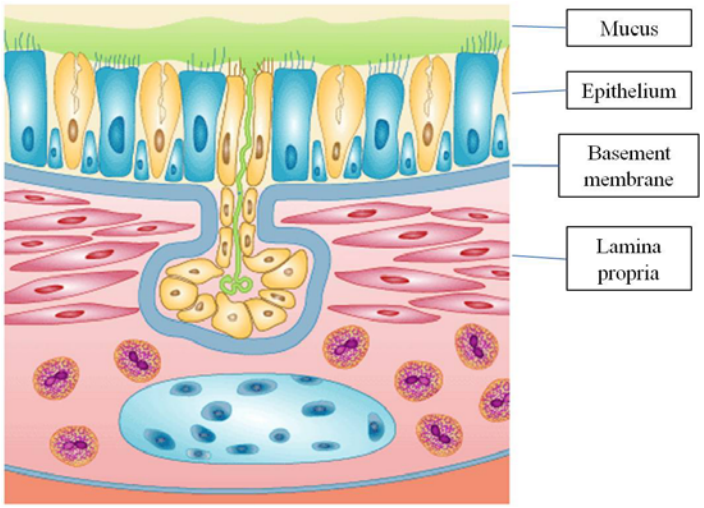

Figure 4. Schematic representation of the respiratory mucosa. Starting from the lumen several layers can be distinguished: a mucus layer (i) lying on top of a ciliated pseudostratified epithelium (ii). Underneath the epithelium a basement membrane (iii) separates the epithelium from the underlying lamina propria (iv). Adapted from (1).

1.2.1. Mucus

The epithelial surface of the respiratory tract is covered by a layer of airway surface liquid (ASL), mainly produced by the goblet cells and submucosal mucous and serous glands. The ASL is composed of a mucus layer overlying a watery periciliary liquid (PCL) layer. The low viscosity aspect of the PCL hydrates cilia and allows them to beat in a coordinated way. Interestingly, epithelial cells maintain ASL composition and volume by exchanging Cl^- and Na^+ ions through ion canals. All these elements are part of what is called the mucociliary clearance apparatus, a defense mechanism which captures and expulses foreign particles (146, 298, 327). Movement of tracheal mucus in healthy adult humans has been found to be on average 4-20 mm/min (305).

In humans, the mucus network contains two major structural types of mucins: MUC5AC and MUC5B, which are mainly produced by goblet cells and submucosal glands, respectively. In this context, it is also important to note that both secretory and membrane-associated forms of mucins exist (297). In bovines, respiratory mucus likely contains similar structural elements (164, 335). The specific interactions of these mucins with foreign particles is not yet fully elucidated. Likely, such interactions are mediated by glycan-binding lectins. Indeed, in mucin protein backbones many serine and threonine residues are covalently linked with O-glycosidic carbohydrate structures (62). Several proteins with proven antimicrobial functions reside within the mucus network including lysozyme, lactoferrin, secretory IgA, defensins, secretory leukocyte peptidase inhibitor (SPLI), lactoperoxidase, CCSP, SP-A and SP-D (62, 146, 156, 318, 356).

Like the respiratory tract, also the genital tract is covered by a thick mucus layer. Whilst the majority of mucus lining the vaginal cavity finds its origin in the gland-like crypts of the cervical canal and is transported towards the vulva, ruminants contain both *glandulae vestibulare minors* and *majors* in the vestibule of the vagina for additional on-site mucus production (79, 108, 331). Functions of cervical mucus are protection of the female reproductive tract from pathogenic invasion (cfr. respiratory mucus) and fluid loss and moreover regulation and facilitation of sperm transport to the upper reproductive tract (139). Incoming pathogens have to devise ways to overcome this vivid barrier to reach the underlying epithelium.

1.2.2. Epithelium

1.2.2.1. Respiratory epithelium

We will focus in this section on the upper respiratory tract, as this is the major entry route for herpesviruses.

The airway epithelium is a dynamic epithelium likely renewing every 30-50 days (62). The airway tract can be subdivided into two zones. The first zone, in which inhaled air is cleaned, moistened and transported towards the distal airways, is called the conditional zone and reaches from the *pars respiratoria* of the nasal cavity over the nasopharynx, larynx, trachea and bronchi to the large and terminal bronchioles. Through most of the conditional zone, the airways are lined by a ciliated pseudostratified columnar epithelium. All cells are in contact with the basement membrane, however, basal cells do not reach the airway lumen. This organization, called pseudostratified, gives the impression that nuclei are not aligned in the same plan and are multilayered (Figure 4). At the end of the conditional zone (i.e. the last branches of bronchi and the bronchioles), a second zone termed the respiratory zone commences. At the level of the respiratory zone an extensive oxygenation of the blood takes place. Typically, at this junction the epithelium changes from pseudostratified to a simple cuboidal epithelium. Several morphologically distinct epithelial cell types are present in respiratory epithelium, although based on ultrastructural, functional and biochemical criteria these may be classified into three categories: basal, ciliated and secretory (172, 327, 337). In the context of this thesis, only the main cell types present in ciliary pseudostratified columnar epithelium will be considered in brief.

Ciliated cells are the main cell type within ciliated pseudostratified columnar epithelia. They arise from either basal or secretory cells. Typically, they possess up to 300 cilia/cell projecting into the lumen, have a small basal cell side and wide apical side; and contain numerous mitochondria apically. Their main function consists in directional transport of mucus from the lungs to the throat by ciliary beating in coordinated waves. This mucociliary clearance is an important initial defense mechanism against incoming foreign particles (195, 327).

Goblet cells are periodic acid Schiff's (PAS) positive, columnar epithelial cells that secrete, together with the submucosal glands, mainly high molecular weight mucus glycoproteins ('mucins') but also lipids and smaller glycoproteins. This mucus layer serves as the body's first line of defense against microorganisms but also assists in nutrient transport and viscosity

regulation (366). Discharge of mucus from the membrane-bound electron-lucent mucous granules is accomplished remarkably rapidly in response to a wide variety of stimuli, including proteinases, irritant gases, inflammatory mediators, reactive oxygen species, nerve activation and changes in the biophysical environment. Importantly, goblet cells have a direct role in epithelial homeostasis as they possess the potential to differentiate into ciliated cells (298, 327). However, columnar cells are documented to lack sufficient telomerase activity to restore a fully differentiated epithelium (141).

Brush cells are a sparse, randomly distributed, special type of projection-containing cells found in the alimentary and respiratory tract. Their 'brush'-like appearance originates from the presence of multiple large thick microvilli on the apical side of the cell. Although their function is not fully elucidated yet, they are believed to function mainly as chemosensory cells. Also, absorptive and secretory functions of brush cells are described (288, 309, 327).

Basal cells are considered to be the resident epithelial stem cells as they play an indispensible role in epithelium homeostasis and tissue renewal post injury. Indeed, their potential to flatten out and cover the BM when contacts with neighboring columnar cells are lost is considered as a defense mechanism (96). The basal cell has a sparse electron-dense cytoplasm containing bundles of cytokeratin, a small polyhedral shape and does not reach the airway lumen. On the contrary, it is the only cell type within the respiratory epithelium that is firmly attached with its wide basal side to the BM via necessary hemidesmosomes (discussed below). Hence, basal cells function as anchors for more superficial cells in the epithelium by connecting with them through desmosomes (discussed below). They also modulate the regulation of neurogenic inflammation, the inflammatory response, transepithelial water movement, oxidant defense and formation of lateral intercellular spaces (98, 195, 327).

In addition, a variety of immune cells, inflammatory cells and phagocytic cells migrate to and remain within the epithelium or transit through to the lumen (184). An overview of immune cells and this transmigration process is provided in part 1.2.5. and 1.3.3. of this thesis.

1.2.2.2. Genital epithelium

Structures involved in the pathogenesis of herpesviruses will be considered below.

The vulva is formed by the *labiae vulvae* which physically seal the external opening of the genital tract. They are covered externally by skin epithelium, a keratinized stratified squamous epithelium, that is richly supplied by several apocrine and sebaceous glands. Internally, they are lined by a stratified squamous epithelium, much like the vagina. The vaginal cavity encompasses the vestibule (*vestibulum vaginae*), the vagina and the ectocervix (*portio vaginalis cervicis*). The entire cavity is lined by a nonkeratinized stratified squamous epithelium except in the anterior portion of the bovine vagina which shows a stratified columnar epithelium interspersed with goblet cells. The transformation zone, also called squamocolumnar junction, represents an abrupt transition between ectocervix and endocervix and is characterized by a transitional epithelium. The endocervical canal, termed endocervix, is leading towards the uterus and consists of a highly active simple columnar epithelium with numerous glands (14, 79, 170, 283). Finally, the endometrium, as part of the uterus, is lined in most mammals by a simple cuboidal or columnar epithelium but may be stratified or pseudostratified in ruminants (14). Interestingly, the different types of epithelia mentioned above are affected differently by reproductive hormones and differ in susceptibility to infection by different organisms. In humans, *Candida albicans* and *Trichomonas vaginalis* colonize the vagina whereas *Chlamydia trachomatis* and *Neiseria gonorrhea* settle within the endocervix. The transformation zone is the main target for human papillomavirus (HPV) (170, 283). The three types of epithelia that are present in the lower genital tract will be discussed in brief below.

Nonkeratinized stratified squamous epithelium

Three layers can be found within this epithelium: basal cells (*stratum basale*), parabasal cells and intermediate cells (*stratum spinosum*) and superficial cells (*stratum superficiale*). Basal and parabasal cells are round cells with small nuclei. Intermediate cells are larger than parabasal cells and have rounded edges. Superficial cells are fully matured and have pyknotic nuclei or lack nuclei. Often these cells are filled with glycogen and are eventually sloughed off. Only basal and parabasal cells have the ability to divide (14, 102, 331). Interestingly, estrogen induces proliferation, maturation and desquamation, and superficial cells predominate during periods of high estrogen levels. During episodes of high progesterone levels, partial maturation is observed and especially intermediate cells are present (170).

Transitional epithelium

The original neonatal position of the squamocolumnar junction changes over time, especially in humans. Reproductive hormones strongly influence tissue morphology, size and activity. As the endocervical mucosa is relatively stable in length and anatomically restrained because of its strong attachment to a muscular wall, endocervical expansion under influence of reproductive hormones can only occur downwards onto the ectocervix. Consequently, a protrusion occurs into the vaginal cavity and endocervical epithelium becomes exposed to the vaginal environment. This phenomenon is general referred to as cervical ectopy and is most prevalent in adolescents, pregnant women and women using oral contraceptives (170, 258). Moreover, two remarkable findings can be observed in the transitional epithelium. First, the exposed simple columnar epithelium changes into squamous epithelia most likely due to a pH-induced reduction in buffering capacity of overlying mucus, estrogen influences and growth of vaginal bacterial flora. This remodeling event is called squamous metaplasia. Second, squamous epitheliazation takes place whereby tongues of squamous epithelium from the native squamous epithelium of the ectocervix are thought to grow underneath reactive columnar epithelium within the transformation zone for replacement. Thus the resulting squamous epithelium in the transformation zone might originate from exposed subcolumnar cells (squamous metaplasia) or from remodeling events out of the ectocervix (squamous epitheliazation) (153, 170, 258, 283).

Simple columnar epithelium

This epithelium consists of a highly polarized, single layer of mucin-secreting cells. Underneath and adjacent to columnar cells, several subcolumnar or reserve cells are present which are believed to multiply and differentiate into squamous epithelium upon certain stimuli. The endocervical epithelium is elevated into several folds and forms crypts or pseudoglands (14, 39, 170).

As in respiratory epithelium, different immune cells are present for immune surveillance (see 1.2.5.).

1.2.2.3. Cell-cell and cell-matrix adhesions

Epithelial barrier integrity is maintained by the physical interactions of intercellular junction molecules on opposing epithelial cells. They do not only maintain integrity but are also required for regulating molecular and cellular traffic. During tissue remodeling events such as wound healing and tissue repair, these junctions can be quickly disassembled and reassembled to facilitate these processes. There are two types of adhesion mechanisms (Figure 5). A first group consists of weak, unorganized intercellular interactions, called non-junctional, and simply link adjacent interacting plasma membranes close enough to provide traction and to allow transmembrane adhesion proteins to interact but still allowing cell motility. A second group are called junctional, referring to their highly organized structure and their tight anchorage to the cytoskeleton, and generally immobilize the cell. It is believed that non-junctional cell-cell adhesion proteins initiate cell-cell adhesions, which are then oriented and stabilized by the assembly of true junctional intercellular adhesive structures (9). Based on functionality, junction molecules can be classified as either occluding, anchoring or communicating (Figure 5). There are four major types of cell-cell adhesions: occluding tight junctions (*zonulae occludens*); anchoring adherens junctions (*zonulae adherens*) and desmosomes (*maculae adherens*); and communicating gap junctions. The first three are usually present as well organized tripartite complexes. Next to cell-cell contacts, cells also need to interact with the underlying extracellular matrix (ECM). Inside-out and outside-in signaling between epithelial cells and the connective tissue happens through two kinds of anchoring junctions namely hemidesmosomes and focal adhesions. All junction complexes are roughly constituted by three major types of proteins: (i) essential structural proteins, required for initiation of junctions; (ii) plaque proteins associated with the cytoskeleton; and (iii) regulatory signaling/polarity proteins. Tight junctions, adherens junctions and focal adhesions interact with actin filaments while desmosomes and hemidesmosomes connect to intermediate filaments (9, 60, 123, 265, 312).

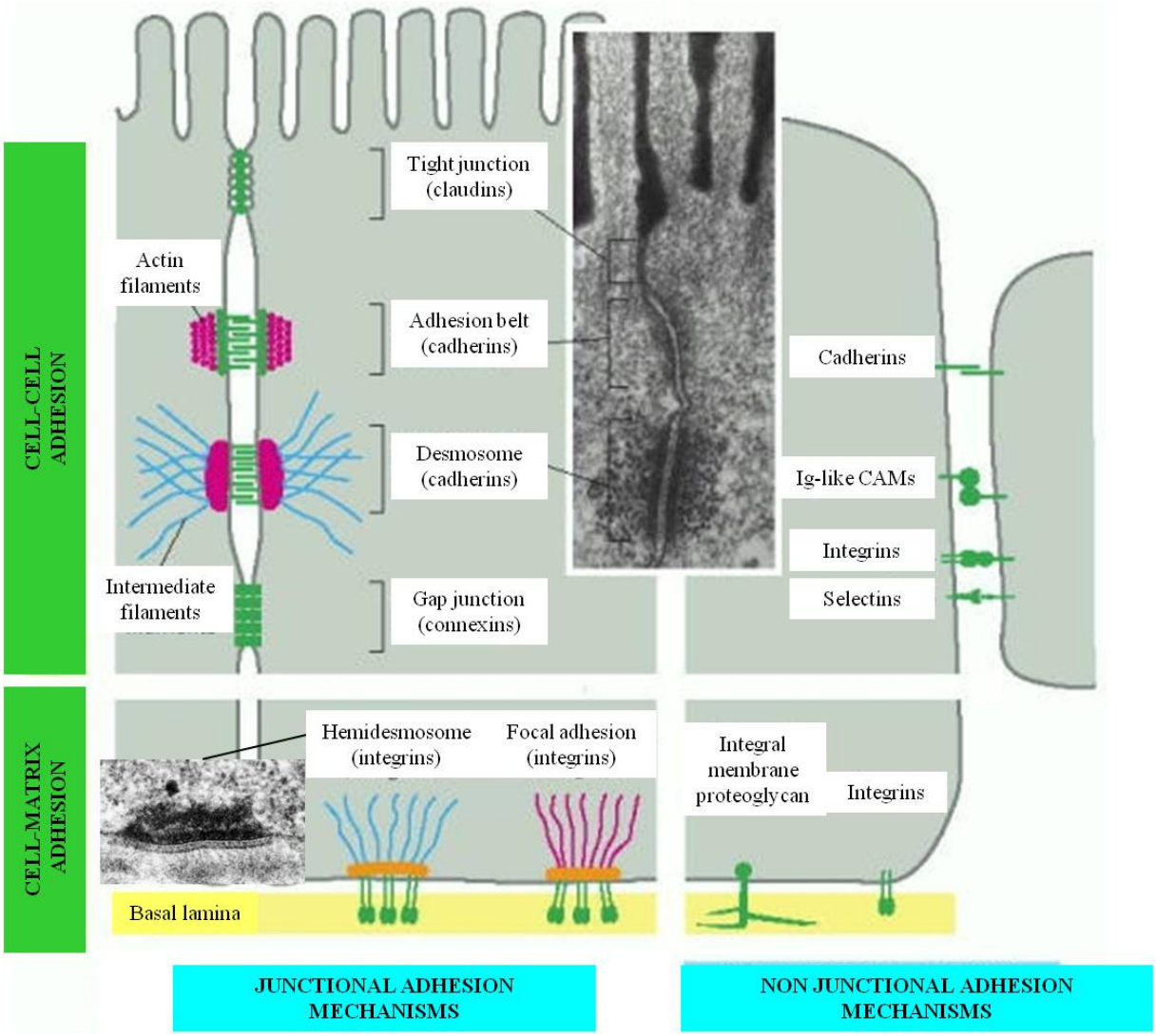

Figure 5. Junctional and non-junctional adhesive complexes in mammalian cells. Adapted from (9, 344).

Tight junctions

First described by Farquhar and Palade in 1963, tight junctions are the most apical structure of the tripartite junction complex bordering the apico-baso-lateral membrane (101, 265). In the tight junction a near-continuous plasma membrane contact zone in between adjacent cells with negligible intercellular space is present, hence their alternative name *zonulae occludens* (9). They function as gate and selective regulators of paracellular flux of both ions and small molecules (116). In addition, they regulate cell differentiation, proliferation and adhesion (312, 344).

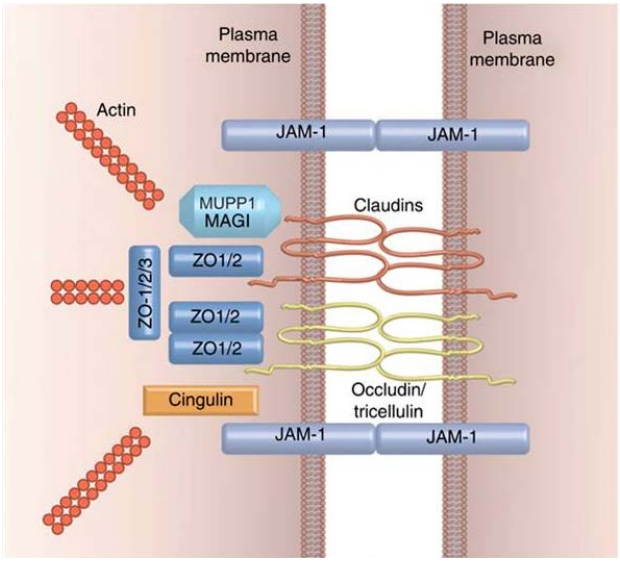

Figure 6. Schematic representation of a tight junction (265).

There are three different structural transmembrane proteins that have the ability to mediate cell-cell contacts including the IgG-like family of junctional adhesions molecules (JAMs), the occludins family and claudins family (265, 312, 344) (Figure 6). Recently, a novel structural protein was discovered with structural similarity to occludin, called tricellulin. However, the latter is only enriched at tricellular tight junctions (167). Both occludins and claudins are Ca^{2+}-dependent proteins and have four-membrane spanning regions, two extracellular and one intracellular loop; and both N-terminal and C-terminal domains are cytosolic. Occludins were the first discovered proteins associated with tight junctions and although present in most of them, their function remains obscure. Claudins are on the other hand better characterized. So far, 24 members have been described with different tissue and organ specificity (116, 265). The EC loops within occludins contain few charged amino acids compared to those of claudins. This implies that because of their charge, claudins selectively regulate ion transport. Changing the charge within claudins has been shown to alter barrier ion specificity. JAMs are Ca^{2+}-independent glycosylated proteins containing one transmembrane domain, two extracellular V-type Ig domains and one intracellular C-terminus. They are tethered to claudins via both ZO-1 and MUPP-1 and selectively recruit regulatory intracellular proteins such as cell polarity proteins to the tight junction complex. The cytosolic plaque proteins associated with the structural proteins are zonula ocludens (ZO)-1, -2 and -3; and MUPP-1. These adaptor proteins serve as links between the integral TJ proteins and the actin cytoskeleton and recruit cytosolic molecules implicated in cell signaling. Interestingly, the formation and maintenance of tight junctions is dependent on the formation and maintenance

of adherens junctions (101, 112, 196, 265, 312, 344). Recently, a study performed by Maiers and co-workers identified α-catenin as a binding partner of ZO-1 thereby linking the tight junction barrier machinery to the adherens junction adhesion complex (223).

Adherens junctions

Underneath tight junctions, adherens junctions are the second element of the tripartite junction complex for linkage of neighboring cells (265). Their principal function is to anchor cells to each other but they also coordinate movement of cells, cell polarity and metabolism (60, 147, 161, 168, 265, 336). Recently, loss of anchoring junctions, both adherens junctions and desmosomes, was found to play an important role in metastasis during tumor progression. Indeed, cells in the primary tumor, lose epithelial characteristics such as cytokeratin and E-cadherin expression and gain mesenchymal markers including N-cadherin and vimentin; a mechanism also known as epithelial-to-mesenchymal transition (EMT) (136). Two basic adhesive units exist within adherens junctions: the nectin-afadin complex and/or the classical cadherin-catenin complex (265) (Figure 7).

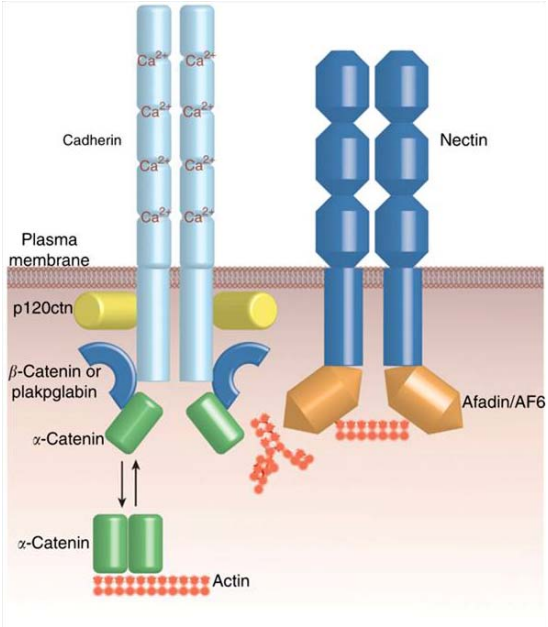

Figure 7. Structural overview of adherens junctions (265).

The nectin IgG-like adhesion protein family consists of 4 members, nectin-1 to -4 and functions Ca^{2+}-independent. They provide the first scaffold upon which adherens junctions, tight junctions and desmosomes are eventually formed. Three extracellular IgG-like loops,

one transmembrane region and a cytoplasmic tail constitute this adhesion receptor. Nectins form lateral homodimers that can engage in both homophilic and heterophilic adhesion with other nectins or nectin-like receptors (168, 265). Interaction of nectins with the actin cytoskeleton is mediated via their binding to afadin, a ubiquitous expressed adapter protein (161, 336). The classical cadherin-catenin complex contains as major structural protein cadherin, often E-cadherin. E-cadherin has an extracellular region containing five tandemly repeated domains (EC1-EC5), a single transmembrane region and a cytoplasmic region. In contrast to nectins, E-cadherin interaction is Ca^{2+}-dependent and mainly results in homodimers. Catenins including α-, β-, and p120 catenin form a complex with E-cadherin and interact via eplin with actin filaments. All catenins, with exception of the structurally unrelated α-catenin, contain a central Armadillo domain. γ-catenin, also known as plakoglobin, can substitute for β-catenin in adherens junctions but is usually present in desmosomal junctions. As mentioned before, nectin-based junctions form first and their formation is followed by formation of the E-cadherin-based junctions (60, 147, 161, 234, 265, 266).

Desmosomes

The third component of the tripartite complex boarding the apico-basolateral membrane are desmosomes (265). They do not only provide mechanical stability because of their hyperadhesive potential but also function as communicating junction with neighboring cells. In addition, they participate in cell signaling, proliferation and differentiation (36, 52, 60, 119, 131). Some well known autoimmune diseases like pemphigus foliaceus and pemphigus vulgaris wherein autoantibodies directed against desmoglein 1 and desmoglein 3 respectively cause a blistering disease, point out their main role in epithelial integrity (52). The structural proteins in desmosomes are transmembrane desmosomal cadherins like desmoglein and desmocollin (Figure 8).

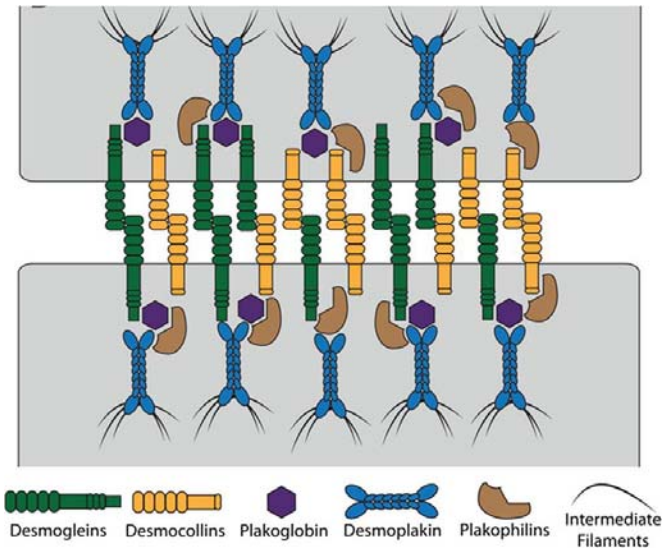

Figure 8. Schematic composition of a desmosomes in between two adjacent cells (36).

These cadherins bind to cytoplasmic armadillo family proteins such as plakoglobin and plakophilin, which in turn interact with desmoplakin, a plakin family member, for successful attachment to the cytoskeleton. Unlike tight junctions and adherens junction, desmosomes connect with intermediate filaments. Electron microscopical (EM) analysis revealed that each desmosomal plaque shows a complex structure and organization containing an apparent thick outer dense plaque and a translucent inner dense plaque (36, 52, 60, 119).

Hemidesmosomes

These epithelial specific cell-matrix adhesion complexes directly link the cytoskeleton to the extracellular matrix. In addition to their anchoring function, they play a role in cell division, differentiation and migration. Hemidesmosomal structures contain the integrin α6β4, the type XVII collagen BP180, the tetraspanin CD151 and the two plakin family members plectin and BP230 (212, 213, 345) (Figure 9). Integrins are the major adhesion receptors for cell-matrix adhesion. They are constituted of an α subunit and a β subunit and regulation of integrin activation is believed to happen through interaction of the β subunit tail. Integrins are normally kept in a low-affinity state by both a clasp in the membrane-proximal region of integrin α and β subunits as well as a clasp in the transmembrane domain in the outer leaflet of the membrane. Disruption of those clasps activates integrins (190).

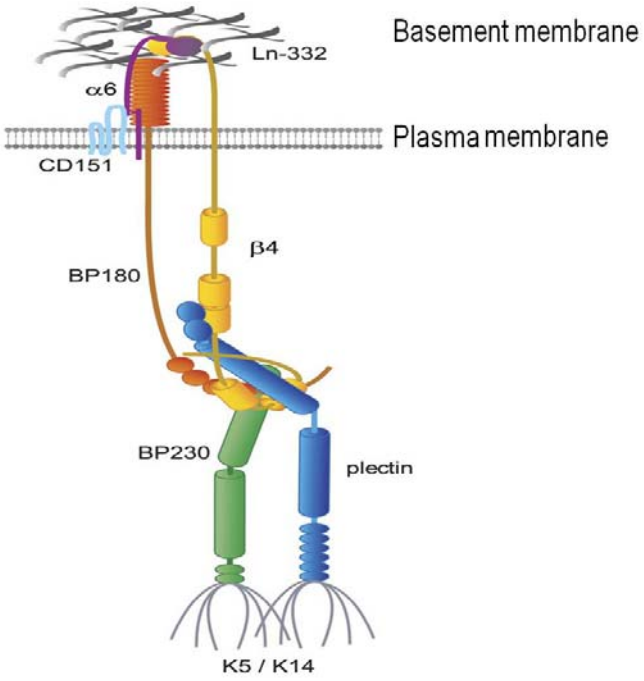

Figure 9. Schematic drawing of the structure and components of hemidesmosomes present in pseudostratified epithelia, linking intracellular keratin filaments (K) via α6β4 integrins to Laminin-332 (Ln-332) (227).

At present, 24 different integrin combinations have been identified in various tissues of which nine have been described to be present in epithelial cells. Nevertheless, two specific types α3β1 and α4β6 are the major anchors for extracellular matrix adhesion (190, 195). As stated above, α4β6 functions in mature hemidesmosomes and acts as a major linker to laminin-332 (Ln-332), a component of the ECM, although XVII collagen BP180 has been described to bind Ln-332 as well. CD151 is required for formation of hemidesmosomes. It plays an indispensible role because of its association with the laminin-binding integrin α3β1 in 'pre-hemidesmosomal' structures. During maturation and association of the former complex with α6β4 integrins, α3β1 is recruited into focal adhesions or is redistributed to cell-cell contacts whilst CD151 remains part of the mature hemidesmosomes (30, 213). Similar as in other junctions, both plakin family members plectin and BP230 function as intermediates to link integrins to the cytoskeletal intermediate filaments such as keratin. BP230 also associates with BP180 (30, 213, 227). Growth factors such as epidermal growth factor (EGF) can trigger hemidesmosome disassembly by inducing phosphorylation of the β4 intracellular domain (227).

Focal adhesions

These anchoring complexes are transient and self-assembling. Beyond their scaffolding function, they also serve as sensory structures through intracellular signaling cascades upon stimuli of the surrounding microenvironment (30, 42, 72, 129, 248). The major component of focal adhesions are integrins (described above) which are responsible for linking the ECM to the cellular actin skeleton (219). Over 180 adhesion-associated proteins have thus far been described, although for most of them their exact role remains elusive. Some of them are however well characterized, such as talin, filamin, migfilin, kindlins, paxillin and vinculin amongst others (Figure 10). Talin promotes integrin clustering and subsequent activation by binding to the β cytoplasmic tail of integrins. In addition, talin links actin to integrins (30, 129). On the contrary, filamin is a strong inhibitor of integrin activation by both competing with talin for integrin binding and by binding migfilin, an agonist of integrin activation, and displacing migfilin from integrin β cytoplasmic tails (72). Kindlins interact with the integrin β cytoplasmic tail but also interact with the actin skeleton via recruitment of filamin/migfilin complexes. They are believed to function as critical regulators of integrin activation (248). Paxillin and vinculin act as cross-linkers for several proteins within focal adhesion complexes (42). At present, on the basis of all these data, kindlins are speculated to recruit migfilin to the integrin β cytoplasmic tail, where migfilin displaces the inhibitor, filamin, resulting in talin binding and integrin activation (30, 42, 72, 129, 248).

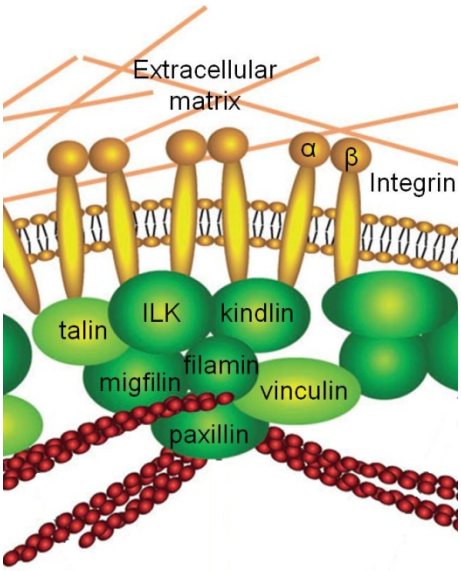

Figure 10. Schematic diagram illustrating a focal adhesion complex (ILK, integrin-linked kinase). Adapted from (248, 365).

Gap junctions

These adhesive molecules form conduits that provide a direct way of communication between cells for passive diffusion of ions, metabolites, water, second messengers and electrical impulses (123, 325, 326). Their main structural protein belongs to the connexin family of which six connexin subunits make up for one hemi-channel (connexon). A full gap junction is established when two hemi-channels of adjacent cells unite. The connexin subunit consists of a four transmembrane spanning region, two extracellular and one cytoplasmic loop (325, 326). Regulation of gap communication is governed by several regulatory enzymes including protein phosphatases and protein kinases (e.g. c-Src). Also, some structural proteins such as ZO-1 and microtubules are described to interact with connexins. Essentially all mammalian tissues contain at least a few connexons and some of them are tissue specific. Formation and degradation of gap junctions is accepted to be a very dynamic process. It is described that the half-life of connexons is less than 2–5 h in cultured cells and tissues (23, 123, 154, 325, 326). In vertebrates however, another group of proteins have been identified to be associated with gap junctions namely pannexins, the orthologues of innexins in invertebrates. But so far, no gap junctional communication has been demonstrated by native pannexins (122) (Figure 11).

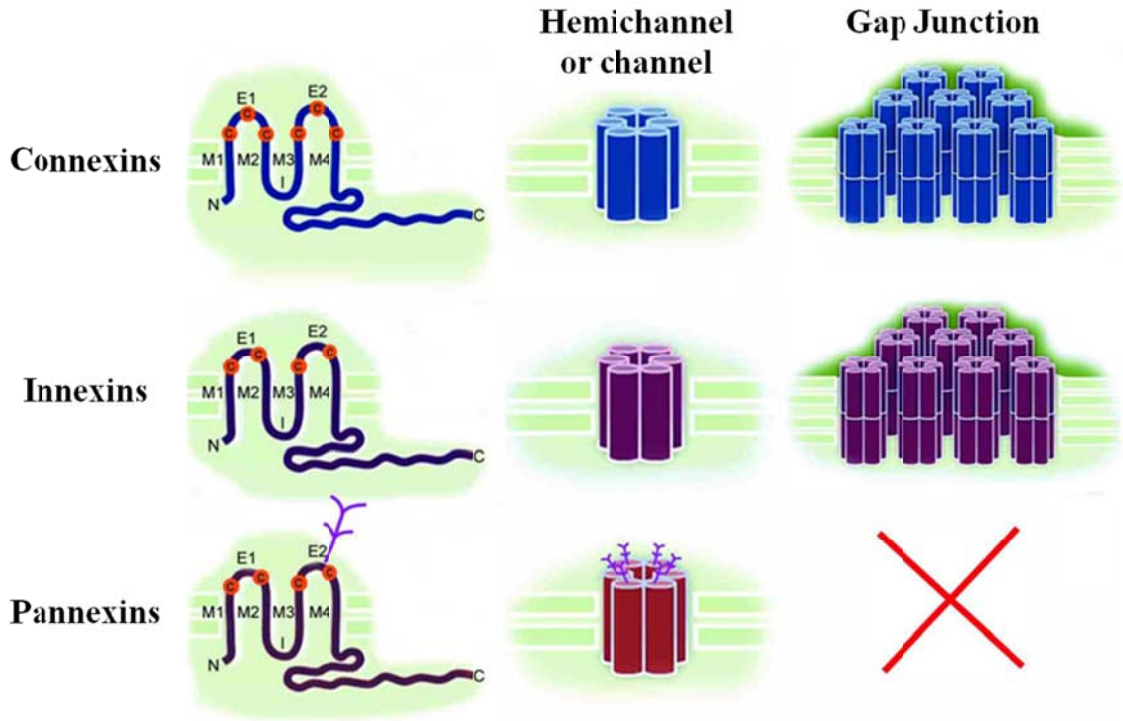

Figure 11. Families of gap junction proteins in both vertebrates (connexins and pannexins) and invertebrates (innexins). These proteins consist of a four transmembrane spanning region (M), two extracellular (E) and one cytoplasmic (I) loop (154).

1.2.3. Extracellular matrix

All cells need a framework to be able to exhibit their main functions including cell metabolism and polarization. In the structure, several proteins and polysaccharides are present. Within the mucosa, the extracellular matrix (ECM) consists of 2 structural entities namely the basement membrane and the lamina propria (9).

1.2.3.1. Basement membrane

The basement membrane (BM) (Figure 12) is an amorphous sheet-like scaffold of 50-100nm first identified by transmission electron microscopy (180). The BM is considered to be a specialized form of ECM and reasons are threefold (i) it contains the same structural elements as normal ECM, (ii) it has a higher density than normal ECM and (iii) on the contrary, is always associated with cells. BM's are usually found basolateral of epithelium, endothelium, fat cells, muscle cells and nerve axons; and separate these structures from the underlying stroma in any tissue (9, 180, 302). Although the basic elements are similar for most tissues, a heterogeneity in BMs is present due to variations in amounts of components, splice variants, age and so one (200, 208). The main function of this thin, rugged network is to provide structural support and compartmentalization within tissues. In addition, they regulate cell behavior as the BM displays adhesion receptors and harbors a repertoire of matrix-bound growth factors (180, 369). With a pore size of around 50nm, only small molecules are able to diffuse freely across this rigid selective barrier (4, 5). Nonetheless, immune cells have to be able to rapidly cross this barrier as part of our immune system (302, 313). The exact mechanism behind this traversing event is briefly considered in section 1.3.3. Crossing of the basement membrane of large molecules requires ECM breakdown and this is nearly always mediated by enzymatic activity (41). These proteolytic enzymes, also known as proteases, are also required for normal physiological functions such as remodeling events during wound repair or angiogenesis. Tissues normally have a well-balanced homeostatic mechanism involving protease inhibitors to prevent uncontrolled breakdown of the ECM. In many disease states, there might be a shift however favoring protease activity and remodeling of ECM. In this context, the best studied pathology during recent years is probably tumor invasiveness in which metastatic cells remodel ECM to gain access to blood vessels (118, 233). Proteases can be subdivided into 4 major classes: serine proteases, cysteine proteases, aspartyl proteases and matrix metalloproteases. Mainly serine proteases and matrix metalloproteases are involved in

ECM breakdown as they function at neutral pH whereas aspartyl and cystein proteases are active at low pH and mainly act intracellularly (18).

TEM revealed the presence of two layers within the BM: the *lamina basalis* (basal lamina, *lamina densa*) and the *lamina reticularis*. A small region of about 40nm in between basal sides of basal cells and the *lamina densa*, contains the extracellular portion of cell adhesion molecules such as integrins. This layer has been confirmed by immune EM applying epitope-specific antibodies and is called *lamina lucida* (33, 239). These anchoring cell-matrix molecules containing integrins were discussed above. The predominant components of the *lamina basalis* are intertwined meshworks of polymeric laminins and type IV collagens. As such, both constitute the main frame of the BM in a self-assembly process for which binding to cellular receptors such as integrins or dystroglycan is a prerequisite. This network is further bridged by various non-covalent interactions with nidogens and perlecans (Figure 6). All the components are secreted by epithelial cells. The *lamina reticularis* on the contrary is formed by bundles of type III collagen reticular fibers and thus belongs to the connective tissue. Minor components of the basement membrane zone include agrin, SPARC/BM-40/osteopontin, fibulins, type XV collagen, type XVIII collagen and fibronectin (9, 61, 95, 180, 208, 302, 370). We will discuss briefly below some of the main components of BMs including type IV collagen, collagen type VII, laminin, heparan sulfate proteoglycans (HSPGs) and nidogen/entactin.

Collagen type IV, is a major element of the BM and defines BM structure and function. Three collagen polypeptide chains, called α chains, are rich in proline and glycine and constitute together a long, stiff, ropelike superhelix, known as the typical collagen molecule. There exist up to 6 different α chains although they can only assemble into three different heterotrimers (200). These tissue-specific heterotrimeric protomers contain a central discontinuous triple-helical domain rich in Gly-X-Y motifs. In addition, an N-terminal 7S domain and a C-terminal globular non-collagenous 1 (NC1) domain are present. Eventually these protomers assemble into polymeric meshworks through interactions of the N-terminal 7S domains (302). Many different cells including tumor cells have been shown to bind to collagen IV, mediated by integrin and non-integrin receptors (189).

Collagen type VII is a specific element of anchoring fibrils, a part of a supracellular anchoring network responsible for attaching the epithelium to the BM. Collagen VII binds to laminin 5 proteins within the *lamina densa* and to collagen I proteins in the underlying *lamina*

reticularis. Reduction of collagen VII results in loss of epithelium in several diseases (97, 354).

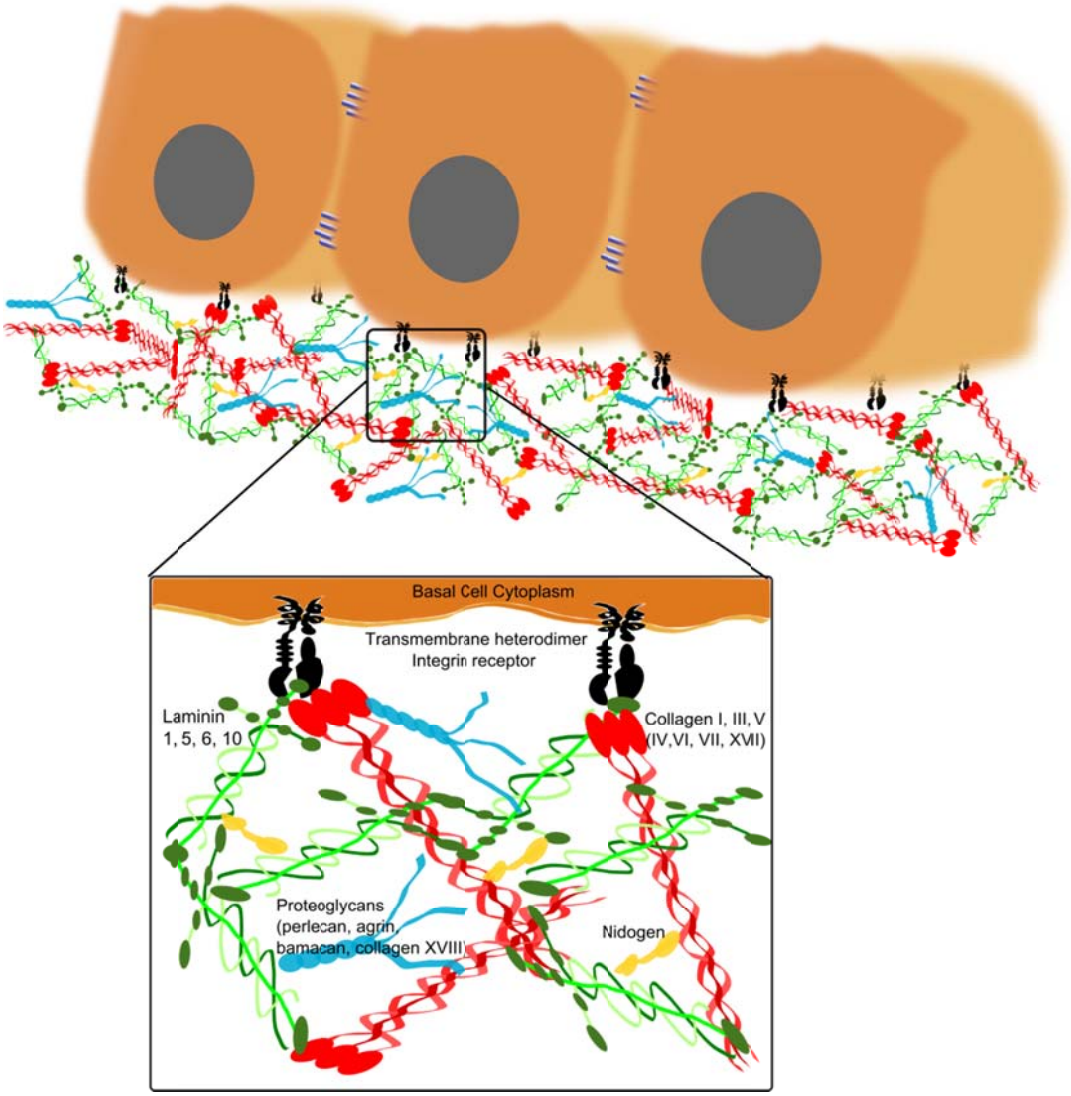

Figure 12. Schematic overview of the basement membrane and its main extracellular matrix components.

The heterotrimeric laminins are of outermost importance for BM formation and maintenance; and form like collagen a network. Three laminin chains (α,β,γ) form an asymmetric cross-like subunit via disulfide-bonds (302). The long arm of the cross (~80 nm length), composed by all three chains, consists of an α-helical coil, whereas the three short arms (35–50 nm) are composed of one chain each. This non-collagenous protein contains multiple binding domains including network domains on each of the three short arms for interactions with ECM components; and the globular domain (G-domain) present at the end of the long arm for

binding to cellular receptors. At present, a total of 16 isoforms have been identified. Laminins -111, -332 and -511 are present in epithelia (9, 158, 200).

Heparan sulfate proteoglycans are heterogenic molecules. To be typed as a proteoglycan, at least one of the sugar side chains has to be a glycosaminoglycan (GAG). All GAGs are covalently bound, via a tetrasaccharide link, to a serine amino acid in the core protein, the central polypeptide chain of proteoglycans (9). Much of the volume of the *lamina densa* is probably derived from the elaborate presence of these proteins as they form a highly hydrated gel (208). In addition, proteoglycans play a structural role, embed growth factors including fibroblast growth factor 2 (FGF-2), vascular endothelial growth factor (VEGF) and platelet-derived growth factor (PDGF), sequester extracellular ions, regulate cell differentiation and confer charge-dependent selective filtration properties to BMs. Some of these roles have been shown for perlecan, probably the best studied member as the other proteoglycans are less well characterized. Perlecan, together with nidogen, is responsible for non-covalent linkage of the collagen and laminin networks (32, 95, 208, 302).

Members of the nidogen family are composed of a series of sulfated monomeric glycoproteins. They do not have the potential to self-assemble into polymers. Three globules G1, G2, G3 and one rod-like part, possessing different domains, make up the typical three globular shape of nidogen (9). Nidogens have strong affinity for collagens and laminins and therefore are considered as typical linker molecules that bridge both networks. In addition, they form complexes with perlecan and fibulins. Nidogens or entactins are highly susceptible to proteolytic degradation, however linkage to laminin provides a mutual protection. Removal of nidogen is believed to initiate BM disintegration (32, 95, 200).

1.2.3.2. Lamina propria

The lamina propria consists of an intricate network of collagen fibers, elastin fibers, fibronectin and proteoglycans; located underneath the BM. The density of this connective tissue varies according to the tissue but harbors local cells (e.g. fibroblasts), mucus-producing glands, blood- and lymphatic vessels, and nerve endings (9). The main types of collagen fibers within the lamina propria are type I, II, III, V and XI. They provide tissue structure and resistance to tensile forces. Increased deposition of collagen type III and V, together with laminin and fibronectin deep into the *lamina reticularis* of the BM is a classic histological

marker of asthma (293). Elastin fibers, bundles of monomeric units consisting of elastin cores within sheaths of microfibrils, are an extremely important functional element for tissue motion including contraction and relaxation. In the respiratory tract, several coils of elastin within the lamina propria function in unison with bigger longitudinal elastic fibers present in a lamina elastica underneath the lamina propria (9). Fibronectins, large and essential multidomain glycoproteins display multiple adhesive properties and function as key links between cells and their extracellular matrices thereby attaching the *lamina densa* to the lamina propria (151). Proteoglycans have been described above. A clear distinction however has to be made in this context between cell surface proteoglycans, ECM-associated proteoglycans in general and specific proteoglycans residing inside the BM such as agrin and perlecan (208, 302).

1.2.4. Mucosal innervation

Mucosa function is regulated by several types of nerves including sensory, parasympathetic and sympathetic nerves. Regulation of vasomotor function in the superficial lamina propria, stimulation of submucosal gland activity and secretion, and mediation of innate and acquired immune responses, all depend upon proper tissue innervations (16, 17, 80). Sensory neurons encompass calcitonin gene related peptide (CGRP), gastrin releasing peptide (GRP), tachykinins (neurokinins) substance P and neurokinin A (NKA) and possibly other peptides. Parasympathetic neurons consist of acetylcholine or vasoactive intestinal peptide (VIP) and nitric oxide containing neurons. Two important subpopulations of the sympathetic neurons are norepinephrine with or without neuropeptide Y (17, 80).

Afferent sensory innervation of the nasal respiratory mucosa is constituted of two major trigeminal derivate fiber systems, consisting of unmyelinated C-fibers and myelinated A_{delta}-fibers (17, 165). Except for a specialized region in the caudal third of the nasal cavity, termed the olfactory neuroepithelium which possesses sensory olfactory receptor neurons that subserve olfaction, most sensory and proprioceptive information from the nasal cavity relays through trigeminal afferents (63, 228). Sensory terminations in or near the epithelium of the larynx, trachea and large bronchi are afferent vagal nerve fibers (348). The terminal fibers or free nerve endings are extensively branched and detectable near to arteriovenous anastomoses, venous sinusoids, submucosal glands, in loose connective tissue but also in between epithelial cells (16). In the rat nasal septum, a dense network of unmyelinated nerve

fibers is located near the basement membrane of respiratory and squamous epithelium, and some fibers send projections between epithelial cells to reach up to the tight junctions (330). Several reports mention the existence of dense innervation of the respiratory epithelium of the upper respiratory system with numerous free intraepithelial nerve endings (148, 202, 218, 231). Also the mammalian vagina is richly imbued with sensory, parasympathetic and sympathetic nerve fibers. Human vaginal innervation is believed to mainly consist of sympathetic and parasympathetic axons with only smaller contributions by sensory fibers (133). An investigation of the anatomical distribution of nerves in the human vagina provides no evidence for nerves extending in the epithelium. The majority of nerves appeared in both the superficial and deep submucosa (274). However, one report describes the presence of free intraepithelial nerve endings in the human *introitus vaginae* region (155). Also, in the rat vagina free or specialized nerve endings were observed immediately beneath and within the epithelium (340).

Efferents of the parasympathetic and sympathetic nervous system has nerve bundles running through the mucosa (126). Electron microscopy showed that choline acetyltransferase immunoreactive (ChAT-IR) nerve fibers possess terminations within the epithelium. Some of these nerve endings were in close contact with goblet cells (183). In addition, parasympathetic VIP-IR fibers in rat nasal mucosa show a similar distribution than ChAT-IR fibers in the epithelium (182).

1.2.5. Mucosal immune cells

Mucosal surfaces are highly vulnerable to colonization and invasion by many microorganisms. It is not surprising that these surfaces possess intrinsic defense mechanisms including innate and adaptive immune responses. The mucosal immune system can be grossly subdivided in inductive sites, where naïve T and B lymphocytes are stimulated by antigens, and effector sites, where the effector cells after extravasation and differentiation exert their function. Inductive sites are often organized mucosa-associated lymphoid tissues (MALT) and regional draining lymph nodes, whereas the effector sites are widespread in the periphery and distributed in different histological compartments, including lamina propria and epithelia of various mucosae (198, 363). We will discuss in this section briefly the different immune cells that are present within respiratory and genital mucosa. An extensive description of mucosal immunology falls out of the scope of this thesis and can be found elsewhere (51).

Lymphoid cells

Lymphocytes consist of a heterogeneous population of cells including cytotoxic CD8 T cells, helper CD4 T cells, B cells and natural killer (NK) cells (2). Upon infection of peripheral tissues, a cascade leading to the priming of T cells initiates, and acts to control pathogens. Activated T cells undergo migrational imprinting within the draining lymph nodes which will result in memory T cells that provide local and systemic protection. The combination of both these migrating and resident memory T cells will confer the host a long-term peripheral immunity, especially at the surfaces that form pathogen entry points into the body (120). Intraepithelial lymphocytes (IEL) populate all the body's surfaces. They represent one of the largest lymphocyte populations. Many IEL are unconventional as they, in contrast to conventional T cells, express antigen receptors with a limited diversity and are kept in a heightened state of activation, allowing quick and efficient action-reaction. Intraepithelial lymphocytes may be composed of antigen experienced memory-effector T cell subtypes bearing the αβ or the γδ T cell receptor (TCRαβ+ or TCRγδ+) (51, 252). Although lymphocytes are very skilled in adaptive immune responses, a recently identified branch represents a family of innate lymphoid cells (ILC) that play diverse roles in immunity and inflammation. The prototypic ILC is the natural killer (NK) cell, which serves as an effector lymphocyte through secretion of IFN-gamma and its cytolytic capacity (25). The purpose of the mucosal B cell system is to collaborate with secretory epithelia to provide a first-line defense through active export of secretory immunoglobulin A and to some extent secretory IgM antibodies (31). Antigen-triggered maturation of germinal center B cells into memory B lymphocytes and plasmablasts will take place in lymphoid tissues. This maturated and differentiated B cell subpopulation will recirculate through peripheral blood into different tissues including mucosae, where long-living plasma cells produce antibodies (276). Intraepithelial plasma cells are usually positioned in the basal layers of the epithelium whereas plasma cells in the lamina propria are more diffusely scattered (225).

Myeloid cells

Myeloid cells originate from hematopoietic stem cells in the bone marrow and encompass monocytes, macrophages, dendritic cells and granulocytes. They represent the major leukocytes in the peripheral blood (185). Monocytes are maturated terminally into macrophages in various tissues. Macrophages are highly skilled as phagocytic cells with key

features such as prominent phagocytic vacuoles, secondary lysosomes and pseudopodia. They readily clear pathogens, cellular debris and non-microbial foreign material which makes them essential for maintaining tissue homeostasis (121, 185). Moreover, conventional macrophages possess the intrinsic capacity to produce pro-inflammatory cytokines, such as TNF-α, IL-1 and IL-6, and also upregulate co-stimulatory molecules (278). They are both present in the epithelium and lamina propria of mucosae although they are found in greatest numbers in the lamina propria (159, 259). Monocytes may also differentiate into dendritic cells (DC). Dendritic cells, as professional antigen presenting cells (APC), serve as an essential bridge between innate and adaptive immunity (328). Mucosal DC recognize and respond to pathogens through engagement of pattern recognition receptors. Upon activation, these cells migrate to the draining lymph nodes and subsequently induce adaptive immune responses. The specialized functions of DCs include the potency to establish an antiviral interferon response to viral nucleic acids, the ability to capture organisms in the lumen, the capacity to cross-present antigens from other infected cells, and the ability to initiate IgA class switching in B cells. The epithelial residents of dendritic cells are Langerhans cells, which serve as true "sentinels" of the mucosa (240, 328). Genital mucosa contains at least two important subsets of dendritic cells including Langerhans cells and dendritic cells that express the C-type lectin receptor DC-Sign. Langerhans cells, characterized by CD1a expression, Langerin and Birbeck granules; form a dendrite network both extending within the epithelium and out in the lumen for efficient scavenging. However, they are not present in simple columnar epithelium (78). On the other hand, DC-Sign^{+} DC's are mainly localized subepithelial (88). Within respiratory mucosa of the rat trachea roughly 20% of resident DC were found to interdigitate within the resting epithelium under physiological conditions, with the remaining 80% residing within the subepithelial layers (171).

Granulocytes are the first cells to be recruited to the local sites upon pathogen invasion and act as a crucial first line of defense in immune responses (121). Mature mast cells are well-known for their contribution in allergic reactions through the release of histamine and ''slow reacting substance of anaphylaxis'' following crosslinking of cell-bound IgE by allergens. In addition, mast cells participate in innate immune responses and may also contribute to adaptive immune responses because of their capability to produce mediators that can promote migration of APC (149). The majority is located within the lamina propria of the respiratory tract with only a minority of mast cells located in the epithelium (48, 224). Another population of granulocytes are neutrophils. These innate immunity cells quickly migrate

towards and accumulate at the site of inflammation and are considered pathognomonic features of both acute and chronic inflammatory conditions (109). Neutrophils possess the capacity to perform a variety of antimicrobial functions such as degranulation and phagocytosis. Pathogens stimulate resident macrophages to produce macrophage-derived chemokines which contribute to the recruitment or chemotaxis of neutrophils. As a consequence, blood neutrophils traverse the vascular endothelium at the site of inflammation and reach the lamina propria. It has been described that under certain physiological or pathological conditions, neutrophils cross the epithelium into the lumen. In general, this response peaks by 24-48 hours after initiation. At the site of inflammation, neutrophils produce on their turn monocyte chemoattractants for a second-wave of immune cell (macrophages) influx (58, 109, 324).

1.3. Microbial interactions with the basement membrane (BM) barrier

(*Adapted from Steukers, L., Glorieux, S., Vandekerckhove, A.P., Favoreel, H.W., Nauwynck, H.J. Diverse microbial interactions with the basement membrane barrier. Trends in Microbiology 20(3), 147-155.)*

During primary contact with susceptible hosts, microorganisms face an array of barriers that thwart their invasion process. Passage through the basement membrane (BM), a 50-100 nm thick critical barrier underlying epithelia and endothelia, is a prerequisite for successful host invasion. Such passage allows pathogens to reach nerve endings or blood vessels in the stroma and to facilitate spread to internal organs. During evolution, several pathogens have developed different mechanisms to cross this dense matrix of sheet-like proteins. To breach the BM, some microorganisms have developed independent mechanisms, others hijack host cells that are able to transverse the BM (e.g. leukocytes and dendritic cells) and oncogenic microorganisms might even trigger metastatic processes in epithelial cells to penetrate the underlying BM.

A first step during infection is the attachment of microorganisms to various host proteins, which could include basement membrane (BM) proteins. Generally, adhesion of bacteria and fungi occurs through binding of adhesive molecules, called adhesins, to host proteins. Most adhesins are extensively described and some of the motifs participating in these processes have been identified (194). In comparison, knowledge about adhesion of viruses with BM components is scarce. Upon adhesion, microorganisms have evolved cunning techniques to overcome the BM barrier, which otherwise hampers their invasive process. Breakdown of the

BM, via activation of microbial and/or host proteases, in order to cross the BM has been shown for many bacteria, fungi and, recently, for some viruses. Pathogens might also gain access to the connective tissue by hijacking host cells, particularly local immune cells, to cross the BM. This chapter provides an overview on recent insights in different pathogen-BM interactions during host invasion, discusses these findings and projects how they could contribute to the design of novel strategies to interfere with microbial invasions and pathology.

1.3.1. Adhesion of pathogens to BM components

A crucial initial event in the pathogenesis of many microorganisms is adhesion to host tissues. Major players during these early steps in infection are microbial adhesive cell surface molecules, termed adhesins (194). Microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) are adhesins that attach to extracellular matrix (ECM) including the BM. Many MSCRAMMs are capable of binding more than one ECM component, and a single strain often possesses several different proteins that bind the same host component. Although current data about viral ECM adhesion and adhesins is still scarce, their importance in virulence has been shown for most bacteria and many fungi. For example in many animal models of staphylococcal infections, CNA, a collagen binding adhesin of pathogenic *Staphylococcus aureus*, increases disease severity. The ability to interact with collagen has been suggested to grant these bacteria a clear advantage in pathogenesis (372). For bacteria, adhesins are roughly subdivided according to the appendage morphology: there are fimbrial or non-flagellar adhesins (chaperone-usher pili, curli, type IV pili, type III secretion needle and type IV secretion pili) and non-fimbrial adhesins (autotransporters; outer membrane, secreted and biofilm-associated adhesins). Pili are further classified according to physical properties, antigenic determinants, adhesion characteristics, characteristics of the major protein subunits or assembly pathways (10, 107). Although adhesins form a heterogeneous group with diverse architecture, domain content and binding mechanism, some do possess similarity in structure, ECM-binding domain organization and function (214, 308). ECM-binding MSCRAMMs of many pathogenic Gram-positive species are cell wall-anchored surface proteins (CWPs). Common features of CWPs are an N-terminal signal peptide followed by a so-called A-region or -domain, segments of repeated domains and a characteristic C-terminal sorting signal. The sorting signal contains an important cell wall-

anchoring site or LPXTG motif, which covalently binds to the cell wall (263, 277). However, recently, the collagen-binding adhesin Slr of Gram-positive *Streptococcus pyogenes* has been shown to lack the LPXTG motif, but uses a cell wall-anchoring TLIA lipobox instead (26). The structure and organization of viral and fungal adhesins to ECM is at present less well documented, although there is proof of diversity in fungal adhesins (308, 364). Some fungal adhesins show structural and/or functional similarities with bacterial adhesins (308, 364). Agglutinin-like sequence (Als) adhesins of *Candida albicans* are composed of a signal peptide, an N-terminal region, a nonrepeat Thr-rich (TR) region, a central region with a variable number of repeats, and a Ser/Thr/Asn-rich C-terminal domain which anchors the CWP via a glycophosphatidylinositol (GPI) remnant (47, 364). In many cases, ECM-binding domains recognize carbohydrate residues or oligosaccharides, but not exclusively as many of them also bind host protein binding sites (76, 268, 277). Besides this direct microbial adhesin-ligand binding, microorganisms have developed other interesting indirect adhesive approaches. *Haemophilus influenzae*, *Moraxella catarrhalis* and *Shigella spp.* prevent cell detachment of infected cells from the BM, through stabilization of focal adhesions. This strategy counteracts rapid exfoliation, which is an effective intrinsic defensive system of intestinal epithelium (192). *C. albicans* promotes laminin 5 and type IV collagen protein secretion to enhance binding to the BM (55). In the following paragraphs, adhesion of bacteria, fungi and viruses to the major BM components collagen, laminin, proteoglycans, entactin/nidogen, BM-40 and fibulin will be discussed in more detail. Bacteria, fungi and viruses also show binding capacity to fibronectin, a minor component of the BM zone, extensively described elsewhere (151).

Collagen, the superhelix

Numerous bacteria are able to interact with collagens via their proper adhesins (143, 199, 321). For many bacterial species, the ability to interact with collagen in the BM is a prerequisite for invasiveness. For example, PilA from *Streptococcus agalactiae*, causing meningitis in newborns, has been found to bind collagen I, which promotes interaction with integrins and subsequently promotes penetration of the blood-brain barrier (BBB) (15).

Some fungal pathogens also interact with collagens during host invasion using distinct fungal receptors. *Aspergillus fumigatus* contains a sialic acid-specific lectin that interacts with collagen type I and IV (342). Similarly for *C. albicans*, the Als3p glycoprotein, a major player

in *C. albicans* pathogenesis, has been shown to be responsible for binding collagen IV (269). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and glycoprotein gp43 of *Paracoccidioides brasiliensis* bind type I collagen. Gp43 also binds type IV collagens and laminin, probably via a sialic acid recognition system, similar to *A. fumigatus* (342). However for most fungi, adhesins and adhesive mechanisms are still largely unknown (47, 364).

So far, viral binding to collagen has not been described.

The multidomain glycoprotein laminin

As described for collagen, many different bacteria and fungi are able to interact with laminins via adhesive proteins (10, 34, 144, 285, 353). Most adhesins recognize multiple molecules. However, there are adhesins that bind laminin but not collagen. Lsa24 and Lsa27 of *L. interrogans* (353) Lmb of *Streptococcus agalactiae* (285) and others act as specific laminin-binding adhesins. Recently, ErpX of *Borellia burgdorferi* was found to have a unique mode of laminin binding through a hydrophobic segment at the center of the bacterial protein. This protein motif has not been identified yet in other bacterial laminin adhesins (34).

For human papillomaviruses (HPV), recent work has put laminin 5 forward as a possible basal ECM receptor. This interaction localizes virus particles to the basal surface of epithelial cells where they can reach their entry receptor, integrin $\alpha_6\beta_4$, the physiological binding partner of laminin 5 (193). However, another recent publication demonstrated that different HPV types show different binding characteristics (37) (Figure 13). The nonstructural protein NSP4 of rotavirus plays a key role in development of severe gastroenteritis by binding ECM proteins laminin β3 and fibronectin. Moreover, rotavirus induces phosphatidylinositol 3-kinase (PI3K) activation in intestinal cells, causing upregulation of integrin expression, prolonged attachment of infected cells to collagen and increased virus production (29, 142).

The heterogenic molecule: proteoglycan

The ability to interact with proteoglycans, often heparan sulfate proteoglycans, is widespread in viruses and bacteria (19, 49). A clear distinction has to be made in this context between cell surface proteoglycans, ECM-associated proteoglycans in general and specific proteoglycans residing inside the BM. Despite the numerous reports on pathogens interacting with ECM-

associated proteoglycans in general (19, 49); so far, no specific pathogen BM agrin nor pathogen BM perlecan interactions have been described.

Currently, only one specific BM proteoglycan–pathogen interaction is known. The proteoglycan bamacan is a cellular ligand of vaccinia virus neurovirulence factor N1L. This interaction promotes viral growth and might contribute to virulence of the virus (253).

Entactin/nidogen, BM-40/osteonectin and fibulins: versatile ECM proteins

Members of the nidogen family are composed of a series of sulfated monomeric glycoproteins. Three globules G1, G2, G3 and one rod-like part, possessing different domains, make up the typical three globular shape of nidogen (180, 208, 302). BM-40 is a glycoprotein of the ECM that binds calcium, collagen and hydroxyapatite and regulates the cell–matrix interaction (180, 208, 302). All fibulins contain epidermal growth factor-like repeats and a unique fibulin-type module at its C terminus that define this family (61). Up till now, only one bacterium has been reported to use nidogen as a potential ligand for ECM-binding. SgrA of *Enterococcus faecium* has been identified as a bacterial receptor for nidogen and fibronectin (152). The opportunistic bacterium *Finegoldia magna* depends on BM-40 interaction for colonization and survival (113). Serum opacity factor is a streptococcal receptor for fibulin-1 (61).

No other bacteria, fungi or viruses are known to bind nidogen/entactin, BM-40 or fibulins during host invasion.

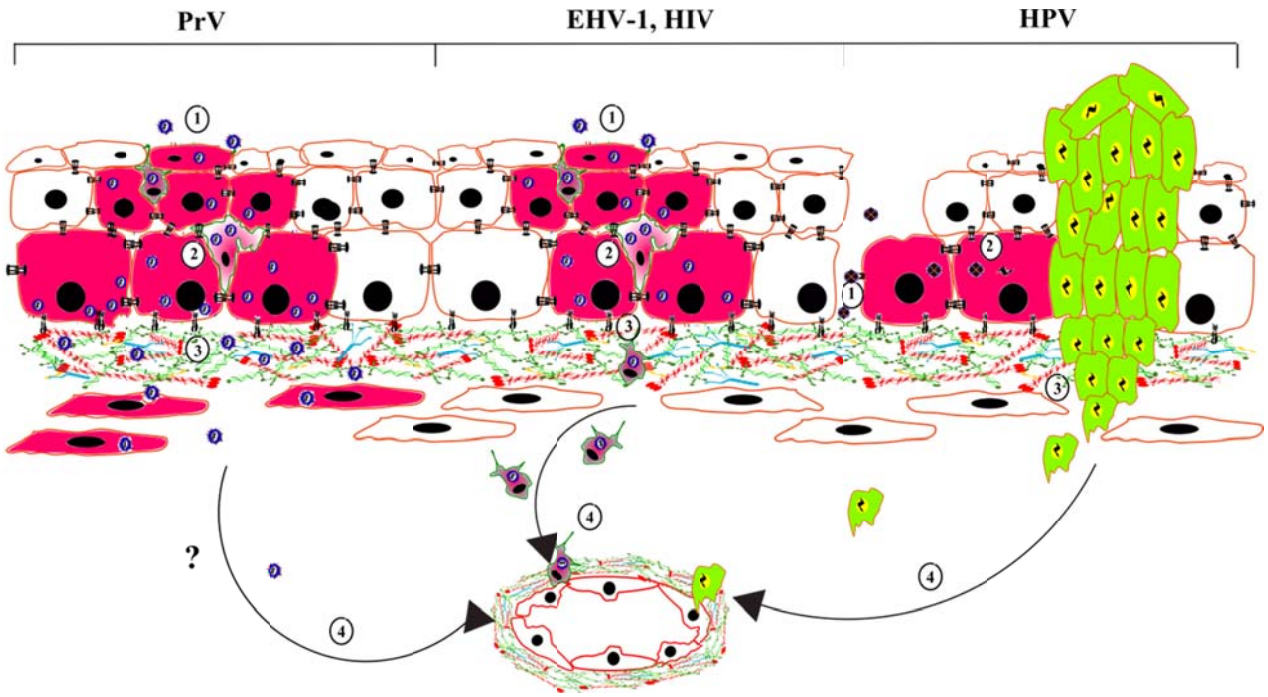

Figure 13. Different viral interactions with the BM. (1) Before entry into cells, viruses either attach directly to cell surface receptors (e.g. herpesviruses) or via intermediate binding to an exposed BM component in epithelial microlesions (e.g. human papillomavirus, HPV). (2) Viral replication and local dissemination (infected cells are in pink). Local immune cells may be infected (e.g. herpesviruses and HIV). (3) Viruses gain access to the stroma by breaching the BM. This may happen in a protease-mediated way (e.g. pseudorabies virus, PRV), via hijacking of immune cells to transverse the BM [e.g. equine herpesvirus 1 (EHV-1) and HIV] or via viral-driven metastasis out of a viral-induced tumor (e.g. HPV, green cells). (4) Finally, viruses may spread in the host by reaching blood or lymph vessels.

1.3.2. Pathogen-driven breakdown of the BM

Disruption of the BM in disease states often involves proteolytic enzymes (41) and an overview of the general characteristics of the different protease types is given elsewhere (18). Many pathogens possess the ability to produce or modulate ECM-degrading enzymes. Regulation of ECM degrading enzymes aids pathogens in invading deeper tissues, thereby enhancing dissemination throughout the host. Besides this direct effect of pathogens on BM-degrading enzymes, pathogens might also indirectly affect such enzymes. Indeed, during inflammation of infected tissues, local immune cells produce an array of these proteolytic enzymes. This indirect activation of proteases falls out of the scope of this chapter and can be found elsewhere (350, 362).

Several bacteria encode or modulate BM-degrading proteases, either directly or by engaging host-derived systems.

Various bacterial pathogens including *Bacteroides fragilis* and *Clostridium perfringens* (281) encode or modulate matrix metalloproteinases (MMPs). Other bacteria degrade the BM barrier by encoding or modulating serine proteases. Indeed, many bacterial pathogens, such as *Enterobacteriaceae*, *Fusobacteriaceae*, *Helicobacteriaceae*, *Legionellaceae*, *Mycobacteriaceae*, *Neisseriaceae*, *Pasteurellaceae*, *Peptostreptococcaceae*, *Porphyromonadaceae*, *Pseudomonadaceae*, *Spirochaetaceae*, *Staphylococcaceae* and *Streptococcaceae*, modulate the plasminogen (Plg)/plasmin system (22) and the structural and functional aspects of this system are described elsewhere (311). Briefly, through the activity of the two main physiological plasminogen activators, urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA), plasminogen is converted into plasmin which degrades laminin and fibronectin and activates precursors of MMPs. Several bacterial pathogens express plasminogen receptors thereby recruiting plasminogen to the bacterial surface which leads to enhanced plasminogen activation. Additionally, some bacteria bind and/or induce uPA or tPA (22, 89) and/or express bacterial plasminogen activators. Moreover, some bacteria inactivate plasmin inhibitors (204).

Some bacterial species modulate multiple proteases to cross the BM. In addition to modulating the plasminogen/plasmin system, *Streptococcus pyogenes* also expresses a cysteine protease (40). *Fusobacterium nucleatum* can invade the BM in a strain-dependent manner, by binding plasminogen (71) and pro-MMP-9 and stimulating MMP-9 and MMP-13 secretion (138). *Helicobacter pylori* induces expression of the cysteine protease cathepsin X in gastric epithelial cells and macrophages. In epithelial cells, *H. pylori* induces morphological and motility changes, partly by MMP-9 (201) and probably also by other proteases. The mechanisms by which *H. pylori* induces expression of proteases in epithelial cells and macrophages is unknown. The proteolytic activities are suggested to play a role in gastric tumourigenesis (201). *H. pylori* also increases the expression of the uPA system in gastric epithelial cells (187). *Mycobacterium tuberculosis* (55, 255) and *Neisseria meningitidis* (89, 314) modulate both MMPs (MMP-8 and MMP-9 respectively) and the plasminogen/plasmin system. Both morphotypes of *Peptostreptococcus micros* possess plasminogen receptors. For *P. micros*, both bacterial (streptokinase) and human plasminogen activators (uPA) can activate plasminogen to plasmin. Rough morphotypes also possess chymotrypsin-like and gelatinase serine proteases (132). *Porphyromonas gingivalis*

upregulates MMPs, modulates the plasminogen/plasmin system and expresses cysteine protease gingipains. Gingipains contribute to BM penetration, either directly or by modulating MMP-2 and MMP-9 (11). *Pseudomonas aeruginosa* expresses a MMP and modulates the plasminogen/plasmin system (70). *Treponema pallidum* expresses a MMP-like and serine protease (163), induces MMPs and modulates the plasminogen/plasmin system (22). *Vibrio spp.* express both MMPs and serine proteases (319).

Protease activity has also been implicated in tissue penetration by pathogenic fungi. Different fungi have been associated with multiple BM-degrading proteases. However, knowledge on substrate specificities and their contribution to virulence and pathogenesis is rather poor. During *Aspergillus spp.* infection, a serine protease, MMP and aspartic protease have been identified (209). *Candida spp.* activate host MMP-9, decrease tissue inhibitor of metalloproteinase 2 (TIMP-2) secretion (55), secrete aspartic proteases and unidentified MMP and serine protease activity (273). *Cryptococcus neoformans* expresses a serine protease (294). A total of 53 cDNAs encoding proteases were shown in *Paracoccidioides brasiliensis* including one unidentified gelatinase (collagenolytic protease) and an extracellular serine-thiol protease (282). *P. brasiliensis* also induces MMP-9 (267). Also *Nectriaceae*, *Saccharomycetaceae* and *Trichocomaceae* induce host MMPs (83).

Viruses have also been reported to modulate host-derived proteolytic activity to alter barrier properties of BMs, thereby enhancing viral dissemination (Figure 13). Most viral-induced alterations of BMs involve MMPs. The latent membrane protein-1 (LMP-1) of Epstein-Barr virus (EBV) induces MMP-9 and uPA (368). Hepatitis B virus x protein (HBx) drives MMP-14 expression (206). HPV has been reported to induce MMP-2, MMP-9 and MMP-14 (43). The glycoprotein K1 of Kaposi's sarcoma-associated herpesvirus (KSHV) modulates the production of MMP-1, MMP-2 and MMP-9 (284). Apart from the involvement of viral-induced proteolytic activities in EBV-, hepatitis B virus-, HPV- and KSHV-induced metastasis, it is presently unclear whether this induction plays a role in viral pathogenesis. A similar mechanism is observed in human T-cell leukemia/lymphoma virus type I (HTLV-1)-associated adult T-cell leukemia/lymphoma, where MMP-9 expression is increased in HTLV-1-infected malignant cells (20, 256). HTLV-1 encephalitis is associated with MMP-2 and MMP-9 (20). Other viral infections have also been implicated in neurological conditions due

to their involvement in BBB impairment by damaging the vascular BM. Human cytomegalovirus (HCMV) infection of human microvascular endothelial cells (HMEC) induces collagenase type IV secretion, which may lead to BM degradation and subsequent release of infected endothelial cells into the circulation and access into the CNS (279). Viral hijacking of immune cells might also modulate vascular permeability. *Bunyaviridae* (Andes virus or ANDV), Dengue virus, HIV and West Nile Virus (WNV) enhance dendritic cell (DC) maturation, MMP-9 expression and plasma vascular leakage (229). MMP-9 is also upregulated both in the periphery and brain upon WNV infection and is partly localized to the brain's blood vessels. WNV may enter into the brain either directly through the BBB or be carried within infected leukocytes (described in the next section). WNV also upregulates MMP-1 and MMP-3 (351). Coronaviruses can also induce MMPs in susceptible cells and have been associated with multiple sclerosis-like disease in rodents. However, the role of MMPs in coronavirus CNS infection is unknown (90). Finally, it has been postulated that bovine herpesvirus 5 (BoHV-5) entry into the CNS is facilitated by leukocytes and MMP-9. However, induction of MMP-9 expression by BoHV-5 has not been directly demonstrated so far (44).

Reports describing the involvement of serine protease activity in viral-induced BM distortion are scarce. As described above, EBV also induces uPA (368). An unidentified trypsin-like serine protease is involved in BM crossing by the porcine pseudorabies virus (PRV) in porcine nasal respiratory explants (128).

In summary, several bacteria, fungi and viruses enhance invasion through the BM barrier by (mis)using proteolytic systems via encoding and/or modulating host-derived proteases.

1.3.3. Hitchhiking across the BM

During physiological processes, such as development and immune surveillance, and during the pathology of many diseases, such as metastatic cancer, cells frequently traverse the BM barrier. Transmigration across the BM is a three-stage process (Figure 14). First, invadopodia-like protrusions perforate the BM. Then these protrusions elongate in the degraded zone and infiltrate in the underlying compartment. The rod-like shape of invadopodia is believed to allow for focal delivery of proteases to restrictive areas of the BM (313).

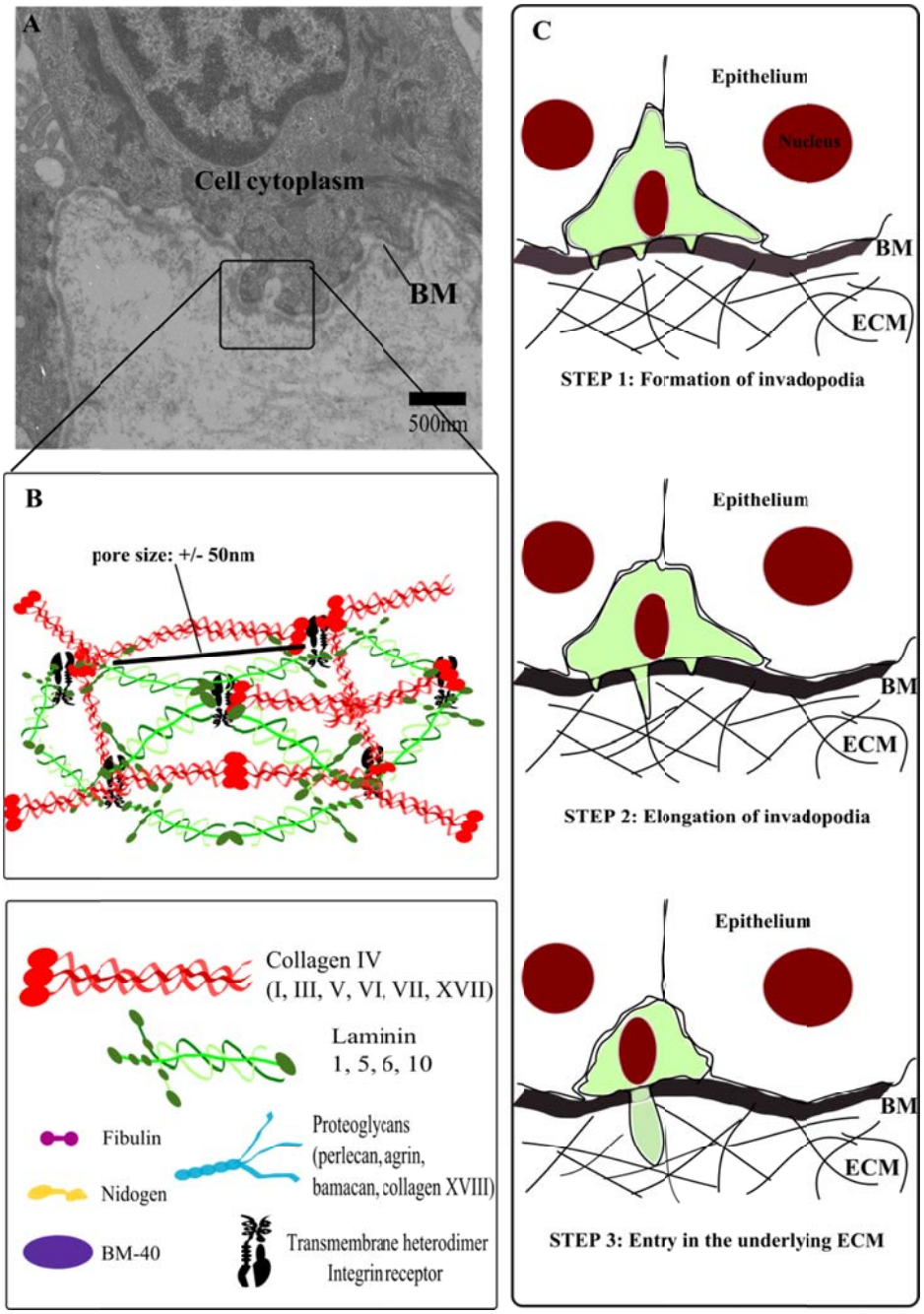

Figure 14. Immune cell trafficking through the basement membrane (BM) network. (A) Transmission electron micrograph of the BM zone. (B) Schematic illustration of a BM network and its main components. Laminin polymerization is believed to initiate the BM scaffold organization. Deposition of this polymer leads to association with a type IV collagen network. The other components of the BM interact with the laminin polymer and the type IV collagen network to organize a functional BM on the basolateral aspect of cells. (C) Despite the small pore size of the BM, immune cells scanning for signs of infection routinely traverse it. The BM transmigration program is a conserved mechanism. First, immune cells adhere to the matrix in an integrin-mediated manner. Subsequently, proteases degrade the BM before actin polymerization extends cell protrusions through the hole. Finally, the cell body moves behind the actin-rich protrusion.

Although the primary function of immune cells is to sample pathogens to initiate an immune response, over time, several pathogens have developed mechanisms to use these cells as Trojan horses to cross the BM barrier and disseminate throughout the host. Mechanisms of intracellular survival due to inhibition of immune cell activation by altering their phenotype and function has been implied or demonstrated to contribute to cell migration (86).

Several bacteria survive in polymorphonuclear neutrophil granulocytes (PMN) or neutrophils: *Anaplasma phagocytophilum*, *Bordetella pertussis*, *Brucella abortus*, *Chlamydia psittaci, Chlamydia trachomatis, Escherichia coli*, *Francisella tularensis*, *Mycobacterium leprae*, *Neisseria gonorrhoeae*, *Salmonella typhi*, *Salmonella typhimurium*, *S. aureus*, *S. pyogenes* and *Yersinia enterolytica*. This may provide a mechanism by which bacteria infect distant site although this needs further experimental confirmation. *Burkholderia pseudomallei*, *Chlamydia pneumoniae*, *Haemophilus somnus* and *Legionella pneumophila* not only survive but also multiply in PMN. Afterwards, pathogens may enter through the uptake of infected apoptotic PMN, survive and multiply in macrophages, which subsequently may transport bacteria throughout the body (207, 332).

The Trojan horse hypothesis is also valid for DCs. *Coxiella burnetii* survives in DCs during infection (35). DCs also transport intracellular *Listeria monocytogenes*, *M. tuberculosis* and *S. typhimurium* from the mucosal areas towards the draining lymph nodes (85).

Mononuclear phagocytes (monocytes and macrophages) can also be misused by intracellular bacteria to disseminate throughout the host. This has been reported or suggested for uropathogenic *E. coli* (28), *L. pneumophila*, *Salmonella enterica* (355), *S.* Typhimurium (86) and *Y. pestis* (115). Furthermore, *H. influenzae* has been found within mononuclear cells both above and below the BM (103). Although *Bacillus anthracis* is not an intracellular pathogen, it can use alveolar macrophages to cross the airway mucosal barriers (135). Mononuclear phagocyte-facilitated entry of the CNS across the BBB has been described for *Brucella spp.*, *Ehrlichia chaffeensis*, *L. monocytogenes*, *M. tuberculosis* and *Streptococcus suis* type II in swine (86). *L. monocytogenes* invades both directly or carried within infected leukocytes (85).

Several pathogens, such as *Mycobacterium avium* (317) and *Shigella flexneri* (67) predominantly exploit M cells to cross the epithelial barrier into the subepithelial lamina propria and subsequently invade macrophages. As described earlier, *H. pylori* is associated with metastatic processes and spread of malignant cells throughout the body (201).

Some fungi also exploit immune cells to cross host barriers. *Histoplasma capsulatun* causes systemic mycosis, mainly in immunosuppressed patients, and can survive and multiply in PMN (207). The facultative intracellular pathogen *C. neoformans* has been demonstrated to cross the BBB via mononuclear phagocytes (monocytes and macrophages), together with other mechanisms involving free yeasts (45).

Several viruses hijack immune cells to transverse the BM (Figure 13). DCs and Langerhans cells (LCs), an epidermal DC subtype, are located at mucosal or epidermal sites of entry for many viruses, such as herpesviruses, immunodeficiency viruses, HPV, and are thus of key importance in their infection. Initial infection of epidermal cells with herpes simplex virus type 1 (HSV-1) results in infection of resident LCs. After infection, a drop in epidermal LC density and a corresponding increase in the number of langerin-positive cells in the underlying dermis has been noticed, arguing for HSV-induced LC migration from the epidermal layer (91). During primary infection with varicella zoster virus (VZV), another herpesvirus, DCs of the respiratory mucosa can transport VZV to human tonsillar $CD4^+$ T lymphocytes, followed by T lymphocyte-mediated dissemination in the host (3). HIV might use LCs for trans-epithelial transport of HIV to susceptible $CD4^+$ T cells (64). Measles virus (MV) is another virus using DCs to gain access to its main target cells in lymphoid tissues (217). As described earlier, ANDV, Dengue virus, HIV and WNV hijack DCs (229).

HIV may not only use LCs for BM passage, but also mononuclear phagocytes (monocytes/macrophages) which could contribute to their ability to invade the brain. Early in the course of infection, HIV-1 can enter the CNS. HIV encephalitis is characterized by HIV-laden monocytes and macrophage infiltration into the CNS parenchyma. Local inflammation as well as HIV products such as gp120, Nef and Tat, which upregulate MMP-2 and/or MMP-9, lead to breaches in the BBB late in HIV-CNS disease. This enables entrance of free virions into the brain (130, 215). Also CMV-infected monocytes can enter the CNS in a Trojan horse model (86). For WNV, LCs support initial viral replication, followed by replication in lymphoid tissues and dissemination to organs and the CNS (291); WNV may enter into the brain through the BBB either directly or carried within infected monocytes or macrophages (as described earlier) (351).

Recently, it has been shown that equine herpesvirus 1 (EHV-1) might use monocytes, macrophages and lymphocytes as Trojan horses to transport the virus through the BM in nasal

mucosae. EHV-1-infected monocytes, macrophages and lymphocytes were found in the connective tissue below the BM in close proximity to the epithelial plaques, suggestive for leukocyte-mediated viral passage of the BM (349). The closely related EHV-4 did not efficiently infect these local immune cells, which might be the reason why EHV-4-induced viremia is rare (349). BoHV-5 is postulated to use a similar invasive mechanism as EHV-1 and BoHV-5 entry into the CNS is facilitated by leukocytes and MMP-9 (44). Lymphocyte-mediated viral entry of the brain has also been demonstrated for HTLV-1 (20). The association with MMP activity was described earlier.

Some oncogenic viruses such as EBV, hepatitis B virus, HPV, HTLV-1 and KSHV drive tumor invasiveness and metastasis by modulating proteases (Figure 13). However, the role of spread of viral-infected malignant cells in the pathogenesis of these viruses is unclear at the present.

In conclusion, different bacteria, fungi and viruses exploit host cells, particularly immune cells, to cross the BM barrier and disseminate throughout the host.

1.3.4. Concluding remarks and future perspectives

The BM represents a formidable barrier of the body against the outside world. However, it is clear that a wide array of pathogens has developed mechanisms to cross the BM and invade the host. There are several important outstanding questions about this still largely unexplored topic. One aspect of BM passage that we have not discussed here is the role of local immunity in breaking down ECM during a microbial infection. Indeed, immune cells produce a large amount of proteases upon stimulation at the site of inflammation and this might rupture important barriers such as the BM, allowing pathogens access to deeper tissues. However, inflammation increases the risk that pathogens will be neutralized by the immune system.

As the BM represents one of the first barriers encountered by the pathogen, dissecting pathogen interactions with, and mechanisms to cross, the BM may provide interesting leads towards developing novel antimicrobial drugs. However, it is important to keep in mind that the current existing detailed *in vitro* knowledge on this topic does not always translate to the *in vivo* situation as axiomatic truth. Improved *in vitro* models that better reflect the *in vivo* environment will provide excellent tools for identification and characterization of putative adhesion and invasion mechanisms before progressing to the use of animal models.

An obvious target to prevent pathogen infiltration is the adhesion step of pathogens to the BM. Moreover, it is important to know that different microbial invasive strategies might have synergistic effects on one another. Indeed, interactions between adhesion and proteolytic activity-mediating mechanisms, to improve and enhance pathogen invasion, have been described (205). In some bacteria, production of proteases might even depend upon quorum sensing (77). Taken into account, hampering bacterial adhesion might also influence their proteolytic activity. Mechanisms underpinning binding to and breakdown of the BM are generally better understood for bacteria and fungi than for viruses. Hence, it will be interesting to identify possible viral factors that are required for efficient penetration through the BM and ECM. If protease activity plays a role, as described recently for a herpesvirus (128), then the use of protease-inhibitors might be a useful strategy. Still, to further develop the potential of proteases as antimicrobial targets, there is a need for identification of all concerned signals, factors and domains during microbial invasion. Compounds, if locally applied, that interfere with pathogen hijacking of migratory cells or limit interactions of metastatic cells with ECM elements could provide an interesting way to prevent or delay further microbial invasion.

In conclusion, several bacteria, fungi and viruses have evolved different finely tuned techniques to adhere to, break down and/or hitchhike across the BM. Fundamental insights in these invasion mechanisms of pathogens could be a promising road towards new therapeutic approaches against these different types of pathogens.

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CHAPTER 2.

AIMS

Many alphaherpesviruses use the epithelium of the respiratory and/or genital tract as a preferential site for primary replication. Local dissemination is followed by initiation of viremia and subsequent spread to internal organs. In addition, these viruses typically invade nerve endings underneath the epithelium and successfully reach the nervous system. However, a potent selective barrier underneath the epithelium, the basement membrane (BM), hampers free passage of pathogens. Driven by evolution, pathogens have developed various mechanisms to overcome the BM. Some microorganisms have developed independent mechanisms, others hijack host cells that are able to transverse the BM (e.g. leukocytes). However, mechanisms underpinning binding to, breakdown of and passage of the BM are generally better understood for bacteria and fungi than for viruses. A better understanding of viral invasion mechanisms may generate new leads towards the development of novel antiviral strategies.

Bovine herpesvirus 1 (BoHV-1) in cattle and herpes simplex virus 1 (HSV-1) and 2 (HSV-2) in humans are important diseases. Remarkably, there is a striking similarity in between these species-specific alphaherpesviruses. Both species harbor herpesvirus isolates with a preferential respiratory tropism (BoHV-1.1 and HSV-1) and isolates that exhibit a more genital tropism (BoHV-1.2 and HSV-2). A thorough study on mucosal invasion mechanisms of both a veterinary alphaherpesvirus and a human alphaherpesvirus will: (i) point out differences and similarities at the level of the mucosa and (ii) provide new ideas towards the development of alternative preventive and curative approaches for animal and human viruses. The thesis aims to identify common and specific mechanisms of herpesviruses at a critical stage of their pathogenesis: the entry in the host through mucosae.

Therefore, the specific aims of this thesis are:

- First, to establish respiratory mucosa and genital mucosa *ex vivo* models for bovine and human, closely mimicking the natural host. Tissue morphology and viability will be extensively evaluated during the entire *in vitro* cultivation time (Chapter 3).
- To thoroughly analyze the replication characteristics of the alphaherpesvirus BoHV-1 within mucosa explants derived from the upper respiratory and genital tract. Different genital and respiratory subtype strains will be included and compared (Chapter 4).
- To assess whether another bovine herpesvirus, the gammaherpesvirus bovine herpesvirus 4 (BoHV-4), exhibits a similar or distinct mechanism of invasion

compared to BoHV-1. Both viruses will be compared for mucosal behavior in explants derived from same animals (Chapter 5).

- To evaluate human alphaherpesviruses HSV-1 and HSV-2 for their mucosal dissemination characteristics in a human genital mucosa model. In addition, distribution patterns of HSV entry receptors within the female lower genital tract will be considered (Chapter 6).
- Finally, to identify which viral factors are required for plaquewise crossing of the basement membrane by alphaherpesviruses. Therefore, emphasis will be put on two important virulence genes, US7(gI) and US8(gE), in alphaherpesviruses and their potential involvement in efficient mucosal invasion. BoHV-1 and bovine respiratory mucosa explants will be used to model viral passage of the BM (Chapter 7).

CHAPTER 3.

ESTABLISHMENT OF RESPIRATORY AND GENITAL MUCOSA MODELS TO STUDY HERPESVIRUS PRIMARY INVASION

A. In vitro study of bovine respiratory and genital mucosa explants

Adapted from

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3.1. Abstract

An *in vitro* model of the upper respiratory tract and lower genital tract of cattle was developed to study molecular/cellular host-pathogen interactions in various respiratory and genital diseases. Different parts of the respiratory tract including distal part of the nasal septum, ventral conchae and proximal trachea, as well as *vestibulum vaginae*, representing the genital tract, were collected from at least three different animals. We cultivated mucosa explants in air-liquid interface organ cultures for 96h. We performed a thorough morphometrical analysis (epithelial integrity/thickness, basement membrane continuity, and lamina propria integrity) at different time points of *in vitro* cultivation, using light microscopy, transmission electron microscopy and scanning electron microscopy. We applied a terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) staining as a viability test during *in vitro* culture. Bovine upper respiratory tract and genital tract mucosa explants were maintained in culture for up to 96h without any significant changes in morphometry and viability and thus are an ideal tool to study different aspects of mucosal pathologies.

3.2. Introduction

Mucosal surfaces such as the respiratory and genital mucosa are common entry ports for many bovine pathogens. Some pathogens can cause devastating clinical entities on their own (e.g. bovine herpesvirus 1) whereas others are mainly involved in a disease complex (e.g. bovine parainfluenza virus type 3) (7, 19, 31). A common serial event in disease complexes, such as enzootic pneumonia in calves and bovine respiratory disease complex, is that initiators such as stress and/or viral and parasitic infections pave the way for secondary bacterial infections (11, 30). In bovines, bovine respiratory tract disease is responsible for major health problems and economical losses. The most common causes of infectious respiratory disease include bovine herpesvirus 1 (BoHV-1), bovine viral diarrhea virus type 1 and 2 (BVD-1/-2), bovine respiratory syncytial virus (BRSV), bovine parainfluenza virus type 3 (PI-3), *Mannheimia haemolytica*, *Pasteurella multocida*, *Histophilus somni*, *Mycoplasma bovis* and lungworm (7, 11, 19, 21). Genital tract infections of both bulls and cows can have serious repercussions on reproductive health in cattle. Bovine herpesvirus 1 and 4 (BoHV-1/-4), *Tritrichomonas foetus* and *Campylobacter foetus subspecies venerealis* among others show a tropism for the genital tract (5, 6, 19). How these different previously mentioned micro-organisms are able to successfully invade the host at the level of the mucosa remains

for many still elusive and is in general better documented for bacteria and parasites than for viruses. Clearly, the use of living animals to study these host-pathogen interactions is undesirable and raises ethical questions about animal welfare, certainly when accessible, reproducible and alternative models can be developed. Organ cultures of respiratory and genital mucosa mimic the physiological condition of the natural host and thus provide an ideal solution. Such systems allow for multiple and frequent sampling points and in addition, defined single or mixed infections can be undertaken in a controlled manner on tissues all originating from the same animal. Bovine nasal and tracheal mucosa organ cultures have been described in literature (3, 4, 9, 24, 26-28). However, these explant models were only maintained for up to 72h without extensive morphometrical analysis during cultivation and hence we attempted to include additional parts of the upper respiratory tract, including ventral conchae, and maintain the organ culture for at least 96h *in vitro*. Hitherto, explant models of bovine *vestibulum vaginae* have not been described yet.

The aim of the present study was to set up *in vitro* explants models of respiratory tissues and genital tissue of cattle. Distal part of the nasal septum, ventral conchae, proximal trachea and vestibule were selected as representative tissues for the respiratory and genital tract respectively. Explants were cultured on gauze at air-liquid interface for 96h. A thorough structural (morphology) and functional (viability) analysis was performed at 0h, 24h, 48h, 72h and 96h of cultivation. To this end, we applied light microscopy, transmission electron microscopy (TEM) and scanning electron microscopy (SEM) to evaluate different morphometrical parameters (epithelial integrity/thickness, basement membrane continuity, and lamina propria integrity) for tissue preservation. In addition, we used a terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) staining as a viability test by means of fluorescence microscopy.

3.3. Materials and methods

3.3.1. Selection of animals

For the design of the respiratory mucosa explant model, bovine respiratory tissue was obtained at a slaughterhouse. Samples were taken from three animals aged 6 months. The head was cut longitudinal into two pieces, exposing the nasal septum. We cautiously removed nasal septum from the caudal two thirds of the nasal cavity (septum), ventral conchae (conchae), and proximal trachea (trachea).

For the establishment of the genital model, four different cows between 3 and 5 years old were selected. A criterion to include the animals in this experiment was based on female reproductive hormone levels. At slaughter, a thorough palpation and visual inspection of the ovaries was performed to select cows with a clear marked corpus luteum. Moreover, blood was collected at slaughter. On all sera, a progesterone determination was performed to determine peripheral blood progesterone.

3.3.2. Cultivation of tissue explants

A similar set up was used for bovine respiratory and genital mucosal explants. Briefly, tissues were immediately placed in phosphate buffered saline (PBS), supplemented with 1 µg/mL gentamycin (Invitrogen, Paisley, UK), 1 mg/mL streptomycin (Certa, Braine l'Alleud, Belgium), 1 mg/mL kanamycin (Sigma, St. Louis, MO, USA), 1000 U/mL penicillin (Continental Pharma, Puurs, Belgium) and 5 µg/mL fungizone (Bristol-Myers Squibb, New York, USA) for transportation to the laboratory. Respiratory and genital mucosa was stripped from the underlying layers. Small square tissue pieces were made and placed on fine-meshed gauze for culture. The explants were cultivated in serum-free medium (50% DMEM (Invitrogen)/50% Ham's F-12 GlutaMAX (Invitrogen)) supplemented with 0.1 mg/mL streptomycin (Certa), 100 U/mL penicillin (Continental Pharma) and 1 µg/mL gentamycin (Invitrogen) for up to 96h (37 °C, 5% CO_2).

3.3.3. Evaluation of tissue viability

Tissues derived from all animals were monitored for occurrence of apoptosis during *in vitro* culture. DNA fragmentation was evaluated using an In Situ Cell Death Detection Kit (Roche), which is based on terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL). This method is designed as a precise, fast and simple, non radioactive technique to detect and quantify apoptotic cell death at single cell level in cells and tissues. We performed the TUNEL reaction according to the manufacturer's guidelines. TUNEL-positive cells were counted from five randomly chosen fields of 100 cells in the epithelium as well as in the lamina propria with a fluorescence microscope (Leica DM RBE microscope, Leica Microsystems). An analysis was made at 0h, 24h, 48h, 72h and 96h of cultivation.

3.3.4. Evaluation of tissue morphometry

The respiratory explants were checked for sufficient ciliary beating on a daily basis using a light microscope as a first viability parameter. At 0h, 24h, 48h, 72h and 96h of cultivation, explants of both respiratory and genital tissue were gathered for further analysis. A thorough morphometrical assessment was performed by means of light microscopy, transmission electron microscopy and scanning electron microscopy.

Light microscopy

Fixation of explants was performed at the different time points by submerging them in a phosphate-buffered 3.5% formaldehyde solution for 24h. Fixation was followed by paraffin embedding using an automated system (STP 420D, Micron, Praran, Merelbeke, Belgium). Sections of 20 µm thick were successively cut, deparaffinized in xylene, rehydrated in descending grades of alcohol, stained, dehydrated in ascending grades of alcohol and xylene, and mounted with DPX (DPX mountant, BDH Laboratory Supplies, Poole, UK). As a parameter for the effect of *in vitro* culture on the epithelial morphometry, epithelial integrity and/or thickness was measured by means of a haematoxylin-eosin (HE) staining. At magnification 40×, five randomly selected places in five randomly chosen fields were evaluated/measured in each explant. Next, a reticulin staining to evaluate continuity and thickness of the basement membrane was carried out. Five randomly chosen places in 5 randomly chosen zones were measured in each sample. Finally, the structure of the connective tissue was evaluated by means of a Van Gieson staining. In five randomly chosen fields, the relative amounts of collagen and nuclei were calculated in a defined region of interest (roi) by setting a threshold. All measurements and calculations were performed using the Cell F Software linked to a BX61 light microscope (Olympus, Hamburg, Germany) (magnification 40×).

Transmission electron microscopy

We performed a transmission electron microscopical analysis to evaluate the structure of the explant down to the subcellular level. Explants were fixed overnight at 4°C in Karnovsky's Fixative (2% paraformaldehyde and 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer

(pH 7.4)) to ensure that the structure of the specimens was preserved (15). Later, explants were rinsed in 0.1 M sodium cacodylate buffer (pH 7.4) for 8h and underwent an overnight postfixation in 2% osmium tetroxide at 4°C. The samples underwent a stepwise dehydratation in ascending grades of alcohol, an infiltration in a low viscosity embedding (LVR) medium (Agar Scientific) for 2 days, and an embedding in LVR. Lastly, ultrathin sections of embedded material were cut using a diamond knife on an Ultramicrotome Ultracut EM UC6 (Leica Microsystems, Wetzlar, Germany) and stained afterwards with a Leica Microsystems EM staining before analysis on a JEM-1010 transmission electron microscope (Jeol) operating at 60 kV.

Scanning electron microscopy

Genital explants were submitted to a scanning electron microscopical analysis. Niesalla and co-workers described this previously for bovine respiratory mucosa explants (20). After gathering the genital explants at the defined time points, fixation of the explants in a HEPES-buffer containing 2% paraformaldehyde and 2.5% glutaraldehyde solution during 24h was followed by a post-fixation step in an un-buffered 1% osmium tetroxide solution for 2h. Then, the fixed explants were dehydrated through ascending grades of alcohol and critical point dried with CO_2 (CPD 030, Balzers, Sercolab, Merksem, Belgium), mounted on metal stubs, platinum-coated (JFC-1300 Autofine Coater, Jeol, Tokyo, Japan) and examined by a Jeol JSM 5600 LV scanning electron microscope (Jeol). Using 1 500× and 5 000× magnification, the integrity of the epithelium was examined.

3.3.5. Statistical analysis

The obtained data were assessed using SPSS software (ANOVA) to evaluate the variance. The results shown represent means + standard deviation of triple (respiratory model) and quadruple (genital model) independent experiments. Results with P values of ≤ 0.05 were considered significant.

3.4. Results

3.4.1 Progesterone determination

The four other animals included for the establishment of the genital model had a peripheral blood progesterone (P4) level of > 1 ng/mL, suggesting they were in the luteal phase of the reproductive cycle.

3.4.2 Tissue viability

Respiratory model

During 96h of *in vitro* culture, there was no major increase regarding the amount of TUNEL-positive cells in the epithelium. However, for each tissue, at 96h of cultivation, we noticed a small but significant increase in the amount of TUNEL-positive cells in the epithelium. Evaluating the viability of the underlying connective tissue, the percentage of apoptotic cells in all tissues ranged from 2.9 ± 1.7 to 19.3 ± 4.3 at 0h and 96h, respectively (Table 1). The occurrence of apoptosis was more clear in glandular structures.

Table 1. Occurrence of apoptosis in epithelium and lamina propria as a parameter for the effect of in vitro culture on the viability of bovine respiratory mucosa explants. Values are given as means ± SD.

Tissue	Layer	% of TUNEL-positive cells at … h of cultivation				
		0	24	48	72	96
Deeper part of the nasal septum	Epithelium	0.4 ± 0.2	2.6 ± 2.0	2.4 ± 2.6	1.5 ± 1.1	5.5 ± 2.7
	Lamina propria	2.9 ± 1.7	10.5 ± 7.2	11.2 ± 4.3	13.3 ± 6.7	19.3 ± 4.3
Ventral conchae	Epithelium	0.5 ± 0.4	2.0 ± 0.9	1.7 ± 1.6	1.3 ± 0.9	2.8 ± 1.7
	Lamina propria	3.0 ± 2.6	9.9 ± 4.0	11.6 ± 1.8	13.4 ± 4.3	17.5 ± 2.4
Proximal trachea	Epithelium	0.5 ± 0.3	0.9 ± 1.0	1.6 ± 0.7	1.3 ± 0.5	2.7 ± 0.6
	Lamina propria	3.0 ± 2.2	7.2 ± 1.1	9.5 ± 2.5	15.1 ± 1.9	17.2 ± 2.0

Genital model

With increasing time of *in vitro* culture, we saw no major differences in the occurrence of apoptosis in the epithelium. However, a small significant increase in apoptotic epithelial cells was observed at 96h of cultivation. For the connective tissue, we noticed a small increase in the occurrence of apoptosis as culture time passed by (to 7.7 ± 2.7 at 96h). Values of the effect of *in vitro* culture on the viability of bovine genital mucosa explants are given in Table 2.

Table 2. Percentage of TUNEL-positive cells in epithelium and lamina propria as a parameter for the effect of in vitro culture on the viability of bovine genital mucosa explants. Values are given as means ± SD.

		% of TUNEL-positive cells at … h of cultivation				
		0	24	48	72	96
Vestibulum Vaginae	Epithelium	0.3 ± 0.1	0.4 ± 0.3	0.8 ± 0.4	0.3 ± 0.2	1.5 ± 0.5
	Lamina propria	0.9 ± 0.4	3.2 ± 1.5	4.8 ± 3.2	5.8 ± 4.5	7.7 ± 2.7

3.4.3 Ciliary beating

The cilia covering the respiratory epithelium kept on beating during the entire period of *in vitro* culture (up to 96h, the end of the experiment).

3.4.4 Epithelial morphometry

Light microscopy

Respiratory model

We observed no significant changes in epithelial integrity during the entire cultivation period for septum, conchae, and trachea (Figure 1). All samples showed a respiratory epithelium. However, concerning septum and conchae we found some zones possessing a stratified squamous epithelium.

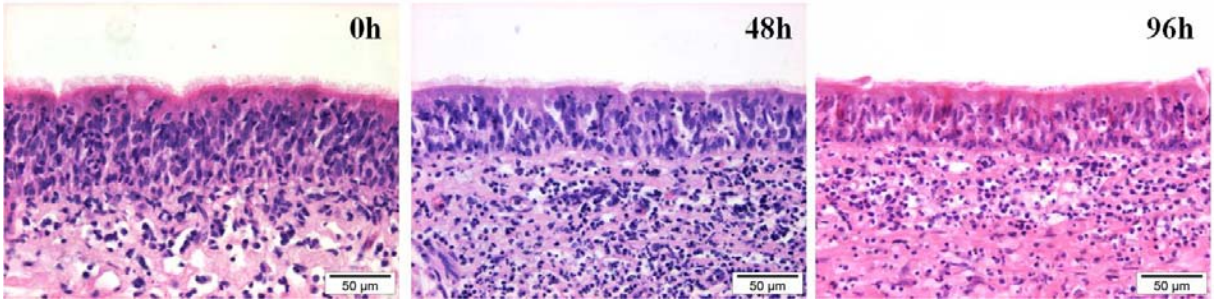

Figure 1. HE-stainings of bovine proximal trachea explants showing preservation of epithelial integrity at different time points of in vitro culture (0h, 48h, 96h).

Genital model

No significant changes were noticed in the measured epithelial thickness of the *vestibulum vaginae* as cultivation time passed by. A clear non-keratinized stratified squamous epithelium was visible for all samples (Figure 2a-b).

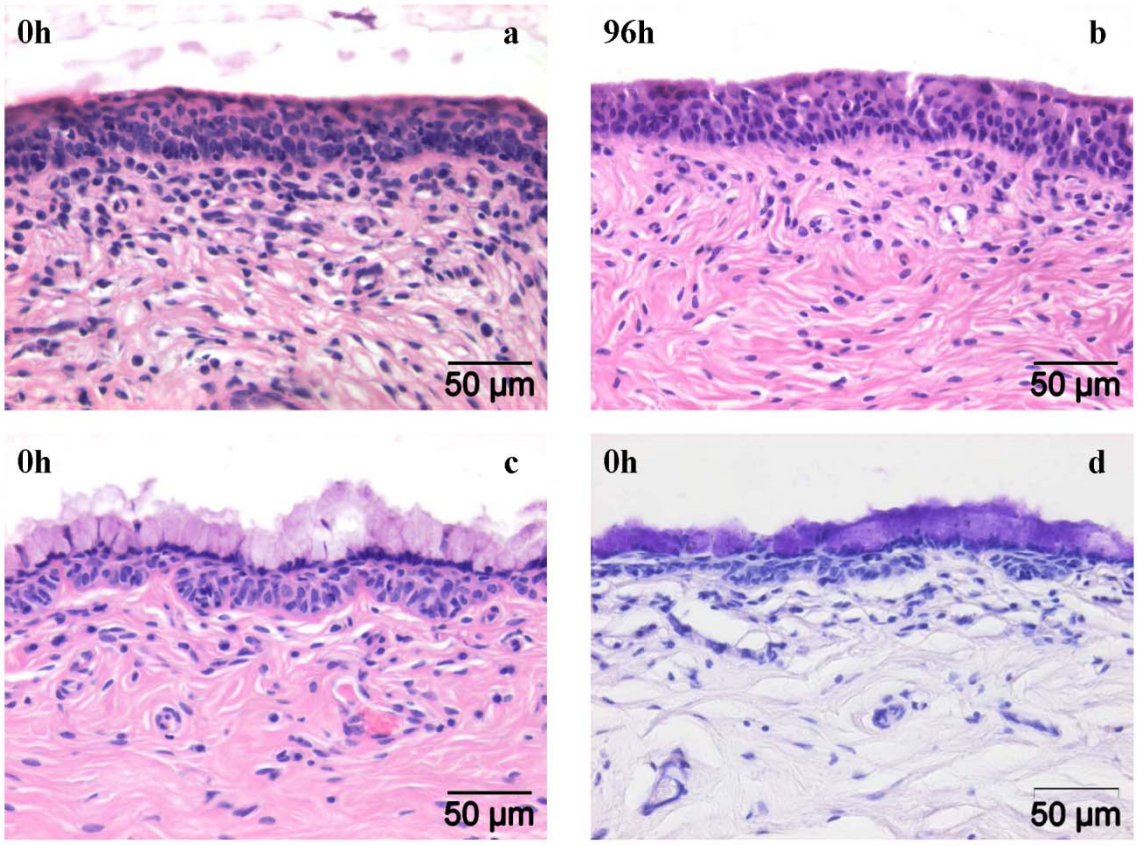

Figure 2. Photomicrographs of bovine stratified squamous epithelium lining the vestibulum vaginae at 0h (a) and 96h (b) of in vitro cultivation (end of experiment) (HE-staining). One cow showed zones containing an active mucus-secreting (PAS +) stratified columnar to cuboidal epithelium, prominent at 0h of in vitro cultivation. This active epithelium is shown in c (HE-staining) and d (PAS-staining).

However, for all cows, a few zones containing a stratified columnar to stratified cuboidal epithelium were observed. Moreover, one cow (1.03 ng/mL P4 level) showed especially at 0h of cultivation some columnar to cuboidal cells in the stratified columnar to cuboidal epithelial zones containing a strong periodic acid + Schiff (PAS) positive substance (Figure 2c-d).

Transmission electron microscopy

Respiratory model

Transmission electron microscopy was applied to evaluate epithelial integrity (Figure 3).

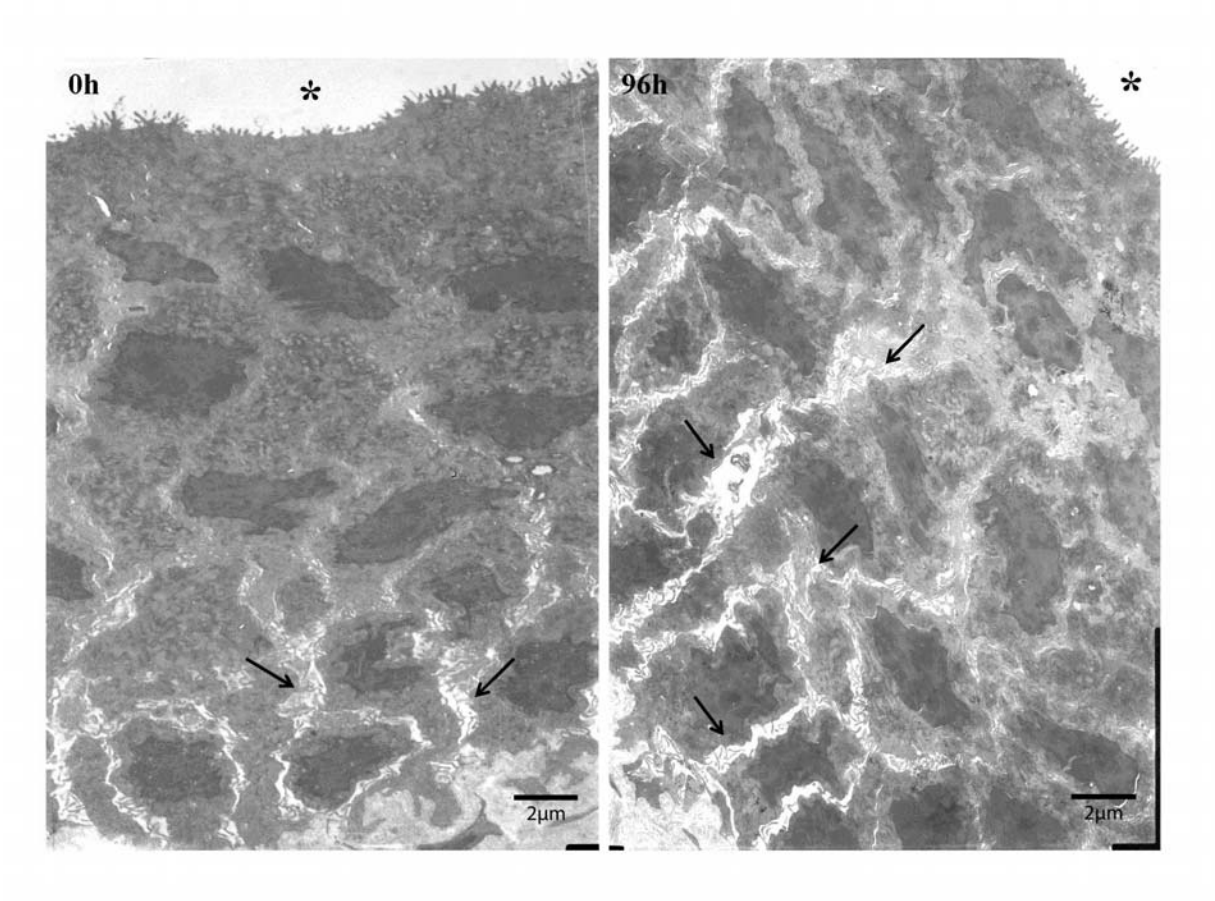

Figure 3. Transmission electron microscopical (TEM) images of bovine respiratory epithelium at 0h and 96h of in vitro culture. Few intercellular spaces between basal cells appeared at 0h of in vitro culture. Starting from 72h of in vitro culture, they became more distinct and came into sight between some apical cells (black arrows). The luminal side of the epithelium is marked by an asterisk. Magnification 3000x.

For all tissues at all collected time points, small intercellular spaces between basal epithelial cells were seen. These observations were not made between apical epithelial cells at 0h, 24h, and 48h of *in vitro* cultivation since apical cells were adjacent. However, it should be noted that starting from 72h of *in vitro* cultivation, intercellular spaces between basal epithelial cells became more distinct, and moreover, few intercellular spaces started to appear between apical epithelial cells. Figure 3 shows intercellular spaces.

Genital model

Overall integrity could be evaluated by means of transmission electron microscopy. During the entire cultivation period (96h) the epithelial structure was maintained (Figure 4a-b). Starting from 0h of cultivation, small intercellular spaces could be observed between some basal cells (Figure 4c). Remarkably, large apical electron-lucent cells randomly spread across the epithelium, were observed starting from time point 0h of cultivation. Conservation of microvilli at 0h, 24h, 48h, 72h and 96h of cultivation was noticed.

Scanning electron microscopy

Genital model

Evaluating the epithelial integrity by scanning the surface of the epithelium, no significant changes were noticed in epithelial morphology during *in vitro* culture for up to 96h. Surface cells had an irregular pavement-like appearance (Figure 4d-e). Moreover, on the surface secretory blebs were visible. All epithelial cells contained stubby microvilli and at the cell borders, clear microridges were visible (Figure 4f). These cell surface structures could be maintained at all time points.

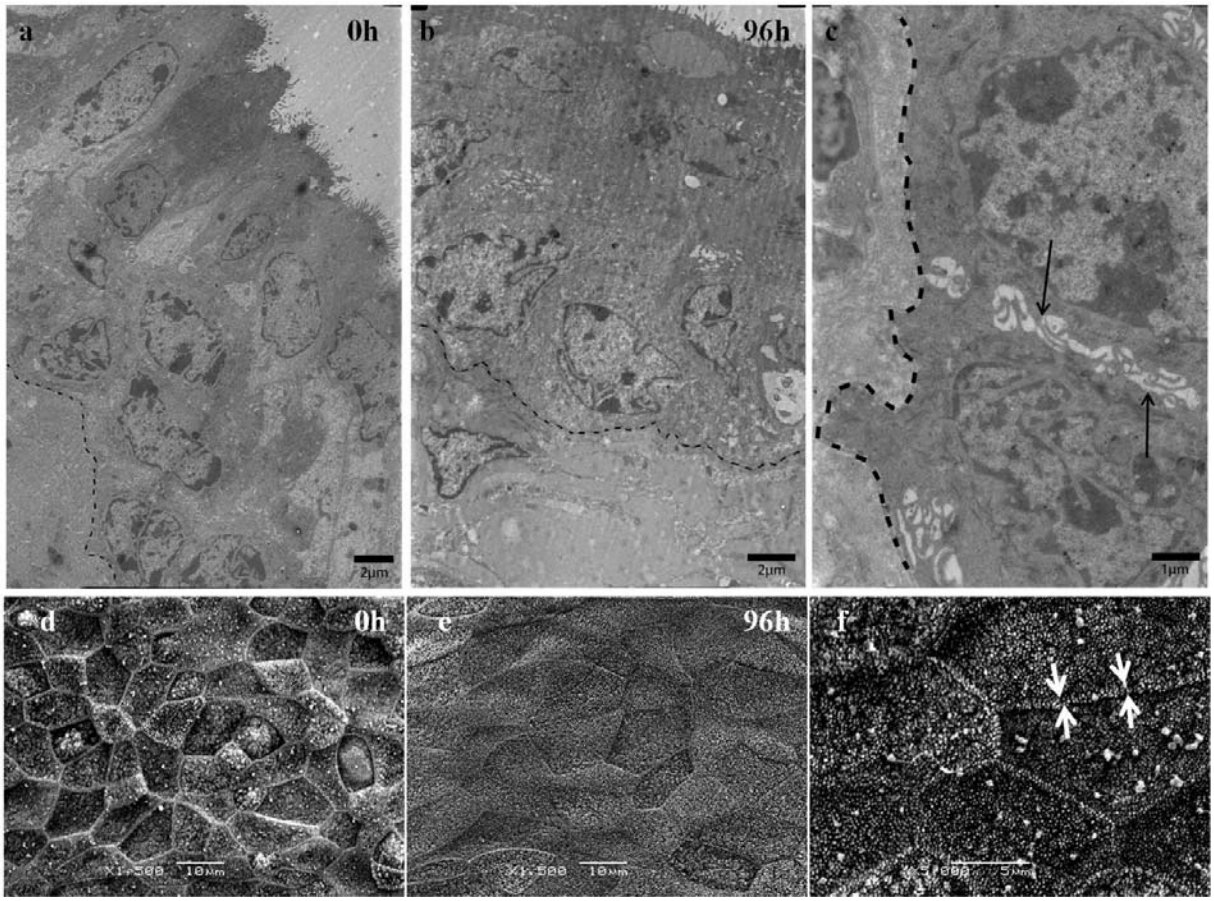

Figure 4. Transmission electron and scanning electron microscopical images of bovine vaginal epithelium. Epithelial integrity and structure was maintained at all time points (0h-96h) as seen with transmission electron microscopy (a-b). Starting from 0h of cultivation, small intercellular spaces between basal cells were observed (c, indicated by black arrows). The dotted line represents the basement membrane (BM). Pavement-like cells containing microvilli were seen at all time points (0h-96h) of in vitro culture when evaluating the epithelial surface by means of scanning electron microscopy (d-e). Cells are aligned with clear microridges (f, indicated by white arrows).

3.4.5 Basement membrane mophometry

Light and Transmission electron microscopy

Respiratory model

No major changes were observed in the thickness of the reticular lamina when evaluating reticulin stainings during the *in vitro* culture of mucosae of the septum, conchae and trachea (Figure 5). At 0h, 24h, 48h, and 72h of cultivation, we noticed a significant difference in *lamina reticularis* thickness when comparing proximal trachea to the deeper part of the nasal

septum and ventral conchae. A smaller thickness of the *lamina reticularis* was observed in proximal trachea (*P* values ≤ 0.05).

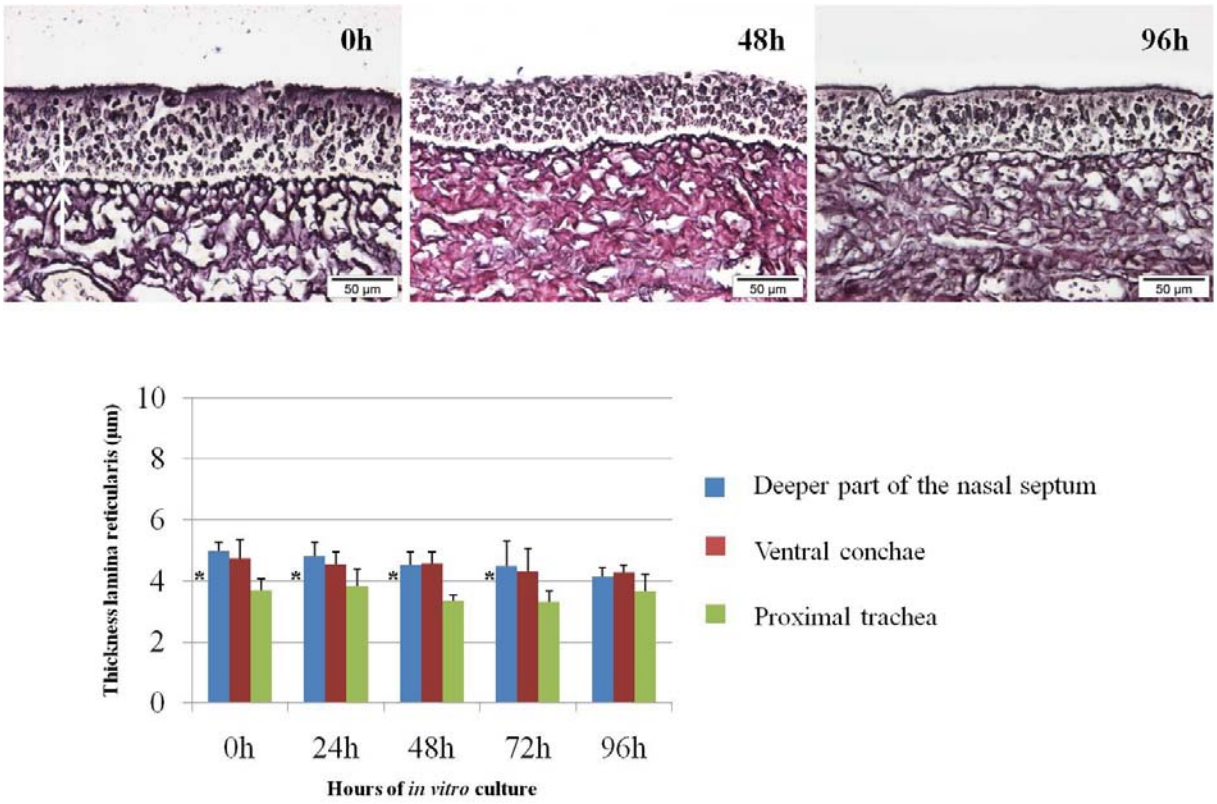

Figure 5. Light photomicrographs of bovine proximal trachea explants at different time points of in vitro culture (0h, 48h, 96h). For analysis of lamina reticularis thickness, a reticulin staining was used (lamina reticularis indicated by white arrows). The graph represents the measured thickness of the lamina reticularis in explants of the deeper part of the nasal septum, ventral conchae and proximal trachea at different time points of in vitro culture. Data are represented as mean values of three calves + SD (error bars) (25 measurements/calf). Data with P values ≤ 0.05 were considered significant (marked by an asterisk).

The continuity of the *lamina densa* of the BM was evaluated by means of TEM. For all explants and at all time points (up to 96h), no significant changes in *lamina densa* integrity were observed (Figure 6).

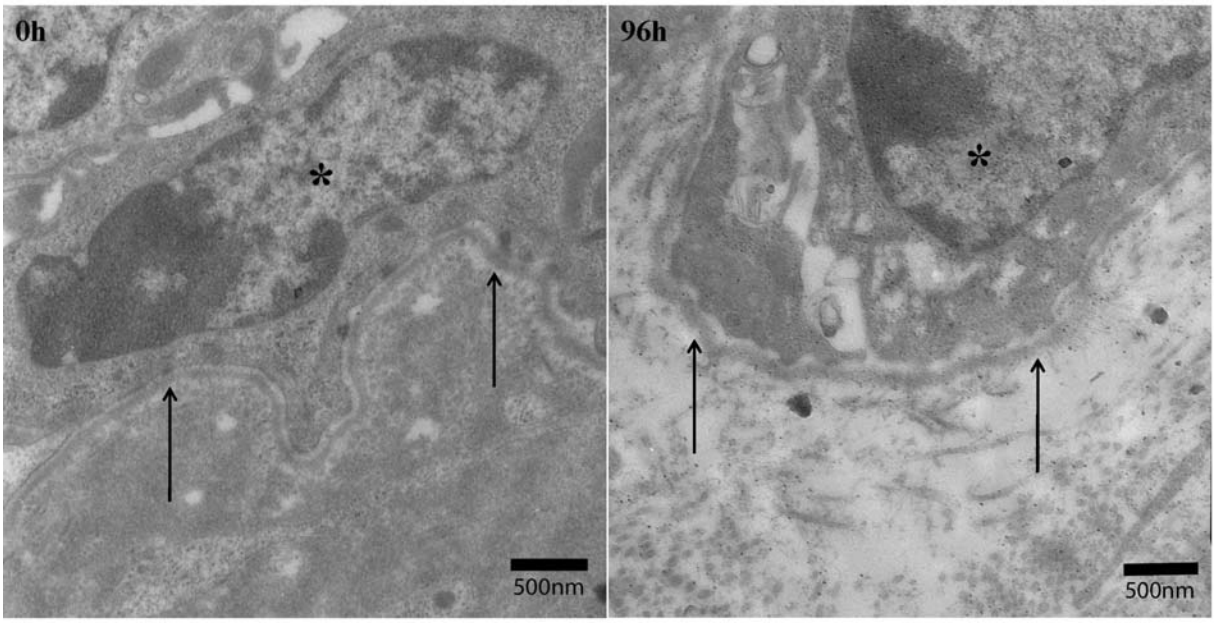

Figure 6. Detailed photomicrographs of respiratory tissue taken by a transmission electron microscope at 0h and 96h of cultivation. The continuity and integrity of the lamina densa of the basement membrane was maintained during the entire experiment (96h) (black arrows). Basal cells of the respiratory epithelium above the BM are visible (asterisk). Magnification 12000x

Genital model

After analysis of the reticulin stained sections, significant changes were not observed regarding the thickness of the *lamina reticularis* during *in vitro* culture (Figure 7a).

By means of transmission electron microscopy, the continuity and integrity of the *lamina densa* of the basement membrane were evaluated. Throughout 96h of *in vitro* cultivation, no significant changes in *lamina densa* continuity and integrity were found (Figure 7b-c).

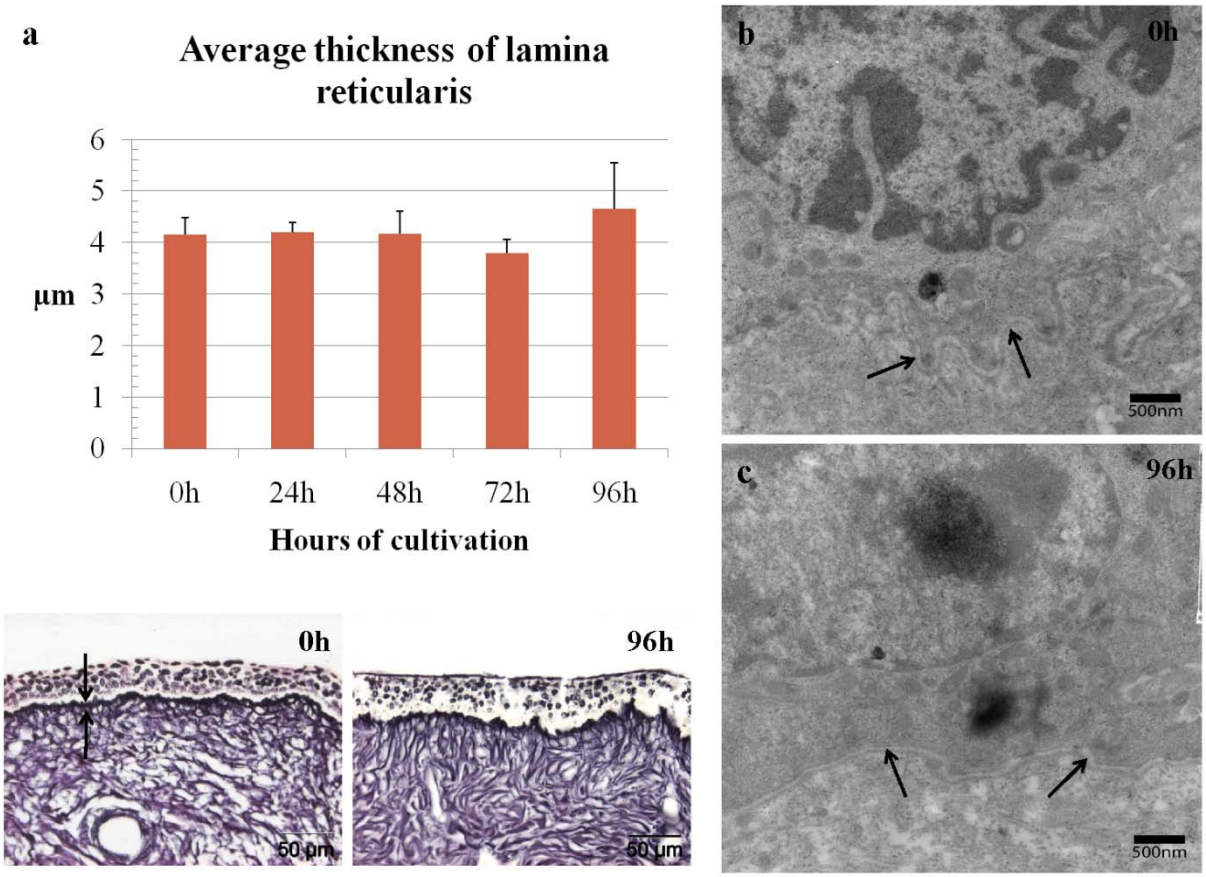

Figure 7. Evaluation of basement membrane (lamina densa and lamina reticularis) continuity and thickness by means of transmission electron microscopy and light microscopy (reticulin staining). Average thickness of the lamina reticularis was monitored throughout in vitro culture (a, black arrows indicate lamina reticularis). No significant changes were observed in lamina reticularis thickness when analyzing reticulin stainings. The lamina densa remained continuous at all time during the entire cultivation period (up to 96h) as shown by the transmission electron microscopical images (b-c, black arrows indicate lamina densa). Data are represented as means + SD (error bars).

3.4.6 Morphometry of the lamina propria

Light microscopy

Respiratory model

When analyzing Van Gieson stainings, no significant changes in relative amounts of collagen and nuclei were observed during the entire cultivation period for all tissues (Figure 8).

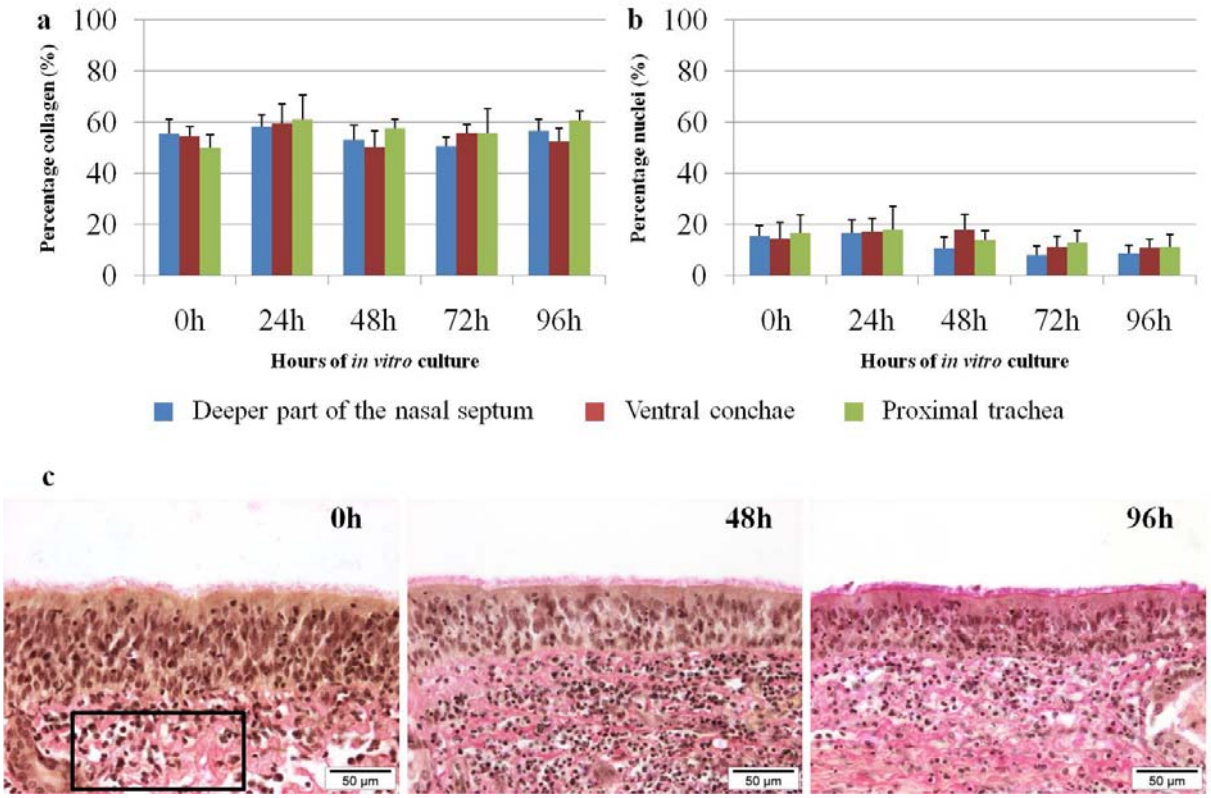

Figure 8. (a) Percentages of collagen within a region of interest and (b) percentages of nuclei within a region of interest in explants of the deeper part of the nasal septum, ventral conchae and proximal trachea at different time points of in vitro culture are given within the graphs. Data are represented as mean values of three calves + SD (error bars) (5 measurements/calf). (c) Light photomicrographs of bovine proximal trachea explants at different time points of in vitro culture (0h, 48h, 96h). Relative amounts of collagen and nuclei within a defined region of interest (roi indicated by black rectangle) were analyzed by means of a Van Gieson staining. By giving different colors to respectively collagen and nuclei (setting a threshold), the percentage of collagen and nuclei was counted within the roi.

Genital model

Using a Van Gieson staining, no significant changes were noticed in relative percentage of collagen and nuclei in the connective tissue with increasing time after sampling (Figure 9).

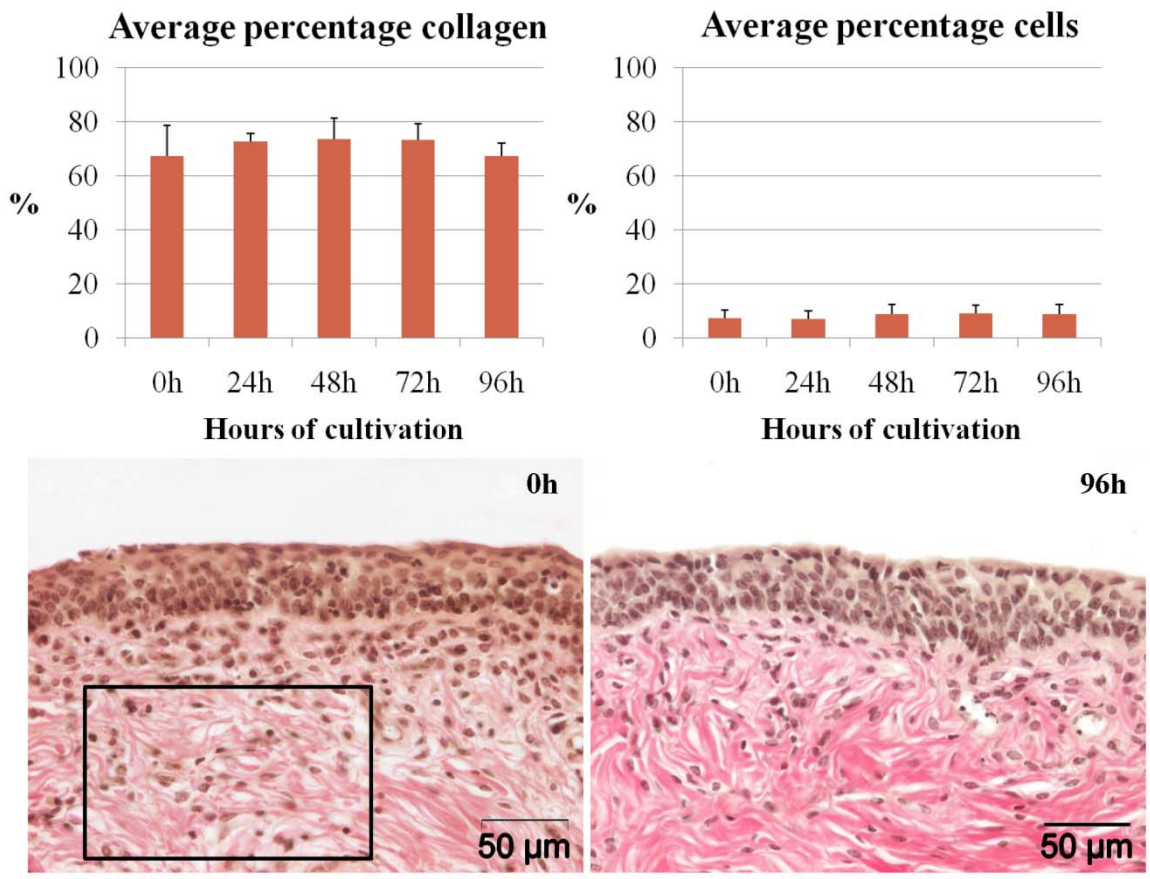

Figure 9. Average percentages of collagen and cells were measured in the connective tissue and assessed on conservation throughout cultivation (up to 96h) using Van Gieson stainings. By giving different colors to collagen and nuclei (setting a threshold), relative amounts of collagen and nuclei were measured within a region of interested (roi) (roi is indicated by a rectangle). Representation of the data is visualized as means + SD (error bars).

3.5. Discussion

Little is known about the events happening upon host entry at mucosal surfaces early in the pathogenesis of several bovine micro-organisms. Nevertheless, getting a fundamental image on how these pathogens behave at their primary replication/colonization site can provide insights for prevention and treatment on a rational basis.

A first aspect of the present study was to establish and optimize a bovine upper respiratory tract mucosa explant model allowing the study of various bovine respiratory pathogens in bovine respiratory mucosa. The use of respiratory organ cultures already proved to be of importance in different scientific areas. Insights in drug transportation, metabolic pathways,

and pathogen-host interactions are attained achievements based on organ culture arrays. The explant model is the perfect compromise between *in vitro* cell cultures and *in vivo* laboratory animals. In cell cultures, no tissue structure is present and therefore essential cell-cell and cell-extracellular matrix contacts are lost. Current *in vivo* model systems often used in alphaherpesvirus studies originated from rodents. However, these models create a heterologous situation since species specific cellular components, which may play a role in viral invasion, are absent. The bovine mucosa explant system represents a homologue model strongly resembling the *in vivo* situation and implements reduction, refinement, and replacement (3R principles). These unique systems may be used for screening of different potent antiviral molecules. Since intraspecies variation is excluded, a specific effect of a certain condition or virus strain related to the mock situation may be examined. Explant models of respiratory tissue have already been described in the literature for use in human (1, 13, 14, 25), rat (8), canine (2), swine (10, 22) and equine models (12, 17, 29). In bovine models, respiratory organ cultures have been described in the literature but these explants were only maintained for up to 72h without extensive morphometrical analysis (3, 4, 9, 24, 26-28). Recently, Niesalla and colleagues (2009) established an *in vitro* bovine respiratory organ culture system to use as a BoHV-1 infection model. Bovine tracheal and nasal mucosae were cultured for up to 72 hours and monitored for viability, integrity, and TNF-α gene expression. However, using latex beads clearance as a viability test only gives an indication about epithelial cell function. There was no information on the viability of the underlying layers. Moreover, a histological assessment of the organ culture using light microscopy and scanning electron microscopy showed evidence of progressive degeneration starting from 24h of cultivation. Although overall integrity of the epithelium was maintained, degeneration of mucus gland structures in the lamina propria, starting from 24h, and separation of collagen fibers from 48h onwards are reported. We believe it is essential to maintain the viability and integrity of the underlying layers when studying a virus-host interaction at mucosal entry ports. Therefore, we modified, optimized, and extended the bovine respiratory organ culture. By applying a precise, non-touch method of the tissues during manipulation and by fine-tuning the cultivation medium, we succeeded in maintaining epithelium integrity and viability up to 96h of cultivation. During *in vitro* culture, intercellular spaces became more clear between basal epithelial cells, and starting from 72h of *in vitro* culture, few intercellular spaces appeared between the adjacent apical cells. These findings were also evident in respiratory mucosa explants of swine and horse. A possible explanation for the gapping is a decreasing strength of cell-cell contacts at different regions in the explants (10, 29).

Regarding BM integrity and continuity, no significant changes were noticed during the entire culture. The trachea showed significant differences in *lamina reticularis* thickness compared to the other tissues at 0h, 24h, 48h, and 72h of cultivation. We can conclude that trachea seems to have a smaller thickness of the *lamina reticularis* compared to septum and conchae. When evaluating the connective tissue, we did not observe major significant changes in tissue morphometry. Concerning viability of the connective tissue, starting from 24h of *in vitro* culture, we noticed an increase in the occurrence of apoptosis, especially inside glandular structures. This could be caused by a lack of sufficient nutrients. However, during the entire cultivation period (96h) occurrence of apoptosis did not exceed 19.3 ± 4.3% and no significant differences were observed between septum, conchae, and trachea. Since we did not notice any severe degeneration of mucus gland structures and collagen fibers, we considered this an acceptable percentage.

The second part of the current study consisted of the development of an organ culture of bovine *vestibulum vaginae*. Hitherto, organ cultures of bovine *vestibulum vaginae* have never been described. Some interesting facts were seen when evaluating tissue morphology. One cow showed highly active columnar to cuboidal cells in the stratified columnar to cuboidal epithelial zones containing a PAS positive substance. Taken into consideration that at the time of sampling this cow was in a transition phase of the reproductive cycle, namely from a progesterone to an estrogen dominance or vice versa (P4 1.03 ng/mL), this mucin production might be an outcome of estrogen influence on the epithelium (18). These hormone related findings are important since herpes virus infections at the genital site are known to be influenced by reproductive hormone levels (16). Starting from 0h of cultivation, few intercellular spaces were visible between basal cells of the epithelium. We made the same observations when establishing the bovine respiratory mucosa explant model. When using transmission electron microscopy, salient large electron-lucent apical cells, randomly spread across the epithelium starting from 0h of cultivation, were noticed. Regli and Kress saw similar cells in the vaginal epithelium of the *Maruspial Monodelphis domestica*, describing the remarkable fact as damaged cells by the previous desquamation process or not yet differentiated cells (23). However, no significant changes in the number of these particular cells were found throughout cultivation. We observed at 96h of cultivation a small increase in the number of apoptotic epithelial cells together with an increase in apoptotic lamina propria cells as culture time passed by. An analogous trend was seen in the viability assessment of upper respiratory tract cultured tissues. Overall, we can state that the newly developed organ

culture of bovine *vestibulum vaginae* was successfully maintained for at least 96h in culture at air-liquid interface without demonstrable changes in tissue architecture or viability.

We can conclude that bovine respiratory and genital mucosa explants can be maintained in culture for up to 96h without any major changes regarding structural integrity and viability Moreover, because of their *in vivo* relevance, these *in vitro* models are highly appreciated since laboratory animal use is diminished and confounding factors such as individual animal variation and environmental factors are excluded. Therein lies their strength, when comparing an array of strains/subtypes on viral behavior at mucosal entry ports.

3.6. Acknowledgements

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B. In vitro study of human genital mucosa explants

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3.8. Abstract

Sexually transmitted infections (STI) globally account for the majority of infectious diseases amongst humans, resulting in substantial medical costs and severe health burden. At present, there is a huge lack in available preventive and/or curative measures for STI. In particular, viral STI, with human immunodeficiency virus (HIV) and human herpes simplex virus (HSV) as prototype members, are difficult to control. There is an urgent medical need for the development of novel anti-STI measures based on targets that have not been identified before. A big gap in our understanding of how these pathogens interact with their host is the mucosal behavior displayed by pathogens and the host invasion mechanisms they apply at these entry ports. Hence, we developed and optimized a human genital mucosa explant model (endocervix and ectocervix/vagina) to mimic the early steps of the pathogenesis of genital infections. During the optimization of this procedure, special emphasis was put on maintenance of tissue morphology as well as tissue viability during this *in vitro* cultivation by applying several techniques.

3.9. Introduction

The lower reproductive tract of humans provides a unique site of host entry for many pathogens. In addition, microbial invasion of genital mucosal surfaces render microorganisms an ideal way for transmission between individuals (16). Sexually transmitted infections (STI) burden human populations not only because of the economical costs and implications on physical health but also because of the psychosocial problems post-diagnosis (19, 22). Globally, an estimated average of 35.3 million people are infected with human immunodeficiency virus (HIV) (31). Human genital herpes, caused by both herpes simplex virus 1 (HSV-1) and 2 (HSV-2), is worldwide one of the most prevalent causes of genital ulcer disease and with a prevalence reaching up to 80% one of the most important STI (1). No less than 610.000 cancers, mainly cervix cancer, of an estimated 12.7 million cancers occurring worldwide in 2008 could be attributed to sexually transmitted infections by human papillomavirus (HPV) (6). The most sexually transmitted bacterial pathogen is *Chlamydia trachomatis* with 3 million new infections each year in the United States (21). Available preventive and/or curative approaches against such infections are very limited and for most of them not existing (2, 11). Especially viral etiologies of STI are difficult to cure. This indicates that there is an urgent need for investigations that identify alternative targets for preventive

and curative measures. A major gap in our understanding of the pathogenesis of many STI is the delineation of events occurring at early stages of infection at the level of the mucosa.

Epidemiological studies have shown that women suffer most often from STI although the reasons for this are not well understood (15). The local microenvironment of the female genital tract has unique features. Two different types of mucosal surfaces can be readily distinguished. The endocervix and endometrium are lined by a polarized single columnar epithelium. Moreover, the endocervix functions as the major site for cervicovaginal mucus production. By contrast, the ectocervix and vagina, nearly indistinguishable from each other, possess a stratified squamous epithelium that lacks polarity (16). The vaginal cavity is a hostile environment for incoming foreign particles. Several elements including (i) an acidic pH, (ii) an endogenous commensal bacterial flora, (iii) a mucus layer with known antimicrobial effects covering the epithelium and (iv) a physical well layered epithelial barrier possessing many resident immune cells, try to hamper free passage of foreign particles (12, 16). It is highly intriguing to gain more knowledge on which mechanisms these pathogens have devised in order to overcome the latter obstacles.

However, investigations leading towards novel information about the role of both endocervical and ectocervical/vaginal mucosa at very early events of STI is difficult to perform in humans. Several alternative *in vivo* models exist that allow in depth study of several STI, including the simian immunodeficiency virus (SIV)/macaque model, as model for HIV, and the HSV/guinea pig model, as model for genital herpes (14, 24). Despite their valuable contributions to the field, these animal models may not accurately reflect the infection of humans at mucosal surfaces. To circumvent this problem, the development of alternative homologous *in vivo*-related models such as tissue-derived organ cultures to model STI is crucial. Unlike the elaborately used cell lines, these models constitute a 3D environment and maintain the natural tissue architecture and microenvironment (27, 28).

In the current study, we optimized a human genital mucosa explant model for future use in studies of pathogenic host entry mechanisms in the female genital tract of humans. Special emphasis was put on maintenance of tissue morphology as well as tissue viability during *in vitro* culture up to 96h: (i) epithelial thickness, *lamina reticularis* thickness and connective tissue composition were evaluated using light microscopy; (ii) epithelial integrity and basement membrane continuity were examined on conservation by means of transmission electron microscopy; (iii) occurrence of apoptosis was monitored by TUNEL-assay.

3.10. Materials and Methods

3.10.1. Patients

Pieces of healthy genital mucosa were gathered from women undergoing a routine hysterectomy (Ghent University Hospital). From all included patients, serum was collected. First, a progesterone and estradiol determination was performed. All persons provided written informed consent and the ethics committee of the Ghent University Hospital approved the study (EC/2010/152). Four different patients of various stages of the menstrual cycle were included for analyzing potential changes in tissue viability and morphology due to cultivation.

3.10.2. Human genital mucosa model set up

A similar set up was used as for bovine respiratory and genital mucosal explants (27). Briefly, tissues were immediately placed in phosphate buffered saline (PBS), supplemented with 1 µg/mL gentamycin (Invitrogen, Paisley, UK), 1 mg/mL streptomycin (Certa, Braine l'Alleud, Belgium), 1 mg/mL kanamycin (Sigma, St. Louis, MO, USA), 1000 U/mL penicillin (Continental Pharma, Puurs, Belgium) and 5 µg/mL fungizone (Bristol-Myers Squibb, New York, USA) for transportation to the laboratory. Genital mucosa was stripped from the underlying layers. Small square tissue pieces were made and placed on fine-meshed gauze for culture. The explants were cultivated in serum-free medium (50% DMEM (Invitrogen)/50% Ham's F-12 GlutaMAX (Invitrogen)) supplemented with 0.1 mg/mL streptomycin (Certa), 100 U/mL penicillin (Continental Pharma) and 1 µg/mL gentamycin (Invitrogen) for up to 96h (37 °C, 5% CO_2).

3.10.3. Tissue viability and morphology analysis during cultivation

An analogous genital mucosa explant model was recently established in our laboratory for bovines. We applied similar techniques including TUNEL-assay, light and transmission electron microscopy for evaluating tissue viability/morphology at different time points of *in vitro* cultivation and are described in section 3.3 of this thesis (27).

3.10.4. Statistical analysis

SPSS software (one way ANOVA, Post-hoc Bonferroni and Tukey's HSD) was used to evaluate the variance. The results are given as means + standard deviation of different (4) independent experiments. Results with *P* values of ≤ 0.05 were considered significant.

3.11. Results

3.11.1. Patients

The 4 donor patients included in this study consisted of a 72 year old woman in post menopause (estradiol: 14.5 pg/mL; progesterone: 0.32 ng/mL), a 65-year old postmenopausal woman (estradiol: <12.0 pg/mL; progesterone 0.36 ng/mL), a 37-year old woman in the luteal phase (estradiol: 102 pg/mL, progesterone: 3.6 ng/mL) and a 45-year old woman in the follicular phase (estradiol: 36.2 pg/mL; progesterone: 1.1 ng/mL).

3.11.2. Viability analysis during tissue *in vitro* culture

Values of the percentage epithelium and connective tissue TUNEL positive cells at the different time points for the different tissues are given in Table 1.

Table 1. Occurrence of TUNEL-positive cells. The TUNEL assay was used as a parameter for the effect of in vitro culture on the viability of human mucosa explants. Values are given as means ± SD of 4 different experiments.

		% TUNEL-positive cells at … h of cultivation				
		0	24	48	72	96
Ectocervix	Epithelium	0.0 ± 0.0	0.1 ± 0.1	0.7 ± 0.8	1.1 ± 1.1	1.3 ± 0.6
	Lamina propria	0.2 ± 0.3	1.1 ± 1.1	2.7 ± 2.7	3.5 ± 2.3	1.7 ± 0.5
Endocervix	Epithelium	0.1 ± 0.1	0.2 ± 0.2	0.4 ± 0.4	0.2 ± 0.4	0.7 ± 1.0
	Lamina propria	0.3 ± 0.3	0.8 ± 0.9	1.4 ± 1.1	1.2 ± 1.0	2.0 ± 0.6

No significant changes were observed in the occurrence of apoptosis, not within the epithelium nor in the lamina propria, during the in vitro cultivation of endocervical and ectocervical mucosa up to 96h (end of the experiment).

3.11.3. Morphology analysis during tissue *in vitro* culture

Light microscopy

Endocervix was found to be lined by a simple columnar epithelium interspersed by mucus-producing cells. Measured values of epithelial thickness ranged from 12.9 ± 0.6 µm at 0h to 12.0 ± 0.5 µm at 96h of cultivation (Figure 1). No differences could be found in between patients due to differences in menstrual cycle stage. No significant changes could be observed due to cultivation of explanted tissues. Ectocervical epithelium consisted of a nonkeratinized stratified squamous epithelium, similar as the vaginal epithelium. At 0h, the epithelial thickness was found to be 194.7 ± 87.0 µm. Measurements performed at 96h of cultivation resulted in an average value of 129.0 ± 106.6 µm (Figure 1). No significant changes in epithelial thickness due to *in vitro* culture were found. However, a substantial SD value was noticed which may be due to the different menstrual cycle stages of the donors. The smallest values were observed in tissues derived from postmenopausal women. Starting from 48h of culture we did see in some explants loss of the luminal layers of the epithelium, mainly *stratum superficiale* and less *stratum spinosum*.

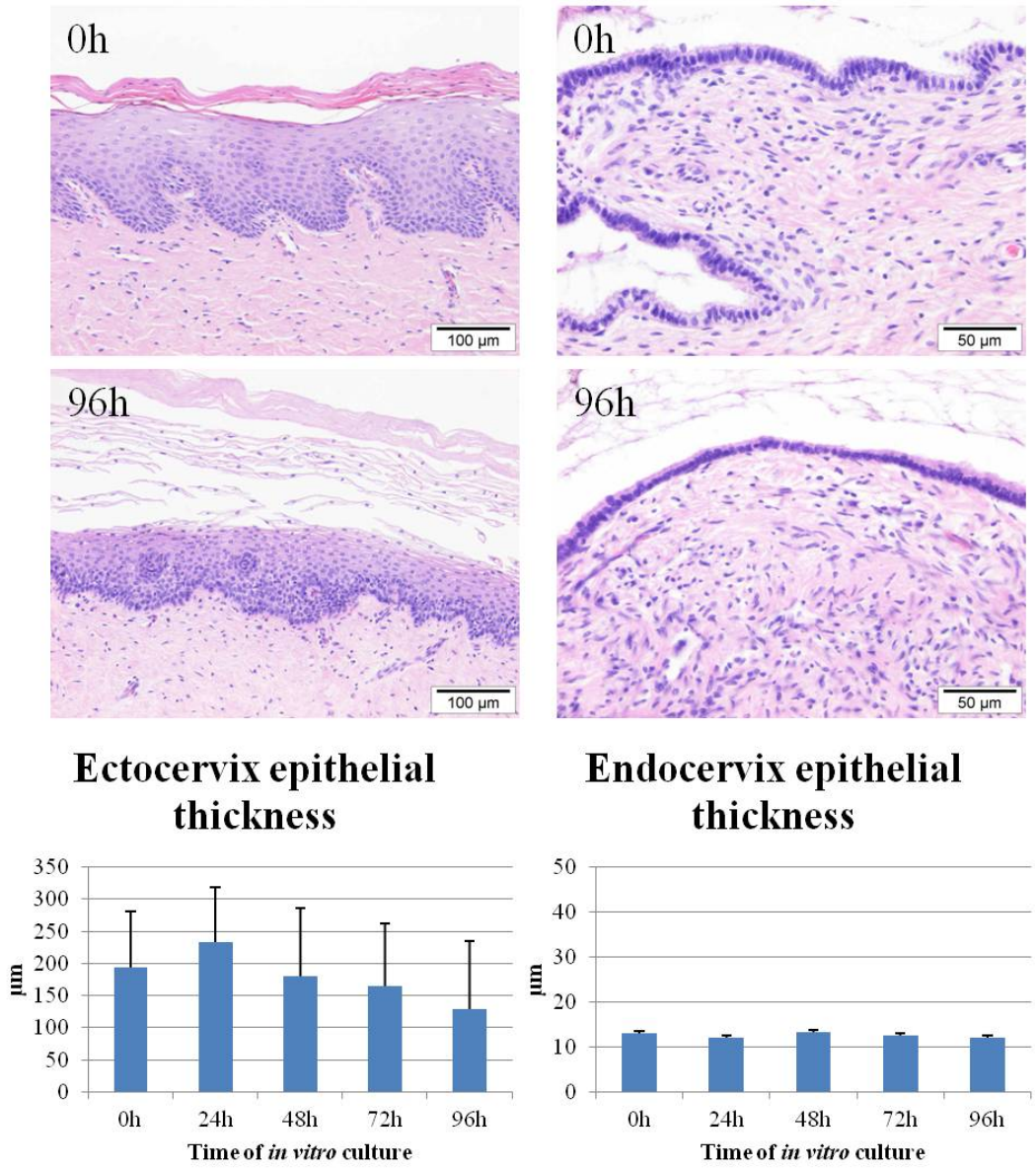

Figure 1. HE staining light photomicrographs of human ectocervical (left) and endocervical (right) mucosa explants at 0h and 96h of in vitro culture. The measurements of epithelial thickness at various time points of tissue cultivation are given as means + SD within the graphs.

Analysis of the composition of the connective tissue revealed no significant differences in percentage of nuclei or collagen due to cultivation. The connective tissue of the endocervix was found to be more cell dense compared to the ectocervix (Figure 2).

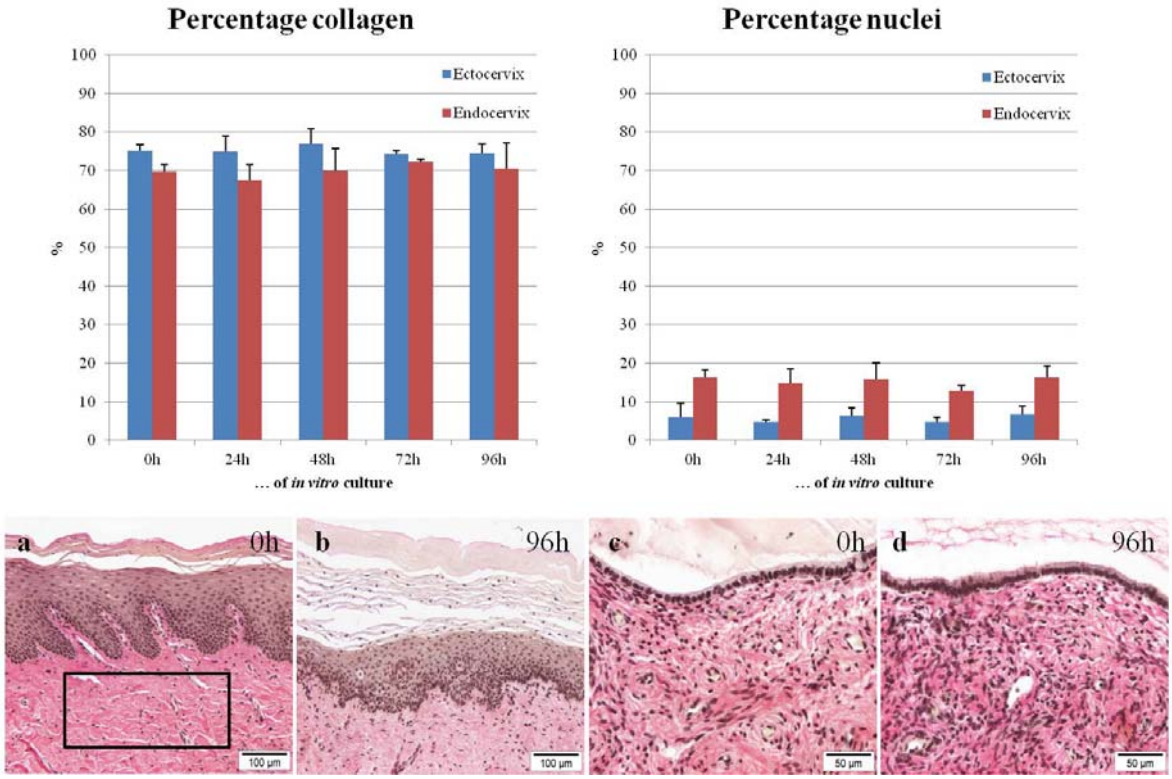

Figure 2. A Van Gieson staining to determine relative amounts of collagen and nuclei within a region of interest (black rectangle) was performed at different time points of cultivation. Values are given within the graphs as means of 4 independent experiments + SD. Pictures of ectocervical mucosa (a,b) and endocervical mucosa (c,d) at 0h and 96h of cultivation respectively are given underneath the graphs.

The *lamina reticularis* thickness of the endocervix and ectocervix could be maintained throughout tissue culture. No significant differences were observed in between time points. Interestingly, we noticed a more profound thickness of the *lamina reticularis* in ectocervical mucosa compared to endocervical mucosa (Figure 3).

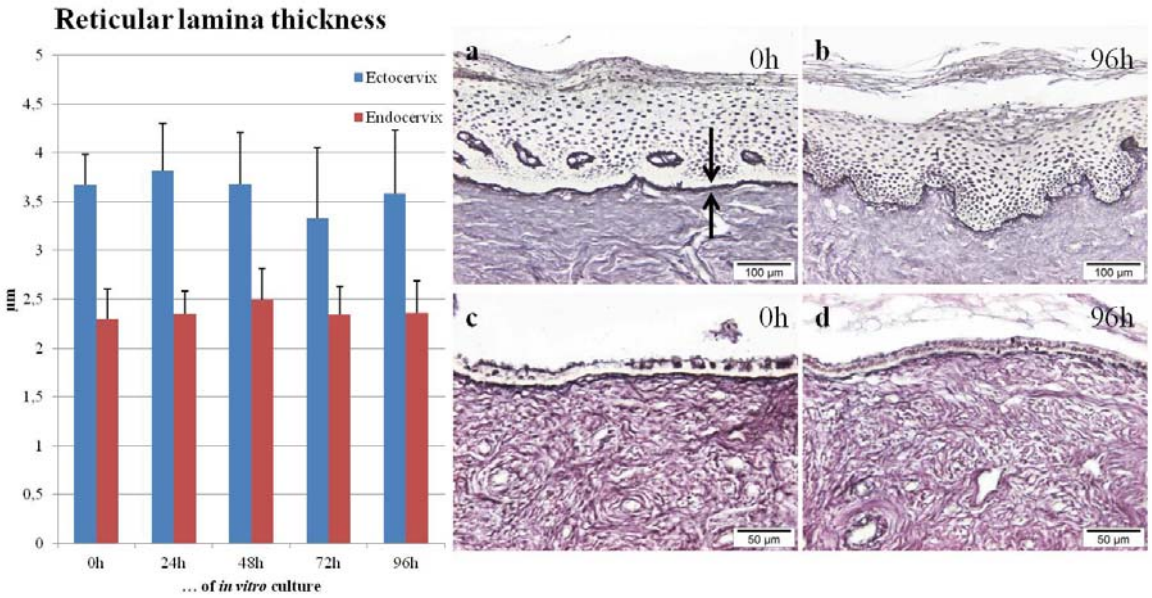

Figure 3. A reticulin staining was performed to determine lamina reticularis thickness (black arrows) in human genital mucosa explants. Ectocervical mucosa (a and b) and endocervical mucosa (c and d), both at 0h and 96h of cultivation respectively. The graph shows the measured values of lamina reticularis thickness at different time points of in vitro culture + SD (5 random measurements in 5 random zones at all time points from 4 independent tissues).

Transmission electron microscopy

Epithelial integrity (Figure 4) and basement membrane continuity (*lamina densa*) (Figure 5) were found to be conserved at all time points for both endocervical and ectocervical tissues, as shown by the representative pictures at 0h and 96h of cultivation.

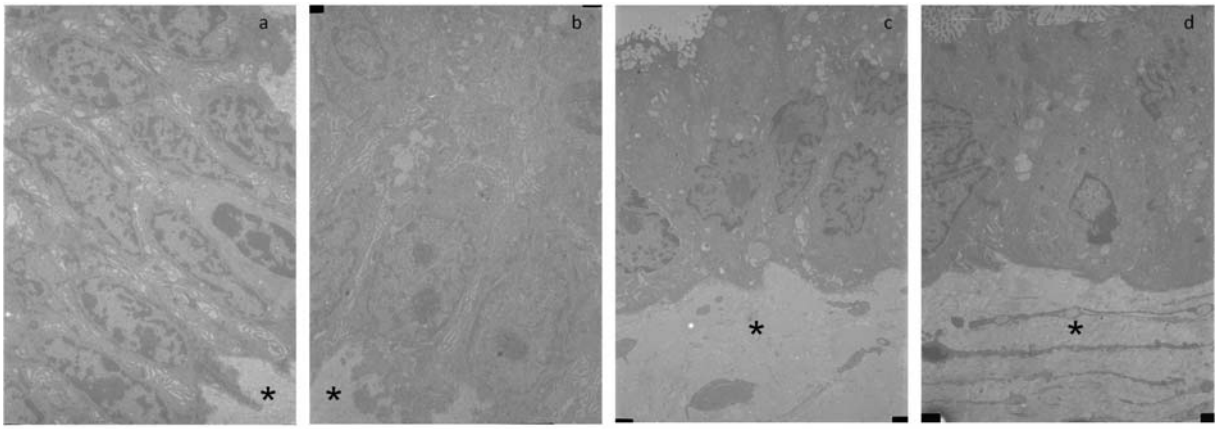

Figure 4. Representative photomicrographs (TEM) of ectocervical basal epithelium (a,b) and endocervical epithelium (c,d) at 0h and 96h of cultivation respectively. The location of the connective tissue is marked by an asterix. (4000 x)

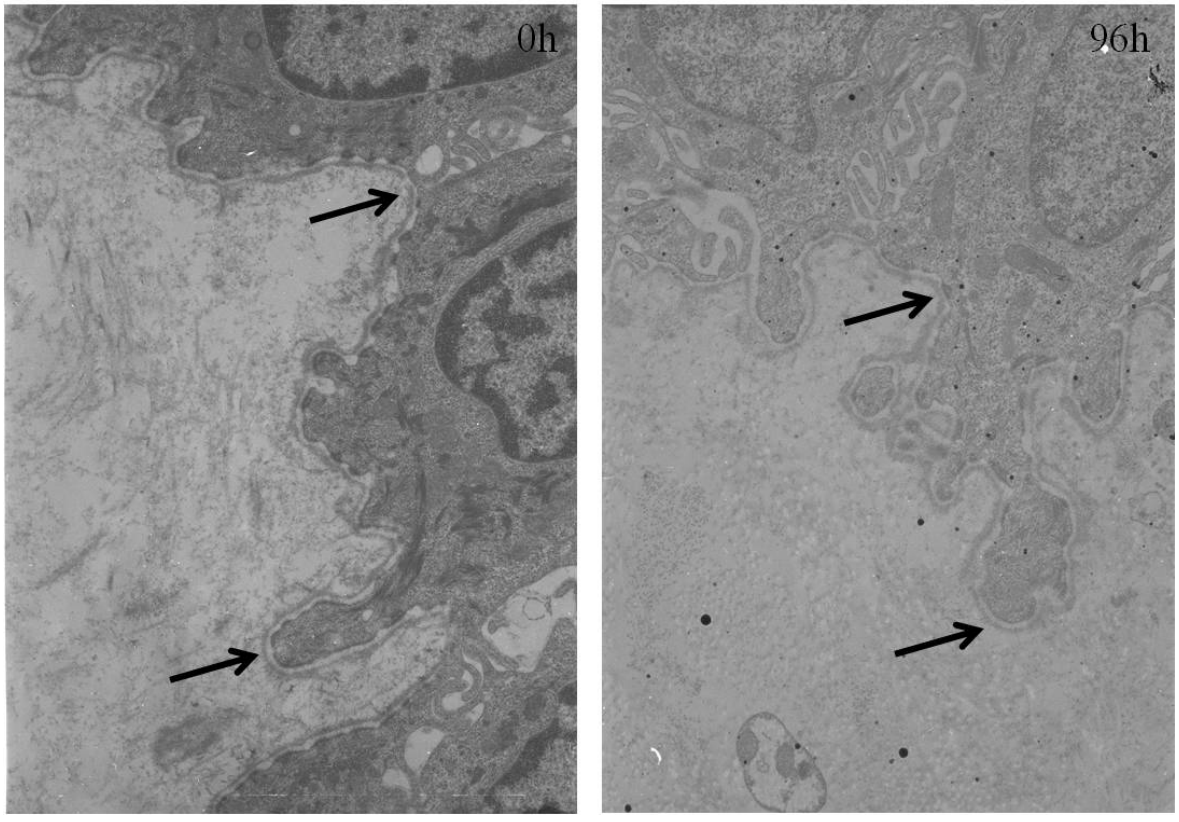

Figure 5. Representative TEM-images of the lamina densa (black arrows) in endocervical mucosa at 0h and 96h of cultivation. (12 000 x)

3.12. Discussion

Here we have described a newly developed and optimized organ culture model, derived from both squamous ectocervical and columnar endocervical tissues, for future host-pathogen studies in the female genital tract of humans. As this model uses tissues rather than a monolayer of cells (primary or transformed), it provides the natural *in vivo* tissue architecture, including epithelium, lamina propria, submucosa and immune cells. Alternative *in vivo* models to study genital infections in humans, such as different rodent models for genital HSV research and the SIV/macaque model for HIV research, have proven their usefulness (8, 14, 24). However, small differences in cellular mediators of infection and invasion, which are often species-specific, exist and may generate inaccurate data when using these models. Different studies point out that extrapolations in between species must be carefully considered. First, one *in vivo* comparative study performed by Linehan and co-workers describes the expression pattern of nectin-1, an HSV entry receptor, in the human and mouse vagina. Interestingly, little extrapolation can be made from one to another. In humans, nectin-1 expression and localization remains identical at the different stages of the menstrual cycle whereas in mice striking differences in expression levels were seen during estrous compared to diestrus (17). Second, although nectin homologues in mice resemble the human forms, mouse nectin-2 is not functional as entry receptor for HSV-1 and HSV-2 whereas human nectin-2 clearly mediates entry of HSV-2 and certain HSV-1 recombinants (30).

In order to avoid such bias, we optimized in the present study homologous *in vivo*-related *in vitro* models for elucidating the host entry mechanisms of genital pathogens in the mucosa. In humans, genital organ cultures derived from ectocervical tissues (3, 4, 7, 9, 18) and endocervical (18, 20, 29) tissues have been described before. Collins and co-workers document the establishment and cultivation of an ectocervical organ culture for a duration of 6 days. Preservation of structure was assessed by means of light microscopy and preservation of function was tested by checking expression levels of several immune cell and epithelial cell markers as well as a cell proliferation marker by means of immunofluorescence microscopy at different time points of *in vitro* culture (3). In addition, trans-epithelial leakage of blue dextran was performed at the last day of culture to check for epithelial functional integrity (10). Endocervix could also be maintained in *in vitro* culture for up to 6 days. As a parameter for functionality, mucus production was checked daily and could still be observed at day 6 of cultivation (18). In most of these studies, no clear information on preservation of basement membrane integrity and connective tissue composition of the cultured tissues is provided.

Given the fact that some microorganisms preferentially adhere to extracellular matrix proteins within the basement membrane and connective tissue, evaluating the preservation of these layers during *in vitro* conditions will be an added value when aiming for pathogen-mucosa interaction studies (26).

Hence, when optimizing the human ectocervical and endocervical mucosa model in this study, special emphasis was put on preservation of viability and morphology of the epithelium, basement membrane and connective tissue during *in vitro* cultivation. Viability analysis of the tissues at different time points of cultivation showed no significant changes in the occurrence of apoptotic cells. Morphological analysis revealed a few interesting facts. First, HE-stainings showed that for ectocervical explants a substantial SD value was noticed which may be due to the different menstrual cycle stages of the donors. The smallest values were observed in tissues derived from postmenopausal women. This is in line with previously published results (23, 25). Starting from 48h of culture we did see in some explants loss of the luminal layers of the epithelium, mainly *stratum superficiale* and less *stratum spinosum*. However, the basal layer stayed intact for the entire experiment. Ectocervical epithelium is accepted to be non-polarized in its outer layers. Intercellular bridges are either weak or non-existing and can explain the easy detachment in *in vitro* culture (24). Collins and co-workers also observed loss of the ectocervical superficial layers starting from 48h of *in vitro* culture. They mention that the basal layers remained intact at all times and that the epithelium started to regenerate after 6 days of *in vitro* culture (3). Second, the *lamina reticularis* was found to be thicker in ectocervical mucosa compared to endocervical mucosa. *The lamina reticularis* thickness is quite tissue-specific and differences may be observed (5). Third, we noticed a more cell-dense connective tissue for endocervical mucosa compared to ectocervical mucosa. Endocervix is known to be anatomically restrained because of its firm attachment mediated by the connective tissue to surrounding muscle fibers (13). In conclusion, we can state that we have set up, optimized and fully characterized both a human genital ectocervical and endocervical mucosa explant model.

3.13. Acknowledgements

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3.14. References

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CHAPTER 4.

BoHV-1 MUCOSAL DISSEMINATION AND INVASION AT HOST ENTRY PORTS

A. Kinetics of BoHV-1 dissemination in an in vitro culture of bovine upper respiratory tract mucosa explants

Adapted from

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ILAR Journal (2012) 53(1).

4.1. Abstract

Bovine herpesvirus 1 (BoHV-1) is a well-known disease-causing agent in cattle. There is little known detailed information on viral behavior with emphasis on host invasion at primary replication sites such as the mucosa of the upper respiratory tract. We previously optimized a bovine upper respiratory tract mucosa explant model and showed that tissues were maintained in culture for up to 96 hours without any significant changes in morphometry and viability. In this study, bovine upper respiratory tract mucosa explants were infected with BoHV-1 (Cooper) and collected at 0, 24, 48, and 72 hours post inoculation (pi). Using a quantitative analysis system to measure plaque latitude and invasion depth, we assessed dissemination characteristics in relation to elapsed time pi and found a plaquewise spread of BoHV-1 across the basement membrane as early as 24h pi, similar to pseudorabies virus (PRV). Moreover, we observed that BoHV-1 exhibited an increased capacity to invade in proximal tracheal tissues compared to tissues of the deeper part of the nasal septum and ventral conchae. Revealing a more distinct invasion of BoHV-1 in proximal trachea, we can conclude that, in order to study an important aspect of BoHV-1 pathogenesis, the bovine upper respiratory tract mucosa explant model is the best suited model.

4.2. Introduction

The revised family *Herpesviridae* incorporates pathogenic members that can cause disease in mammals, birds, and reptiles. This family is the most important out of three families in the new order of the *Herpesvirales* (1). During evolution, these viruses developed different mechanisms to (i) penetrate different mucosal barriers and to reach internal organs via leukocytes and nerve endings (invasion), (ii) evade both specific and aspecific immunity (immune-evasion), and (iii) hide in the infected host (latency) (7-9). Many viruses from the *Alphaherpesvirinae* subfamily use the epithelium of the upper respiratory tract as an important mucosal portal of entry. In contrast to other respiratory viruses, these viruses can penetrate through the basement membrane (BM) after local dissemination. If during this mucosal invasion nerve endings or blood vessels are reached, these viruses can spread in the host, resulting in neuronal symptoms and viremia (14, 15). Bovine herpesvirus 1 (BoHV-1) is an important pathogen of cattle and can cause two major clinical entities: infectious bovine rhinotracheitis and infectious pustular vulvovaginitis/balanoposthitis (2). BoHV subtype 1.1 (infectious bovine rhinotracheitis) spreads in the respiratory mucosa which leads to extensive

tissue destruction with ulceration in the upper respiratory tract. However, subtype 1.2 (infectious pustular vulvovaginitis/balanoposthitis) replicates at the peripheral genital tract and is associated with pustular lesions. From the primary site of replication, the virus will gain access to local sensory neurons, reach corresponding ganglia, and establish lifelong latency. Following viremia, abortion in cows and fatal systemic infections in young calves may occur (13). How BoHV-1, as well as many other mammalian alphaherpesviruses, can penetrate so easily through the mucosal layer, despite the presence of barriers including the BM, is still unknown. Little is known about the mechanisms of replication and invasion in the respiratory tract for BoHV-1. We previously optimized a bovine upper respiratory tract *in vitro* explant model. The aim of the present study was to study the BoHV-1 dissemination kinetics in the upper respiratory mucosa, including nasal septum, ventral conchae and proximal trachea by applying the established *in vitro* model.

4.3. Materials and methods

4.3.1. Experimental design of a respiratory mucosa explant in *in vitro* culture

Bovine respiratory tissue was obtained at a slaughterhouse and the bovine respiratory mucosa explant model was set up as previously described. In brief, samples were taken from four calves aged 6 months. The head was cut longitudinal into two pieces, exposing the nasal septum. We cautiously removed nasal septum from the caudal two thirds of the nasal cavity (septum), ventral conchae (conchae), and proximal trachea (trachea). Tissues were transported to the laboratory on ice in phosphate buffered saline (PBS), supplemented with 1 µg/mL gentamycin (Gibco), 1 mg/mL streptomycin (Certa), 1 mg/mL kanamycin (Sigma), 1000 U/mL penicillin (Continental Pharma), and 5 µg/mL fungizone (Bristol-Myers Squibb). We collected blood to perform a complement-dependent seroneutralization (SN)-test to determine BoHV-1 specific antibody titers as an animal exclusion criterion. Using surgical blades (Swann-Morton), respiratory mucosa was carefully stripped from the underlying layers. Mucosal explants covering a total area of 0.7 cm^2 were produced and placed in six well culture plates (Nunc) epithelium upwards on fine meshed gauze for culture. We added serum-free culture medium (50% DMEM (Gibco)/50% Ham's F-12 GlutaMAX (Gibco)) supplemented with 0.3 mg/mL glutamine (BDM Biochemical), 0.1 mg/mL streptomycin (Certa), 100 U/mL penicillin (Continental Pharma)), and 1 µg/mL gentamycin (Gibco) until

achieving an air-liquid interface. The explants were cultivated for up to 96h at 37°C and 5% CO_2.

4.3.2. Virus inoculation and assessment of dissemination kinetics

Inoculation of the explants with BoHV-1 Cooper strain (Colorado) took place after 24h of cultivation (18). Explants were taken from their gauze and placed epithelium upwards in a 24-well plate (Nunc). After double washing with warm medium, 1 mL medium containing 10^7 $TCID_{50}$/mL virus was added in each well thereby submerging the explant. After incubation for 1 hour at 37°C and 5% CO_2, the explants were washed three times with warm medium and placed back on the gauze.

To monitor kinetics of viral dissemination, explants were gathered at different time points post inoculation (pi). After collection, samples were carefully embedded in methocel (Fluka) and frozen at -70°C. Cryosections were made, fixed in methanol (-20°C, 100%), and kept at -20°C until staining. For the evaluation of penetration through the BM, the BM in BoHV-1 infected explants was stained. Mouse anti collagen VII antibodies (Sigma), directed against anchoring fibrils residing in the BM, and goat anti-mouse Texas Red antibodies (Molecular Probes) were used in a first step to mark the BM barrier. Secondly, a FITC -labeled goat anti-IBR polyclonal antiserum (VMRD) staining was performed to visualize viral proteins. Mounted samples were analyzed using a confocal microscope (Leica TCS SP2 confocal microscope). Thereafter, plaque latitude and invasion depth (distance underneath the BM) were evaluated using the line-tool function of the software program ImageJ. Finally, at 24 and 48h pi, the average number of plaques was measured in the entire evaluated surface of explants derived from either septum, conchae, or trachea.

4.3.3. Statistical analysis

Analysis of variance was performed on the obtained data using SPSS software (ANOVA). The results represent means + standard deviation of quadruple independent experiments performed for the analysis of viral dissemination. Data with P values of ≤ 0.05 were considered significant.

4.4. Results

4.4.1. Evaluation of primary viral dissemination

All four animals showed an SN-titer of < 2 for BoHV-1 specific antibodies and were therefore selected for this study. We observed clear distinct infected cells at 24h, 48h, and 72h pi. BoHV-1 was found to spread in a plaquewise manner in respiratory mucosa (Figure 1).

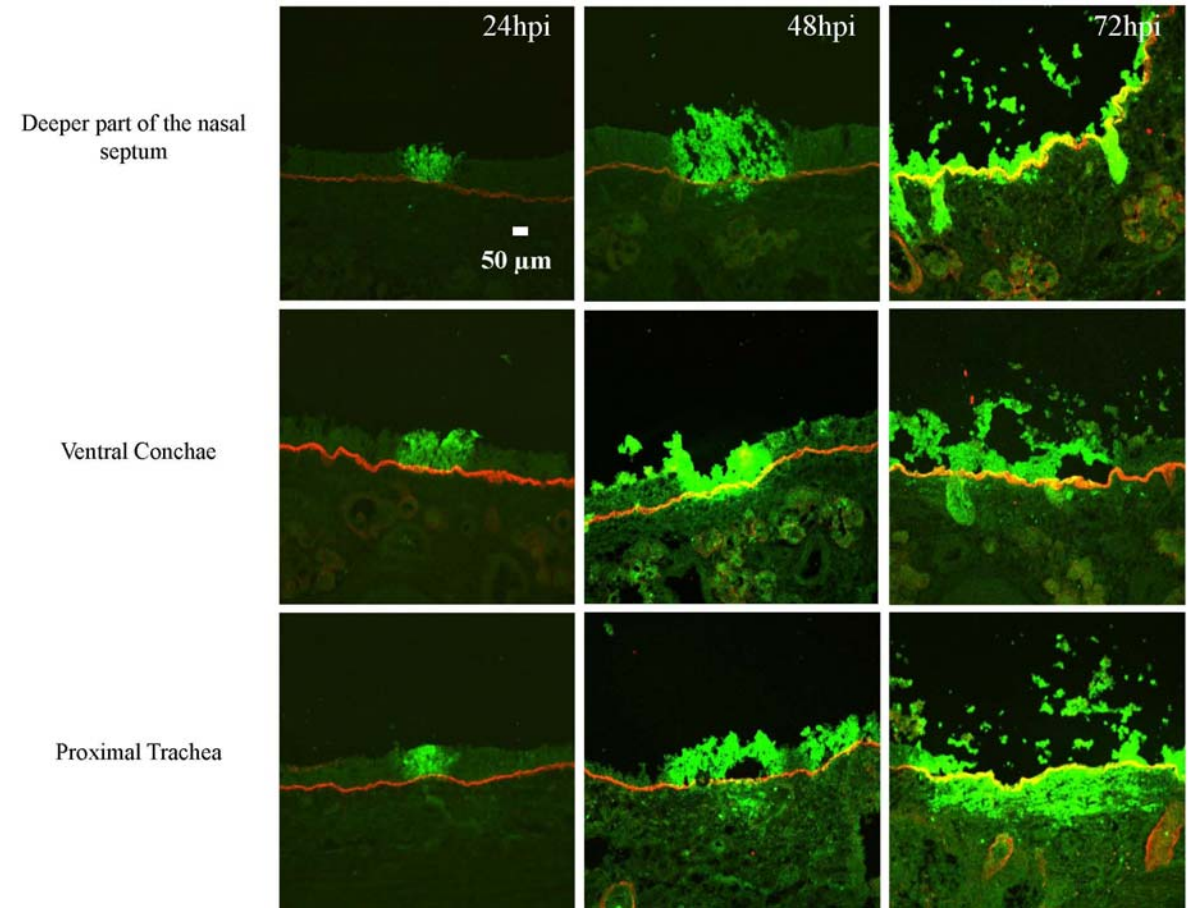

Figure 1. Fluorescent images taken by means of confocal microscopy from different upper respiratory tissues infected with BoHV-1 Cooper strain at 24, 48 and 72h pi. Viral antigen was stained using a FITC®-labeled goat anti-IBR polyclonal antiserum (VMRD). To visualize collagen VII, mouse anti collagen VII antibodies (Sigma) and goat anti-mouse Texas Red® antibodies (Molecular Probes) were used.

Starting at 48h pi and more pronounced at 72h pi, infected epithelial cells loosened and detached from the viral plaque. Both plaque latitude and invasion depth in septum, conchae, and trachea were evaluated at different time points pi.

Plaque latitude

No plaques were visible at 0h pi for all tissues. Individual plaques were measured at 24h and 48h pi Since almost all present epithelial cells in the bovine upper respiratory tract (bURT) mucosa explants were infected, we were not able to measure individual plaques at 72h pi At 24h pi, average plaque latitudes of 142.5 ± 67.4 µm in septum, 170.6 ± 32.4 µm in conchae, and 168.3 ± 43.4 µm in trachea were observed. Plaque latitude increased over time and at 48h pi, we measured average plaque latitudes of 275.5 ± 55.0 µm in septum, 312.3 ± 14.4 µm in conchae, and 317.9 ± 12.1 µm in trachea (Figure 2a). Plaque latitude rose significantly between 0h, 24h, and 48h pi. However, no major changes concerning the latitude were seen when comparing septum, conchae, and trachea.

Plaque depth

Plaque depth underneath the BM was evaluated at 0h, 24h, 48h, and 72h pi. In septum average plaque depths of 0.0 ± 0.0 µm at 0h pi, 1.3 ± 2.2 µm at 24h pi, 14.1 ± 9.0 µm at 48h pi, and 34.6 ± 13.1 µm at 72h pi were observed. Average plaque depths in conchae ranged from 0.0 ± 0.0 to 2.8 ± 2.1 µm, 25.9 ± 7.0 µm and 36.8 ± 11.6 µm at 0h, 24h, 48h, and 72h pi, respectively. When analyzing average plaque depth in trachea, values starting from 0.0 ± 0.0 µm at 0h pi to 7.4 ± 5.4 µm at 24h pi, 50.7 ± 7.3 µm at 48h pi, and 64.7 ± 16.2 µm at 72h pi were observed (Figure 2b). There was a significant increase in plaque depth between 24h, 48h, and 72h pi for all tissues. Except in tracheal tissues, between 48h and 72h pi there was no significant change in plaque depth underneath the BM. There was a clear significant difference when evaluating plaque invasion depth underneath the BM in trachea compared with that in septum (at 24h, 48h, and 72h pi) and conchae (at 48h and 72h pi) (Figure 1).

Plaque number

We did not see any significant changes in plaque number between the deeper part of the nasal septum, ventral conchae, and proximal trachea at 24h and 48h pi. Results of the average number of plaques are given per 5 mm^2. At 24h pi, an average plaque number of 21.2 ± 14.8, 42.3 ± 27.2, and 25.0 ± 0.8 was counted for septum, conchae, and septum. There was an average amount of 31.0 ± 5.6 plaques in septum, 24.5 ± 10.5 plaques in conchae, and 25.0 ± 12.3 plaques in trachea at 48h pi. Moreover, no major differences in average plaque number were noticed when comparing time points 24h and 48h pi for septum, conchae, and trachea.

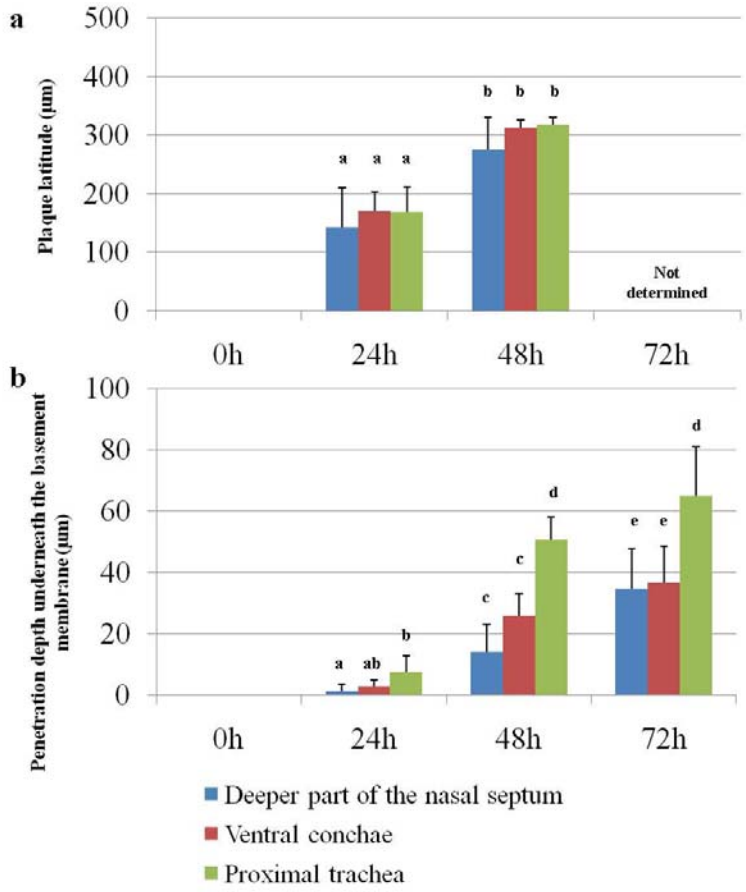

Figure 2. Evolution of BoHV-1 (a) plaque latitude and (b) plaque penetration depth underneath the basement membrane at 0, 24, 48h pi and 0, 24, 48, 72h pi, respectively. Data are given as means + SD (error bars). Significant differences are pointed out by the use of different letters (another letter means a statistical difference). Data with P values ≤ 0.05 were considered significant.

4.5. Discussion

Little is known about the primary replication and dissemination of different alphaherpesviruses at host mucosal entry ports. Nevertheless, getting a fundamental image on how the virus behaves at its primary replication site can provide insights for prevention and treatment on a rational basis. In the present study, a bURT mucosa explant model was set up to study BoHV-1 dissemination characteristics in bovine respiratory mucosa. The use of respiratory organ cultures already proved to be of importance in different scientific areas. Insights in drug transportation, metabolic pathways, and pathogen-host interactions are attained achievements based on organ culture arrays. The explant model is the perfect compromise between *in vitro* cell cultures and *in vivo* laboratory animals. In cell cultures, no

tissue structure is present and therefore essential cell-cell and cell-extracellular matrix contacts are lost. Current *in vivo* model systems often used in alphaherpesvirus studies mostly make use of rodents. However, these models create a heterologous situation where species specific cellular components, which may play a role in viral invasion, are absent.

When comparing dissemination kinetics of BoHV-1 in septum, conchae, and trachea, we made some interesting findings. There was a significantly higher BoHV-1 invasion depth in tracheal tissues when compared to that in tissues of septum and conchae at 48h pi as well as 72h pi. The reason for this difference may be the significantly smaller thickness of the *lamina reticularis* in trachea compared to septum and conchae, suggesting that the virus has to cross a thinner BM barrier in the trachea. Although speculative, an alternative hypothesis can be put forward to explain the rapid and efficient invasion in tracheal tissues. Evans and colleagues reported an attenuated fibroblast sheath located just underneath the BM zone (4). This sheath consists of large flat fibroblasts covering up to 70% of the BM zone and makes contact with the basal lamina. These contacts are mainly situated underneath basal cells, which form the contact between epithelium and BM (3, 6). Attenuated fibroblasts have only been described so far in trachea, intrapulmonary bronchi, and terminal bronchioles in different species (4, 5, 19). Alphaherpesviruses are known for their efficient cell-to-cell spread (14, 15). Since epithelial basal cells and attenuated fibroblast are in close proximity to each other, combined with the observed smaller reticular lamina thickness, this could explain why invasion in trachea is more efficient than in conchae or septum. These findings put emphasis on the involvement of the trachea in the clinical picture (IBR) caused by BoHV-1 and are correlated with the *in vivo* situation (14).

Generally, we set up mucosa explant models of bovine, porcine, and equine in our laboratory to study aspects of viral mucosal invasion. BoHV-1 and pseudorabies virus (PRV) both exhibit a plaquewise spread across the BM in the *in vitro* mucosa explant model suggesting they have minor effort in passing this host defense line (10, 11). This is correlated with the severe upper respiratory tract *in vivo* symptoms of both viruses. On the contrary, equid herpesvirus 1 (EHV-1) spreads only laterally and does not pass the BM. EHV-1 developed another system to invade. It misuses local immune cells as carriers to penetrate the host (12, 16, 17). The attenuated EHV-1 replication characteristics in respiratory mucosa correlate with the mild *in vivo* respiratory symptoms. Moreover, in striking contrast with what was observed for BoHV-1, EHV-1 dissemination characteristics in tracheal tissue did not differ at all from those in other respiratory tissues (17)(A. P. Vandekerckhove, Ghent University, personal

communication). These findings confirm the *in vivo* relevance of the mucosa explant *in vitro* model. During evolution, what is the reason different mechanisms of viral invasion occurred between different alphaherpesviruses? Is this inherent to the species and merely an adaptation of the virus to its host? Or did some strains become more virulent by acquiring advantageous genetic material over time? Further studies using the respiratory mucosal explants may provide answers to these questions.

Conclusion

We conclude that *in vitro* cultured upper respiratory tract mucosal explants are susceptible to BoHV-1 infection and therefore a good alternative for experiments on living animals (the three Rs of Russell and Burch). BoHV-1 was found to spread in a plaquewise manner when investigating the evolution of plaque formation at different time points pi in bURT mucosa explants. The crossing of the BM started from 24h pi onwards and all plaques crossed the BM at 48h pi. Furthermore, BoHV-1 invaded more efficiently in depth across the BM in proximal tracheal tissue. Therefore, the proximal trachea explant model seems to be the ideal tissue model for further investigations of the BoHV-1 stromal invasion mechanism through the BM.

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B. Comparative analysis of replication characteristics of BoHV-1 subtypes in bovine respiratory and genital mucosa explants: a phylogenetic enlightenment

Adapted from

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Veterinary Research (2011) 42(33).

4.8. Abstract

In general, members of the *Alphaherpesvirinae* use the epithelium of the upper respiratory and/or genital tract as a preferential site for primary replication. Bovine herpes virus type 1 (BoHV-1) may replicate at both sites and cause two major clinical entities designated infectious bovine rhinotracheitis (IBR) and infectious pustular vulvovaginitis/balanoposthitis (IPV/IPB) in cattle. Subtype 1.1 is hypothesized to invade preferentially the upper respiratory mucosa whereas subtype 1.2 favors replication at the peripheral genital tract. However, some studies are in contrast with that hypothesis. A thorough study of primary replication at both mucosae could elucidate whether or not different BoHV-1 subtypes show differences in mucosa tropism. We established bovine respiratory and genital organ cultures with emphasis on maintenance of tissue morphology and viability during *in vitro* culture. In a next step, bovine respiratory and genital mucosa explants of the same animals were inoculated with either the BoHV-1.1 Cooper, the BoHV-1.1 Lam, the BoHV-1.2a Schönböken or the BoHV-1.2b K22 strain. A quantitative analysis of viral invasion in the mucosa was performed at 0h, 24h, 48h and 72h post inoculation (pi) by measuring plaque latitude and penetration depth underneath the basement membrane. All BoHV-1 subtypes exhibited a more profound invasion capacity in respiratory tissue compared to that in genital tissue at 24h pi. However, at 24h pi plaque latitude was found to be larger in genital tissue compared to respiratory tissue and this for all subtypes. These similar findings among the different subtypes take the edge off the belief of the existence of specific mucosa tropisms of different BoHV-1 subtypes.

4.9. Introduction

Alphaherpesviruses in general have a broad epithelial tropism. BoHV-1, the known etiological agent of infectious bovine rhinotracheitis (IBR) and infectious pustular vulvovaginitis/balanoposthitis (IPV/IPB) in cattle, may replicate in both respiratory and genital mucosa. However, respiratory and genital infections have been assigned to different BoHV-1 strains in the past (24).

Although there is proof of the existence of the virus as early as 1941, BoHV-1 was firstly isolated in 1955 (10, 15). In the late 1950's both the respiratory disease prototype strain Cooper and the genital disease prototype strain K22 were isolated from field cases of respectively IBR and IPV in the United States (14, 33). Importantly, before 1977, a

continental discrepancy was present. Genital infections were predominant throughout Europe whereas respiratory infections were mainly prevalent at feedlots in the United States and Canada. Some authors believe that the virulent respiratory strains emerged out of the less virulent genital strains. They state that the enhanced virulence of the virus for the respiratory epithelium is a consequence of rapid passages of the virus in crowded susceptible populations present in "feedlots", typically for the United States at that time. Since 1977, severe "North American like" IBR emerged on the European continent (12, 18, 27).

An attempt was made to see if BoHV-1 could be subdivided into distinct types with different tropisms i.e. whether a correlation could be found between IBR and IPV on the one hand and distinct virus subtypes on the other hand. Using restriction endonuclease digestion and reactivity tests to a panel of monoclonal antibodies, a classification was made of different BoHV-1 subtypes. BoHV-1.1 was associated with respiratory disease and abortion whereas BoHV-1.2 was regarded as a genital type (17, 19, 22). Subtype BoHV-1.3 was renamed into BoHV-5 as new findings showed significant differences in genomic and antigenic properties between BoHV-1.3 and other BoHV-1 strains (3, 20). Furthermore, a distinction was made between different BoHV-1.2 subtypes. BoHV-1.2b causes local genital lesions and possibly mild respiratory illness; BoHV-1.2a seems to have both tropism for the genital and respiratory mucosa and is associated with abortion (17, 19, 28). However, this postulation about several subtypes possessing diverse mucosa tropisms has been contested since several studies showed no correlation between the different genotypes and their clinical manifestations (1, 4, 12, 16, 18, 23, 28).

A good way to elucidate the relationship between the distinct viral BoHV-1 subtypes and the clinical entities would be to test isolates representing the different subtypes on similar groups of animals under identical conditions. Practical, ethical and financial reasons make this in vivo approach difficult to perform. For that purpose, suitable *in vitro* systems resembling the *in vivo* situation and implementing the three R's principle of Russell and Burch (1959), are needed to study primary host-virus interactions.

The aim of the present study was to evaluate quantitatively the replication characteristics of different BoHV-1 subtypes in *in vitro* systems of bovine respiratory and genital mucosa explants. Previously, we described the optimization of *in vitro* bovine respiratory and genital organ cultures. In this part of the study, bovine organ cultures of trachea and vestibulum vaginae derived from the same animals were infected with several BoHV-1.1, BoHV-1.2a and

BoHV-1.2b isolates. A quantitative analysis of viral mucosal invasion was performed for the different BoHV-1 subtypes and compared at 0h, 24h, 48h and 72h post inoculation (pi) by measuring plaque latitude and penetration depth underneath the basement membrane (BM).

4.10. Materials and methods

4.10.1. Selection of animals

At the slaughterhouse, three different female slaughter animals between 3 and 5 years old were selected. Criteria to include the animals in this experiment were based on both female reproductive hormone level and BoHV-1 specific serological status. At slaughter, a thorough palpation and visual inspection of the ovaries was performed to select cows with a clear marked corpus luteum. Moreover, blood was collected at slaughter. On all sera, a progesterone determination and a complement-dependent seroneutralization (SN)-test were performed to determine peripheral blood progesterone levels and BoHV-1 specific antibody titers respectively.

4.10.2. Gathering of tissues and preparation of air-liquid interface organ cultures

From three different cows, proximal trachea and vestibulum vaginae were collected at the local abattoir immediately after slaughter. A similar set up was used for bovine respiratory and genital mucosal explants. Briefly, tissues were immediately placed in phosphate buffered saline (PBS), supplemented with 1 µg/mL gentamycin (Invitrogen, Paisley, UK), 1 mg/mL streptomycin (Certa, Braine l'Alleud, Belgium), 1 mg/mL kanamycin (Sigma, St. Louis, MO, USA), 1000 U/mL penicillin (Continental Pharma, Puurs, Belgium) and 5 µg/mL fungizone (Bristol- Myers Squibb, New York, USA) for transportation to the laboratory. Respiratory and genital mucosa was stripped from the underlying layers. Small square tissue pieces were made and placed on fine-meshed gauze for culture. The explants were cultivated in serum-free medium (50% DMEM (Invitrogen)/50% Ham's F-12 Gluta-MAX (Invitrogen)) supplemented with 0.1 mg/mL streptomycin (Certa), 100 U/mL penicillin (Continental Pharma) and 1 µg/mL gentamycin (Invitrogen) for up to 96h (37 °C, 5% CO2). Both respiratory and genital tissue of three cows was used to evaluate BoHV-1 subtype replication characteristics.

4.10.3. Inoculation of explants with different BoHV-1 subtypes

Different strains were used, representing the known different genotypes of BoHV-1. The Cooper strain, which is considered as the prototype 1.1 subtype, was isolated in Colorado (33). Another 1.1 strain, Lam, was isolated in 1972 from an IBR-case in the Netherlands (8, 31). The prototype 1.2 strain K22 is typed as a subtype 1.2b (17). This isolate was obtained from an IPV outbreak in New York (14). The Schönböken isolate is designated as a BoHV-1.2a subtype and was isolated in Germany (6, 13). All strains obtained were from unknown passages. From each of them, a second passage was produced and utilized in our laboratory. After 24h of cultivation, explants were inoculated with the different strains. The explants were taken from their gauze and placed in a 24-well plate after rinsing with warm medium. In each well, 1 mL of virus-containing medium (10^7 $TCID_{50}$/mL) was added. The submerged explants were incubated for 1h (37 °C, 5% CO2). Before the tissues were placed back again on their gauze, they were thoroughly washed. In that way, different explants, both proximal trachea and vestibulum vaginae from the same animal were infected with the different strains. Samples were collected at 0h, 24h, 48h and 72h pi. At every time point, 2 respiratory and 2 genital explants from each animal for each strain were collected. Finally, all gathered explants were embedded in a cryoprotection medium (Methocel®, Fluka (Sigma)) and frozen at -70 °C.

4.10.4. Evaluation of BoHV-1 subtype kinetics

From all collected samples, cryosections were produced, fixed in methanol (-20 °C, 100%) and kept at -20 °C until staining. An immunofluorescence staining was performed to evaluate plaque latitude and plaque penetration depth underneath the BM. Firstly, to stain the BM, mouse anti-collagen VII antibodies (Sigma) and goat anti-mouse Texas Red® (Molecular Probes (Invitrogen)) were used. Next, an FITC®-labeled goat anti-IBR polyclonal antiserum (VMRD, Pullman, WA, USA) directed against viral proteins was applied. Mounted samples were analyzed by means of a confocal microscope (Leica TCS SP2 confocal microscope). Using the software program ImageJ, dissemination characteristics were monitored.

4.10.5. Statistical analysis

The data obtained were assessed using SPSS software (ANOVA) to evaluate the variance. The results shown represent means + standard deviation of triple independent experiments for analysis of viral subtype replication characteristics. The results with P values of ≤ 0.05 were considered significant.

4.11. Results

4.11.1. Progesterone determination and Sn-test

All animals included in the experiment had a peripheral blood progesterone (P4) level of > 1 ng/mL, suggesting they were in the luteal phase of the reproductive cycle. Three animals showing an Sn-titer of < 2 for BoHV-1 specific antibodies were selected for the study of BoHV-1 subtype dissemination characteristics.

4.11.2. BoHV-1 subtype kinetics

Individual plaques were visible starting from 24h pi for all strains in all tissues. All different BoHV-1 subtypes were found to spread in a plaquewise manner in both the respiratory and genital mucosa derived from three animals. The analysis of plaque latitude and penetration depth underneath the BM was performed at different time points pi for all BoHV-1 subtypes on proximal trachea and vestibulum vaginae (Figure 1).

Plaque latitude

Individual plaque measurement was performed at 0h, 24h, 48h and 72h pi (Figure 2a).

In relation to time

At 0h pi, no viral plaques were visible. A significant increase in plaque latitude was observed between 0h, 24h, 48h and 72h for all strains on the different tissues.

In relation to tissue

Interestingly, we observed a significant difference in plaque latitude between proximal trachea and vestibulum vaginae at 24h pi. This was noticed for all different BoHV-1 subtypes. Lateral spread was more clear in vestibulum vaginae compared to the proximal trachea. At 48h pi, the Lam and Schönböken strain still exhibited a higher dissemination capacity in the epithelium of the vagina compared to the trachea whereas Cooper and K22 did not. All strains no longer showed a significant difference in plaque latitude when comparing proximal trachea and vestibulum vaginae at 72h pi (Figure 2a).

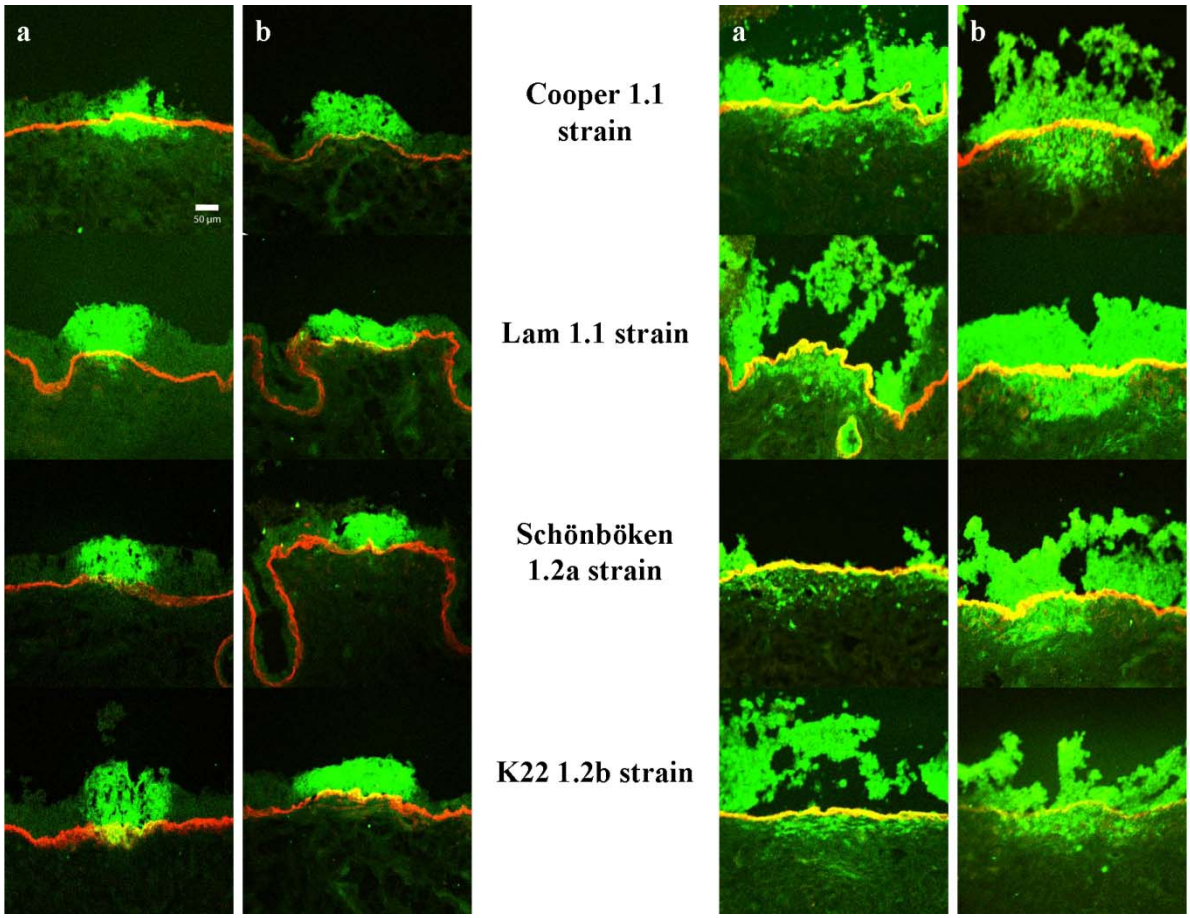

Figure 1. Confocal fluorescent images of bovine respiratory (a) and genital (b) mucosa explants inoculated with different BoHV-1 subtypes at 24h pi (left side) and 72h pi (right side). Viral antigen is colored with an FITC®- labeled goat anti-IBR polyclonal antiserum. Collagen VII is marked with mouse anti-collagen VII and goat anti-mouse Texas Red® antibodies.

In relation to strain

When comparing the strains, no significant differences between the subtypes could be observed at 0h, 24h, 48h and 72h pi. All did exhibit a higher average plaque latitude at 24h pi in vestibulum vaginae compared to proximal trachea (Figures 1-2a).

Plaque penetration depth underneath the BM

The average invasion depth of different BoHV-1 subtypes was measured at 0h, 24h, 48h and 72h pi (Figure 2b).

In relation to time

At 0h pi, no vertical spread was observed for all different BoHV-1 subtypes in both proximal trachea and vestibulum vaginae. All strains included showed an increase in plaque penetration depth between 0h, 24h, 48h and 72h pi in proximal trachea and vestibulum vaginae. This increase was significant for all strains on all tissues between 48h and 72h pi.

In relation to tissue

Both respiratory strains Cooper and Lam showed a clear advantage on proximal trachea compared to the vestibulum vaginae. The average invasion depth was significantly higher in respiratory tissue than in genital tissue and this at 24h (Lam en Cooper) and 48h (Cooper) pi. Interestingly, for both designated genital 1.2-strains Schönböken and K22, we did not see a significant difference when comparing both target tissues. However, we must mention that for both genital strains at 24h pi, not one plaque crossed the BM in the genital tract whereas some plaques did in the respiratory tract. At 48h (except Cooper) and 72h pi, no significant differences were found when comparing all strains on proximal trachea and vestibulum vaginae (Figures 1-2b).

In relation to strain

We observed no significant differences amongst strains at 0h, 24h, 48h and 72h pi. The penetration depth of the Cooper strain in the proximal trachea was significantly higher than the penetration depth of the Schönböken strain in the proximal trachea only at 24h pi.

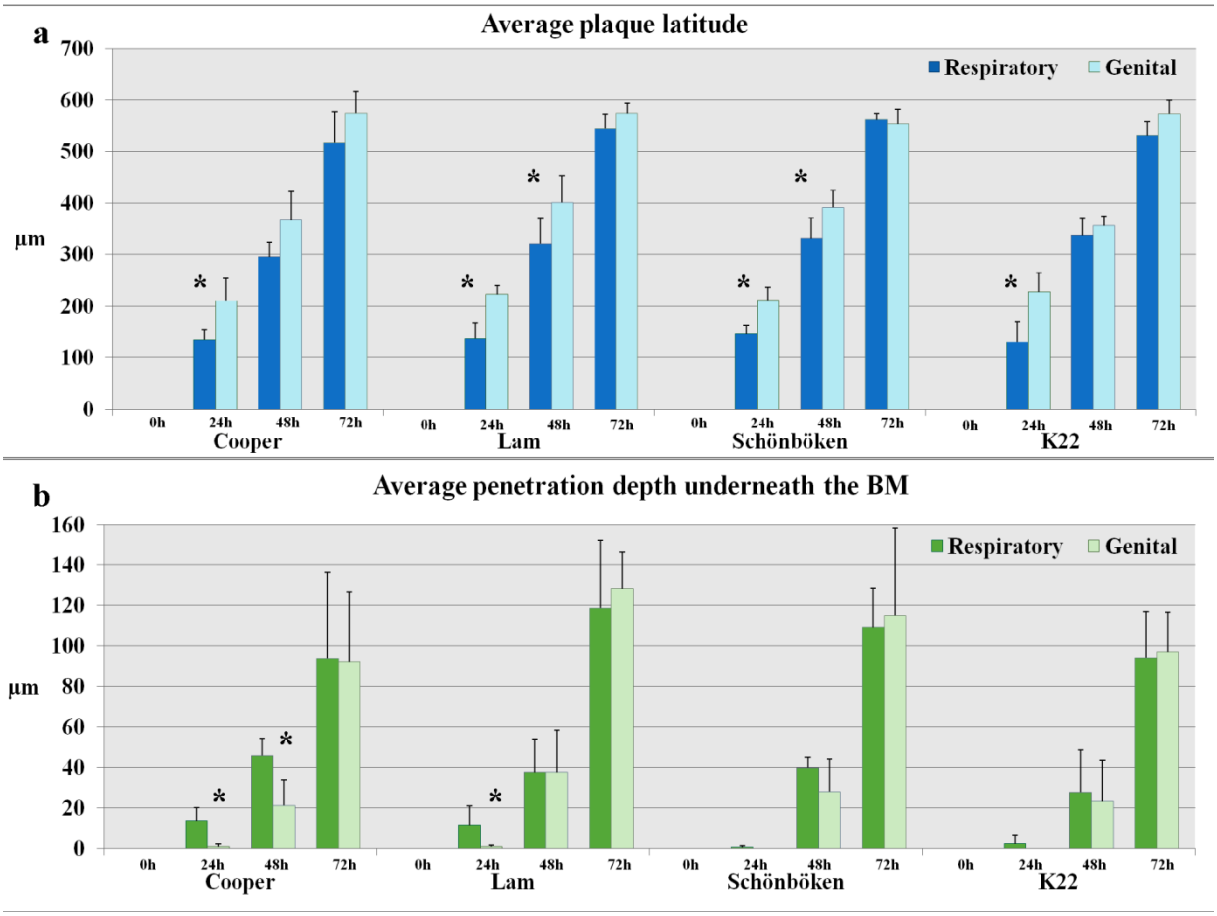

Figure 2. Replication characteristics of different BoHV-1 subtypes in respiratory and genital tissues derived from the same animals. Evolution of plaque latitude (a) and plaque penetration depth underneath the basement membrane (BM) (b) was evaluated at 0h, 24h, 48h and 72h pi. Data are given as means + SD (error bars). Significant differences between respiratory and genital tissue are indicated by means of asterisks.

4.12. Discussion

In this study, we utilized bovine respiratory and genital organ culture systems to reconstruct key elements in BoHV-1 mucosal invasion and moreover, to shed some light on the current classification of different BoHV-1 subtypes with particular tropisms. Because of their *in vivo* relevance, these *in vitro* models are highly appreciated since laboratory animal use is diminished and confounding factors such as individual animal variation and environmental factors are excluded. Therein lies their strength, when comparing an array of strains/subtypes on viral behavior at mucosal entry ports.

Bovine respiratory and genital mucosa explants from the same animals were inoculated with several BoHV-1 subtypes. Both respiratory and genital mucosa were found to be susceptible to infection with all different BoHV-1 subtypes. This dual tropism is also seen in closely related animal alphaherpesviruses with either emphasis on the genital tract such as caprine herpesvirus type 1 and cervid herpesvirus type 2; or on the respiratory tract such as cervid herpesvirus type 1 and suid herpesvirus type 1 (PRV) (5, 21, 25, 29, 30). However, subdivision into different subtypes of the latter viruses never was an issue. Moreover, comparison of the DNA sequences of different BoHV-1 subtypes is generally accepted to show at least 95% homology (7, 26). Such genetic heterogeneity is also observed among field strains of other herpesviruses such as PRV (2, 9, 32).

Next, a thorough analysis on the dissemination characteristics of different BoHV-1 subtypes on both respiratory and genital mucosa divulged important information on the current phylogenetic classification. Looking at average plaque penetration depth, key findings were twofold. Firstly, the so called respiratory subtypes Cooper and Lam invaded significantly deeper in respiratory tissue compared to genital tissue at 24h and 48h pi. However, secondly, in general all BoHV-1 subtypes exhibited a more profound invasion capacity in respiratory tissue compared to that in genital tissue at 24h pi. Massive accumulation of neutrophils and activation of macrophages reach peaks between 24-48h post BoHV-1 infection (11). Thus, the latter findings clearly demonstrate the outright advantage of all subtypes when invading respiratory tissues in pathogenesis. Caprine herpesvirus type 1, which is responsible for genital and respiratory disease in goats, shows a similar advantage on the respiratory tract. In general, infection by the genital route is often locally confined while the virus can spread by viremia after respiratory infection (29). The observed rapid spread through the BM towards blood vessels in respiratory tissues and the avoidance of the local immunity peak, might be an explanation why BoHV-1-induced abortion is mainly seen after a respiratory infection (24). Concerning average plaque latitude, all strains replicated to a similar extent in epithelial cells. It is noteworthy that at 24h pi, plaque latitude was found to be higher in genital tissue compared to respiratory tissue and this for all subtypes. This higher plaque latitude in the genital tract may be a compensation of the virus for the hampered spread into the depth compared to respiratory tissue, as mentioned above. Differences in BoHV-1 spread between upper respiratory and genital tissues could potentially be related to differences in apoptosis during cultivation of these different tissues. The only differences observed in BoHV-1 subtype replication characteristics in the different tissues were noticed at 24h pi. However, at

this point (48h of cultivation), we see very little, if any, differences in the occurrence of apoptosis between the upper respiratory and genital tract. For this, we conclude that the small increase in apoptotic cells throughout cultivation is likely negligible for the replication characteristics of BoHV-1 strains.

Our findings are in line with the vision of McKercher. He believes that virulent respiratory strains arose out of the less virulent genital strains and that in general the virus may be limited in invasion capacity due to biochemical and physiological characteristics of the vagina with emphasis on barrier function (12, 18). We can conclude that respiratory and genital organ cultures are suitable to study BoHV-1 invasion at primary entry ports and that they may be extrapolated to other species and viruses.

Taken together, our findings and the existing knowledge on BoHV-1 classification, clearly take the edge off the belief of the existence of specific mucosa tropisms of different BoHV-1 subtypes and make the phylogenetic BoHV-1 subdivision rather farfetched. The question arises whether this could reflect a difference in virulence between strains, as seen in all other viruses, rather than a difference in mucosa tropism?

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CHAPTER 5.

BoHV-4 USES THE VAGINAL MUCOSA AS POSSIBLE ENTRY AND DISSEMINATES AT SLOWER PACE THAN BoHV-1

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In preparation

5.1. Abstract

Bovine herpesvirus 4 (BoHV-4), a gammaherpesvirus widespread in cattle, mainly remains dormant but can cause significant economical losses as complicating agent in uterine diseases. Together with murid herpesvirus 4 (MuHV-4), BoHV-4 represents one of the best experimental models for studying gammaherpesviruses, which include the human pathogens Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV). Nevertheless, a readily available, *in vivo* related, homologous *in vitro* tissue model for BoHV-4 is still lacking and limits pathogenesis studies. In the present study, we established such a model by inoculating bovine respiratory and genital mucosa explants with BoHV-4 and evaluated whether these tissues support replication and act as primary host entry portals. In addition, we compared the dissemination kinetics of BoHV-4 with those of another bovine herpesvirus BoHV-1. In a last step, we studied the cell tropism of BoHV-4 at the level of the mucosa early upon host entry. Our salient findings are that BoHV-4 productively replicates in mucosal tissues and infects single individual cells in both epithelium and lamina propria of genital mucosa from 12h pi onwards. No signs of infection were observed in the epithelium and lamina propria of the respiratory tract. At both 12h and 24h pi, infected cells are identified as being cytokeratin$^+$ (epithelial cells) and CD172a$^+$ (monocyte/macrophage lineage cells). At 48h pi, clear BoHV-4 epithelial plaques are present in the genital tract. Unlike the aggressive basement membrane passage observed during BoHV-1 host entry, we propose that BoHV-4 uses a much more sophisticated mechanism for invasion consisting of an epithelial and myeloid infection pathway, bearing similarities with MuHV-4 and EHV-1.

5.2. Introduction

Bovines host both alpha- and gammaherpesviruses. Most of these viruses are associated with severe clinical pathologies including infectious bovine rhinotracheitis and sheep-associated malignant catarrhal fever evoked by bovine herpesvirus 1 and ovine herpesvirus 2 respectively. Until now, a lot of effort has been made to acquire more information on the viral molecular biology of many herpesviruses. However, data concerning the pathogenesis of these viruses with special emphasis on primary replication at entry ports within the host is far better documented for alphaherpesviruses than for gammaherpesviruses (4, 11). Several reasons can be put forward to explain this: some gammaherpesviruses can simply not be propagated *in vitro* thus far (e.g. OvHV-2), but most importantly, there is a lack of *in vivo*-

related *in vitro* models valuing animal ethics that allow the study of primary host-pathogen interactions (3). To enable the investigation of bovine herpesviruses with both respiratory and genital mucosa, an *in vitro* model was set up recently (25, 26). As in the case of bovine herpesvirus 1 (BoHV-1), we have shown that BoHV-1 replicates plaquewise in both respiratory and genital mucosa, easily breaches the basement membrane barrier and invades the host. Moreover, all subtypes of BoHV-1 including 1.1, 1.2a and 1.2b replicated to a similar extent in both respiratory and genital mucosa thereby questioning whether specific tissue tropisms truly exist (25). At present, we are investigating which finely tuned mechanisms are necessary for BoHV-1 efficient penetration of the basement membrane barrier allowing pathogen invasion (24).

Another major economical loss in the cattle industry are uterine infections. Although the major causative agents are bacteria, gammaherpesvirus bovine herpesvirus 4 (BoHV-4) has been shown to be a possible (co-)factor in post-partum metritis (29). BoHV-4 is widespread in natural populations and remains in the vast majority of infected individuals latent and asymptomatic. BoHV-4 persists within its host, favorably in peripheral blood leukocytes, lymphoid organs and the nervous system (1, 7, 10). Interestingly, *in vivo* studies have shown that in persistently/latently infected cattle and rabbits especially splenic cells located in the marginal zone, belonging to the non-T non-B cell compartment, harbor BoHV-4. This implies that other resident cells such as monocyte/macrophage lineage cells are a possible site of persistent/latent BoHV-4 infection (17, 21). Subsequently, Donofrio and Santen have shown that cells of the monocyte/macrophage lineage support BoHV-4 persistent infection. A small subset of macrophages survives after BoHV-4 inoculation and can be maintained several passages further. Interestingly, persistent infected macrophages contain both circular and linear BoHV-4 DNA and shed continuously small amounts of progeny virus (7). Information on viral entry mechanisms (16, 18) and immune evasion mechanisms (19) of BoHV-4 is on the increase. In addition, recent viral genome sequencing (31) and construction of a BoHV-4 bacterial artificial chromosome (BAC) (14) have dramatically extended the available tools for viral molecular biology research. Nevertheless, development of novel vaccines and antivirals should be pathogenesis-based but available information is limited until now. Jacca and co-workers put forward a hypothetical summary of what we know so far about the pathogenesis of BoHV-4: BoHV-4 establishes persistence in macrophages. Post-partum, infection of the uterus can be caused by environmental bacteria. In otherwise healthy animals such infection is cleared within 3 weeks. On the contrary, in animals persistently infected with BoHV-4, the

inflamed uterus routes macrophages carrying BoHV-4 to the inflamed endometrium. Once there, replication within macrophages might initiate and progeny virus spreads to endometrial stromal cells, which are highly susceptible for BoHV-4 (5, 15). However, little is known about BoHV-4 primary replication and putative entry sites.

In this study we wanted to assess whether BoHV-4 is using similar primary replication sites namely respiratory and/or genital mucosa as other bovine herpesviruses like BoHV-1 do. In addition, we sought to identify the very first cells that are being targeted by BoHV-4 early in infection. Finally, we compared the replication and dissemination of both BoHV-4 and BoHV-1 in the same animals allowing unbiased comparison.

5.3. Materials and Methods

5.3.1. Donor cows

Otherwise healthy respiratory and genital tissues from adult donor cows, generally aged 2-5 years, were obtained from a local slaughterhouse. In addition, peripheral blood was collected from all cows. Complement-dependent seroneutralization (SN)-tests were performed to determine both BoHV-1 and BoHV-4 specific antibody titers. Three different animals were included based on SN titers which ranged from 16 to 24 for BoHV-1 and < 2 for BoHV-4. Tissues from selected animals were used to set up bovine respiratory and genital mucosa explants.

5.3.2. Bovine respiratory and genital mucosa model

The cultivation of bovine respiratory and genital mucosa was performed similarly as described before (25, 26). In brief, tissues were immediately placed in phosphate buffered saline (PBS), supplemented with 1 µg/mL gentamycin (Invitrogen, Paisley, UK), 1 mg/mL streptomycin (Certa, Braine l'Alleud, Belgium), 1 mg/mL kanamycin (Sigma, St. Louis, MO, USA), 1000 U/mL penicillin (Continental Pharma, Puurs, Belgium) and 5 µg/mL fungizone (Bristol-Myers Squibb, New York, USA) for transportation to the laboratory. Tracheal (respiratory) and vaginal (genital) mucosa was stripped from the underlying layers. Small square tissue pieces (on average 40 mm^2) were made and placed on fine-meshed gauze for culture. The explants were cultivated in serum-free medium (50% DMEM (Invitrogen)/50%

Ham's F-12 GlutaMAX (Invitrogen)) supplemented with 0.1 mg/mL streptomycin (Certa), 100 U/mL penicillin (Continental Pharma) and 1 µg/mL gentamycin (Invitrogen) for up to 72h (37 °C, 5% CO_2).

5.3.3. Virus strains and inoculation procedure

Virus strains used in this study were the V.test strain of BoHV-4 and the Cooper strain of BoHV-1. The BAC-cloned reference strain V.test belongs to the European clade of BoHV-4 strains and was isolated from an infertile bull's testicle (22). Cooper strain, considered as the reference BoHV-1 strain, was isolated in Colorado (30). All strains obtained were from unknown passage. From each of them, a second passage was produced and utilized in our laboratory.

Inoculation of the explants was performed at 24h of cultivation. Respiratory and genital explants were taken from their gauze, placed in a 24-well plate and rinsed with warm medium. Next, explants were either mock inoculated or submerged in 1 mL of BoHV-4-containing medium (10^7 $TCID_{50}$/mL). As a control, different respiratory explants of all included animals were inoculated with 1 mL of BoHV-1 enriched medium (10^7 $TCID_{50}$/mL). For BoHV-1, we limited ourselves to respiratory explants as we have shown before that BoHV-1 replicates to a similar extent in both respiratory and genital tissues (25). The submerged explants were incubated for 1h (37 °C, 5% CO_2). Before the tissues were placed back again on their gauze, they were thoroughly washed. Inoculated tissues were collected at 0h, 12h, 24h and 48h pi. At every time point, 2 respiratory and 2 genital explants from each animal were collected. All gathered explants were embedded in cryoprotection medium (Methocel®, Fluka (Sigma)) and frozen at -70 °C. In addition, at 2h, 12h, 24h, 36h and 48h pi, explant cultivation medium was collected of BoHV-4 inoculated tissues for virus titration. In comparison, explant medium from BoHV-1 inoculated explants was collected at representative time points 2h, 24h and 48h pi and submitted for virus titration.

5.3.4. Replication kinetics of BoHV within respiratory and genital mucosa

Virus titration

Explant cultivation medium was collected at 2h, 12h, 24h, 36h and 48h pi from mock inoculated, respiratory and genital BoHV-4 inoculated explants; and at 2h, 24h and 48h pi from respiratory BoHV-1 inoculated explants for analysis of viral titers. Briefly, 70-80% confluent Madin-Darby Bovine Kidney (MDBK) cells were inoculated for 1h (37°C, 5% CO_2) with serial 10-fold dilutions (10^0 to 10^{-7} in quadruplicate) of BoHV-4 and mock inoculated explant medium. For BoHV-1 titration, confluent MDBK cells were used. Afterwards, MDBK's were overlaid with medium and observed daily for cytopathic effect (CPE) for 7-9 days. Time point 2h was included to determine residual virus titers after inoculation. As a control, BoHV-4 and BoHV-1 inactivation at 37°C, 5% CO_2 in explant medium was examined at 0h, 2h, 24h, 48h and 72h after adding either 10^7 $TCID_{50}$/mL, 10^5 $TCID_{50}$/mL or 10^3 $TCID_{50}$/mL of BoHV-4 or BoHV-1 as starting titers to control wells.

Evaluation of primary viral dissemination in mucosa

At least 100 cryosections (10µm) of the different inoculated explants were produced, methanol fixed for 20 min (100%,-20°C) and subsequently stained for BoHV-4 or BoHV-1 specific antigens. A primary mouse monoclonal IgG2a antibody (Mab35) recognizing an early-late (E-L) glycoprotein complex gp6/gp10/gp17 was used for BoHV-4 (1:1000 in PBS). Classification as being early-late refers to the fact that both a precursor of two components gp10/gp17, expressed in the early phase, and the mature form gp6/gp10/gp17, expressed in the late phase, are recognized (8, 9). Next, a secondary goat anti-mouse IgG2a Alexa fluor® 594 (Invitrogen) (1:500 in PBS) was applied. Staining of BoHV-1 was performed using an FITC®-labeled goat anti-IBR polyclonal antiserum (VMRD, Pullman, WA, USA) (undiluted). Analysis of all IF stainings was performed by using a DM2500(Sim) confocal microscope (Leica). Time points 0h, 12h, 24h and 48h pi were analyzed for BoHV-4 and mock inoculated explants. In comparison, we evaluated the replication of BoHV-1 at three representative time points namely 0h, 24h and 48h pi.

5.3.5. Quantification and characterization of BoHV-4 infected cells

The number of single infected cells was evaluated at 0h, 12h, 24h and 48h pi in at least 100 cryosections of 10µm using a BoHV-4 specific staining described above. In order to determine the nature of single BoHV-4 infected cells early after inoculation, we performed double immunofluorescence stainings using different cell surface markers at 0h, 12h and 24h pi. For each marker we made at least 100 cryosections of 10µm thick for both mock and BoHV-4 inoculated genital and respiratory mucosa, and subsequently fixed them for 20 min in methanol (100%, -20°C). In a first step, we visualized BoHV-4 antigens applying the procedure described above. In a second part of the staining, either $CD3^+$ T lymphocytes (pan bovine T lymphocyte marker), $CD172a^+$ cells (monocyte/macrophage lineage cells), $cytokeratin^+$ cells (epithelial origin) or IgM^+ B lymphocytes (B lymphocyte marker) antibodies were used to identify the type of infected cells. More specifically, a primary monoclonal rat IgG1 anti-human CD3 (AbD Serotec, Biorad-laboratories, Kidlington, UK) (1:100 in PBS) and secondary goat anti-rat Alexa fluor® 488 (Invitrogen) (1:200 in PBS) were applied to stain T lymphocytes. A primary monoclonal mouse IgG1 anti-bovine $CD172a^+$ (VMRD) (1:100 in PBS) or monoclonal mouse IgG1 anti-human cytokeratin (Dako, Glostrup, Denmark) (1:50 in PBS) with both a secondary goat anti-mouse IgG1 FITC® (Abcam, Cambridge, UK) (1:100 in PBS) were used to stain monocytes/macrophages or epithelial cells respectively. A polyclonal sheep anti-bovine IgM labeled with FITC® (AbD Serotec) (1:150 in PBS) was used to stain B-cells. In parallel, similar sections were stained with irrelevant isotype matching antibodies to determine specificity. All incubation steps were performed at 37°C for 1h. In between, thorough washing steps were included. Hoechst was applied in all stainings as a last step in order to visualize cell nuclei. Finally, all samples were mounted with glycerin-DABCO and analyzed by means of confocal microscopy. Two regions of interest were taken into account: (i) epithelium and (ii) lamina propria. Only regions in the middle of explants were considered, excluding the edges and borders of the explant.

5.3.6. Statistical analysis

Data were processed by the SPSS software (SPSS) for analysis of variance (One-way ANOVA). The data are represented as means + standard deviations (SD) of three independent experiments. Results with P values of ≤ 0.05 were considered significant.

5.4. Results

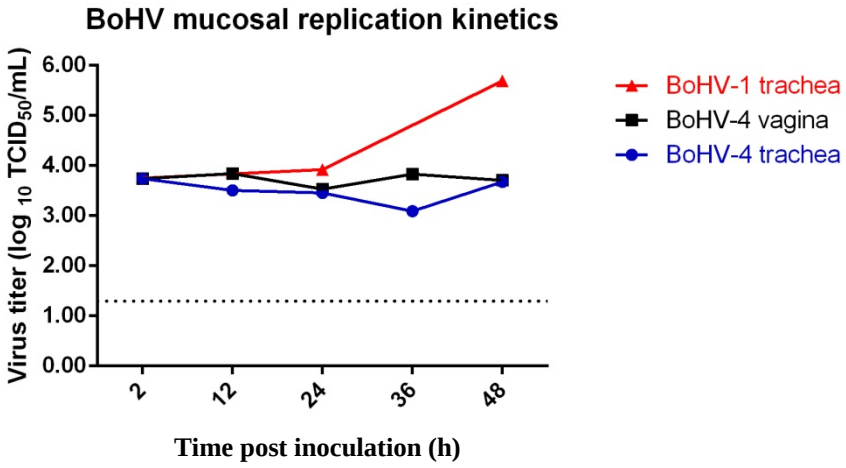
5.4.1. Extracellular virus production shows the ability of BoHV-4 to replicate in respiratory and genital mucosa

As a parameter for productive replication, viral titers were determined in the supernatant of inoculated mucosa explants (Figure 1).

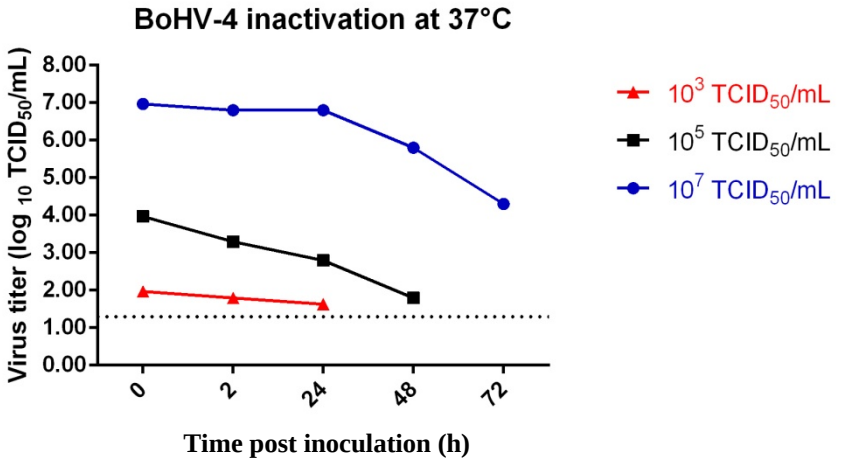
BoHV-4 was detected in both explant medium of respiratory and genital tissues at all time points. As a reference, viral titers indicating BoHV-1 replication in respiratory tissues were included. In addition, normal BoHV-4 and BoHV-1 inactivation curves at 37°C are given in Figure 1.

Residual virus after inoculation, shown by time point 2h pi, was on average $10^{3.7}$ $TCID_{50}$/mL and was found to be similar for both tissues as well as for BoHV-1 and BoHV-4.

A



B



C

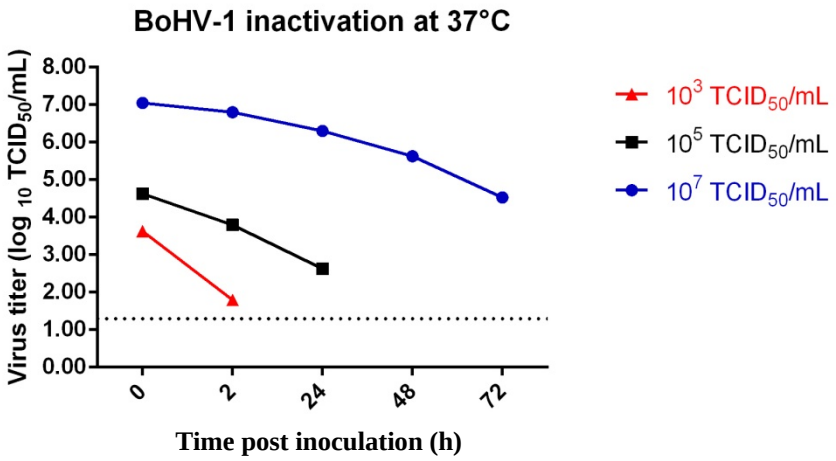


Figure 1. Virus shedding of respiratory BoHV-4-inoculated, genital BoHV-4-inoculated and respiratory BoHV-1-inoculated explants at 2h, 12h, 24h, 36h and 48h pi (A). BoHV-4 (B) and BoHV-1 (C) titer decay at 37°C in explant medium due to inactivation events. Different starting titers were used and evaluation was performed at different time points. The horizontal dotted line represents the detection limit for the titration assay.

No significant differences were observed in between all time points for BoHV-4 titers. However, taken into account the normal inactivation of BoHV-4 at 37°C, the observed titers, especially at 48h pi, indicate virus production rather than remaining residual virus after inoculation. For BoHV-1, titers rise significantly between 24h and 48h pi indicating active viral replication. In addition, at 48h pi, a significant difference is observed in virus shedding between BoHV-1 and BoHV-4 with BoHV-1 reaching titers of $10^{5.69}$ $TCID_{50}$/mL compared to an average of $10^{3.7}$ $TCID_{50}$/mL for BoHV-4.

5.4.2. Single BoHV-4 infected cells are visible at 12h pi mainly in the epithelium, increase over time (24h pi) to become plaques at 48h pi

BoHV-4 dissemination was examined in our recently established bovine respiratory and genital mucosa explant system. To this end, tissues from three different animals were included and inoculated with BoHV-4. Two explants for each tissue were collected at different time points (0h, 12h, 24h and 48h pi).

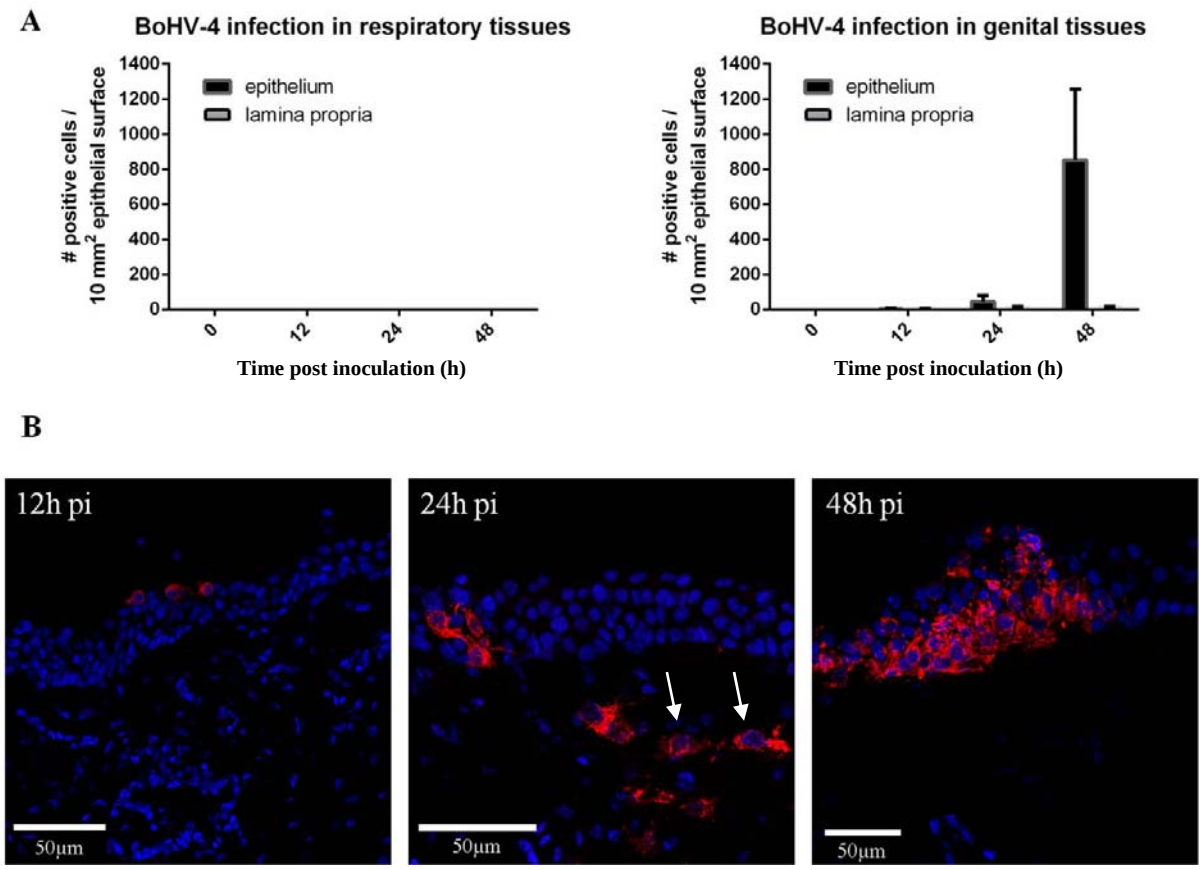


Figure 2. Evaluation of BoHV-4 infection in bovine respiratory and genital mucosa explants at 0h, 12h, 24h and 48h pi. (A) Number of individual BoHV-4 infected cells in both epithelium and lamina propria of respiratory and genital mucosa are given per 10 mm² examined epithelial surface at different time points after inoculation. All data represent means + SD of triplicate independent experiments. (B) Representative pictures of BoHV-4 spread within genital mucosa at 12h, 24h and 48h pi. BoHV-4 single infected cells in the lamina propria are marked by white arrows.

Single infected cells were visible as soon as 12h pi both in epithelium and lamina propria of genital mucosa. At none of the collected time points post inoculation, BoHV-4 positive cells were visible within the respiratory mucosa. The amount of cells increased significantly over time from 0h to 48h pi onwards in the genital epithelium (Figure 2A). At 12h pi, the amount of cells in the epithelium was on average similar as in the underlying lamina propria. However, starting from 24h pi, the majority of cells were detected within the epithelium. At 24h pi, single cells in the epithelium were often clustered in groups of 5-10 cells, seemingly having intercellular contacts (Figure 2B). Nevertheless, these clusters did not have the appearance of an obvious herpes-induced epithelial plaque like f.e. BoHV-1 at 24h pi. However, at 48h pi, distinct BoHV-4 epithelial plaques could be observed without basement

membrane crossing. By applying the measurement tool of the DM2500(Sim) confocal microscope software (LAS AF Lite, Leica), we measured the plaque latitude of several BoHV-4 plaques. On average, plaque latitude was found to be 191.43 ± 76.16 µm. In close proximity of epithelial infection, sporadic single infected cells underneath the basement membrane could be observed (Figure 2B).

5.4.3. BoHV-4 behaves differently at primary entry portals than BoHV-1

Respiratory explants inoculated with BoHV-1 showed clear signs of infection as described previously (25, 26). BoHV-1 induced epithelial plaques were observed starting from 24h pi. At 48h pi, the majority of plaques showed a plaquewise penetration of the basement membrane proving that BoHV-1 is capable of drilling in an aggressive way through this barrier (Figure 3). Interestingly, this is in striking contrast to what is observed for BoHV-4 for which a quite different mechanism of dissemination within tissues of the same animals was observed (Figure 2).

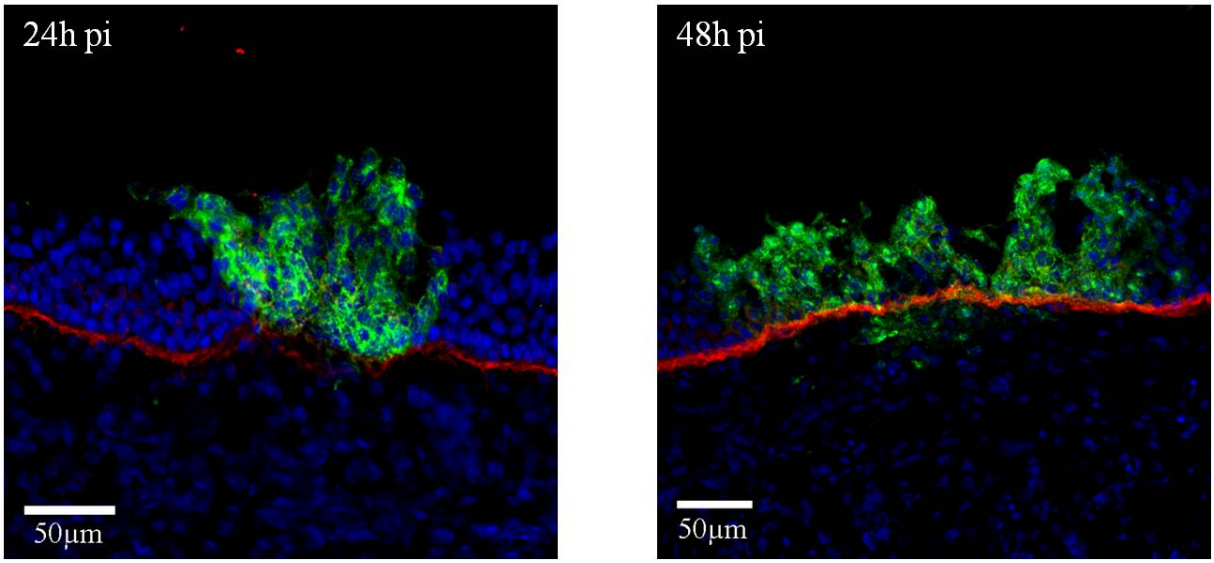


Figure 3. Representative confocal immunofluorescent images showing a different mode of mucosal dissemination for BoHV-1 compared to BoHV-4 within the same animals (for BoHV-4 see Figure 2). BoHV-1 induced plaques were observed at 24h and 48h pi. At 48h pi clear penetration of BoHV-1 (FITC) through the BM (Texas Red) can be observed.

5.4.4. BoHV-4 cell tropism at 12h and 24h after initial inoculation

To gain information on which specific cells are the first cells upon entry being infected by BoHV-4 in the host, we looked at the BoHV-4 cell tropism by means of immunofluorescent double stainings at 12h and 24h pi. We used several markers, staining epithelial cells (cytokeratin$^+$), monocytes/macrophage lineage cells (CD172a$^+$), T lymphocytes (CD3$^+$) and B cells (IgM$^+$), for identification (Figure 4). For BoHV-4, single infected cells in the epithelium and lamina propria were visible at 12h pi in genital tissues. The vast majority of infected cells in the epithelium were epithelial cells (cytokeratin$^+$) whilst cells observed in the lamina propria were mostly CD172a$^+$ monocyte/macrophage lineage cells (Table 1). At 24h pi, nearly all infected cells in the genital epithelium were epithelial cells and a small minority of CD172a$^+$ were observed within the epithelium, often in proximity of infected epithelial cells. Within the lamina propria of the genital epithelium, BoHV-4 infected CD172a$^+$ cells were scarcely present. However, at 12h and 24h pi we neither observed BoHV-4 infection of T lymphocytes nor of B cells. Interestingly, at 12h and 24h pi no infected cells were found in tracheal tissues.

Table 1. Number of marker-positive BoHV-4 infected cells at early time points post inoculation in genital tissues (Epithelial surface covered within a cryosection is on average 0.03 mm^2).

Time point pi	Tissue	Zone	Total amount of marker positive cells / Total amount of BoHV-4+ cells counted in ≥ 100 sections/animal (triplicate experiment)			
			Cytokeratin$^+$	CD3$^+$	IgM$^+$	CD172a$^+$
12h	Vagina	Epithelium	6/6	0/2	0/1	0/8
		L. propria	0/0	0/3	0/1	5/7
24h	Vagina	Epithelium	32/35	0/57	0/87	5/82
		L. propria	0/13	0/8	0/14	14/20

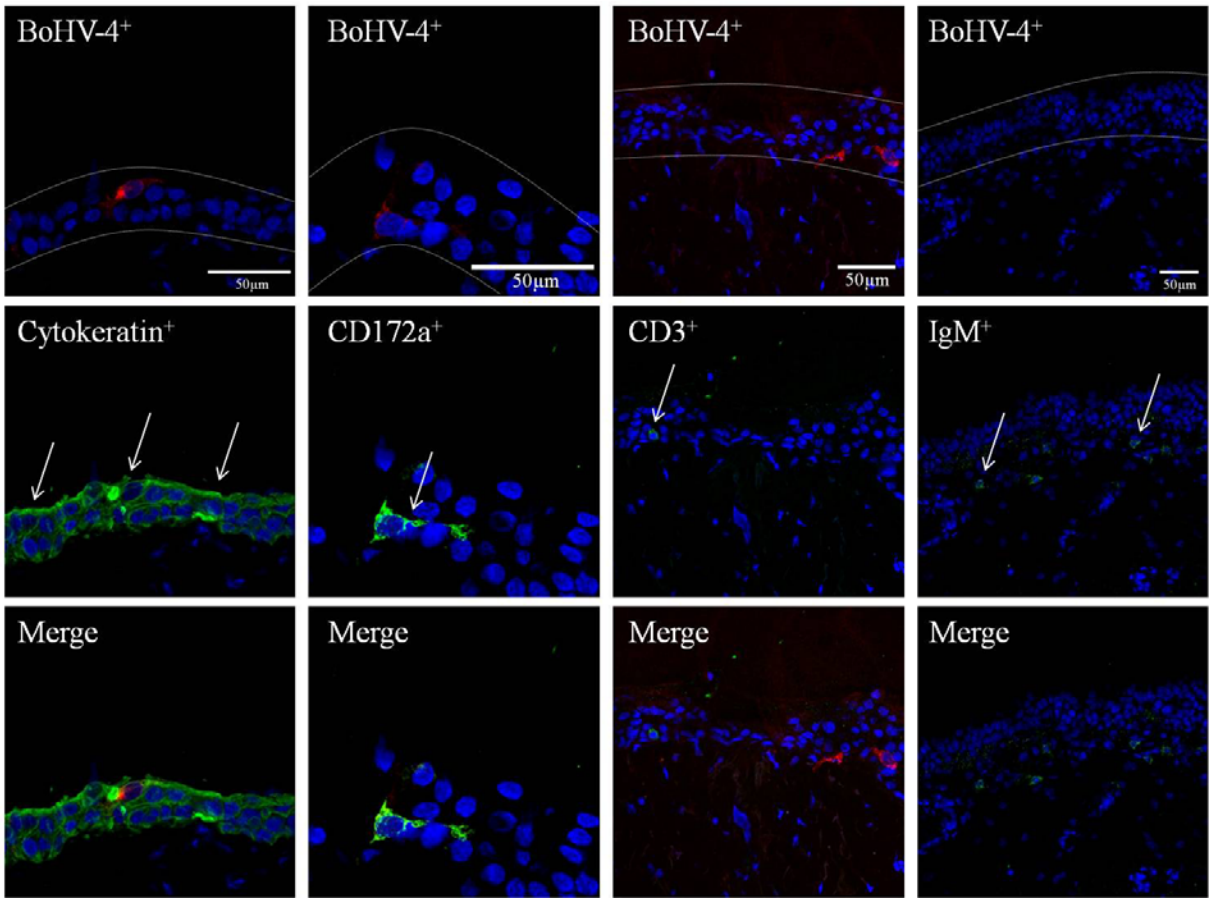


Figure 4. Cell tropism of BoHV-4 at 24h pi in genital epithelium. The row on top shows BoHV-4 positive cells (Texas Red) within the epithelium (dotted line). The middle row shows respectively cytokeratin, CD172a, CD3 and IgM expression (FITC) in different cells (white arrows). The bottom row represents merges of the rows above. Only for cytokeratin and CD172a, double positive cells for the virus are observed.

5.5. Discussion

In general, alphaherpesviruses initiate their pathogenesis at the level of the upper respiratory tract and/or the lower genital tract. Recently, we studied the mucosal behavior of a bovine alphaherpesvirus, BoHV-1 and visualized how BoHV-1 penetrates several barriers upon host entry for invasion (25, 26). Although there is growing evidence that gammaherpesviruses might use similar entry routes as alphaherpesviruses (12), proof of concept is lacking for the majority of them. BoHV-4, an often quiescent bovine gammaherpesvirus present in carrier animals, is thought to reactivate upon stress (e.g. partus) and aggravate uterine infections caused by primary agents such as bacteria (15). Thus even though disease is individually rare, the total burden in populations, by acting as a co-virulence factor, is large. However, no

information exists on how BoHV-4 gains access to its host and which mechanisms it implies to do so.

In this study, we have utilized a previously established bovine respiratory and genital model to assess the potential involvement of these tissues in the early pathogenesis of BoHV-4. To this end, we inoculated several tissues and analyzed kinetically the mucosal behavior of BoHV-4. Moreover, we looked at BoHV-1 replication within tissues of the same animals as a comparative control. First, we observed a lower virus production in BoHV-4 explants compared to BoHV-1 explants, especially clear at 48h pi. BoHV-4 DNA synthesis, unlike BoHV-1, is documented to be dependent on the S phase of the cell cycle (dividing cells). This study describes that in resting cells (G0 phase), BoHV-4 DNA replication was found to be arrested due to a block in replication after early (E) protein expression (28). In general, epithelia are highly differentiated which is accompanied by irreversible cell cycle exit. The only cells still capable of mitosis in epithelia are basal and parabasal cells (20). Hence, this might imply that productive replication of BoHV-4 in mature epithelium happens with low efficiency and is restricted to parabasal and basal cells. These observations can explain the lower viral shedding seen in our experiments. Although no signs of infection were observed in the epithelium and lamina propria of the respiratory tract (our region of interest), we cannot firmly exclude infection of cells and productive replication at the lateral and bottom sides of explants. Indeed, our inoculation procedure allows the virus to come in contact with lateral and bottom sides of explants. As this route of infection does not reflect the *in vivo* situation, we did not take these regions into account. Second, we found that BoHV-4 infects single individual cells in the genital epithelium starting from 12h pi. The majority of these were from epithelial origin (cytokeratin$^+$) and although a clear plaque-like appearance was lacking, they were often found in small clusters at 24h pi. Only from 48h pi, clear BoHV-4 plaques were visible in the epithelium. In addition, from 12h pi onwards few CD172a$^+$ cells were found BoHV-4 positive in the lamina propria, underneath the basement membrane. Over time, the amount of single individually infected cells increased steadily in the epithelium, especially the number of epithelial cells. At 24h pi, cell tropism did not change as still only epithelial and monocyte/macrophage lineage cells are being infected. Interestingly, Frederico and co-workers have recently analyzed how murine herpesvirus 4 (MuHV-4) enters the host via the upper respiratory tract by using a homologous mouse model. They found and proposed that rhadinoviruses (e.g. MuHV-4) in a sequential manner first infect epithelial cells, then myeloid cells and only then B cells (12, 13). In addition, BoHV-4 gp180, a major envelope protein, is

described to function as an O-glycan shield against neutralizing antibodies thereby covering otherwise vulnerable viral epitopes. The finding that this protein, if present, allows efficient infection of glycosaminoglycan $(GAG)^+$ cells like epithelial cells and if absent, gives the virus the ability to more efficiently infect GAG-deficient cells such as monocytes, suggests that gp180 is probably shielding GAG-independent entry epitopes. Several hypotheses can be formulated but as previously described for MuHV-4 and EBV, possibly BoHV-4 binding to and/or infection of epithelial cells allows efficient subsequent myeloid/lymphoid cell infection (2, 12, 18, 19, 23). Therefore in our study, epithelial cells are likely the very first target of BoHV-4, as the vast majority of infected cells at 12h pi were found to be of epithelial origin. For BoHV-4, this epithelial infection occurs in the genital tract thereby pointing out that the virus has a tropism for the genital tract in general (e.g. metritis). Next to that, monocytes/macrophages are being infected and possibly confer the virus the ability to be transported inwards in a Trojan horse-like manner across the basement membrane, a mechanism previously described for alphaherpesvirus EHV-1 (27). We did not observe infection of T or B lymphocytes at 12h and 24h pi despite their presence in the mucosa. B-cell infection for MuHV-4 is described to be a late event in pathogenesis as the infection pathway is timely regulated in a cascade manner, going over epithelial cells and subsequent myeloid cells to B cells. Indeed, Frederico and co-workers identified a binding block to myeloid infection that was overcome by co-culture with epithelial cells, and a post-binding block to B cell infection that was overcome by virus propagation in myeloid cells (12). Therefore, it might be interesting for BoHV-4 to look at later time points in infection to see whether lymphocytes are eventually being targeted by the virus and if a similar epithelial/myeloid/lymphoid infection pathway exist for BoHV-4. Nevertheless, BoHV-4 is extensively documented to persist in macrophages (7, 17). Furthermore, there are currently no clear indications in favor of BoHV-4 infection of lymphocytes (17, 21). Thus, another explanation why no BoHV-4^+ T or B lymphocytes are observed in our study might be that, unlike MuHV-4, epithelial/myeloid to lymphoid spread simply does not occur for BoHV-4.

BoHV-1 and BoHV-4 clearly exhibit a different mechanism of invasion. BoHV-1 causes extensive epithelial plaque formation and exhibits a clear crossing of the basement membrane barrier. On the contrary, BoHV-4 causes subtle epithelial infection and possibly misuses resident individual monocytes/macrophages to be transported across the basement membrane. As explants are gathered from the same animals, any inter-cow variability can be excluded. In addition, we measured plaque latitude for BoHV-4 plaques in the genital epithelium at 48h pi

(191.43 ± 76.16 μm). If we compare this to previously measured plaque latitudes of BoHV-1 plaques in the genital mucosa of similar aged cows at 48h pi (366.29 ± 55.84 μm) (25), clearly BoHV-4 less efficiently paves its way through the epithelium compared to BoHV-1.

Taken together our findings with the hypothesis generated by Jacca and co-workers, we want to speculate about the pathogenesis of BoVH-4: BoHV-4 infects, like some alphaherpesviruses and gammaherpesviruses, the genital tract. Primary replication is established in epithelial cells and results in productive replication of BoHV-4, confirmed by the observed viral shedding in our study. We propose that the virus is transmitted in genital secretions and contributes to its widespread presence in populations. However, compared to BoHV-1, titers are much lower and might indicate a less efficient way of transmission to naïve animals. Whilst dissemination among epithelial cells proceeds through cell-to-cell spread, as virus positive epithelial cells are often found in small clusters and eventually plaques, infection of some resident monocytes, macrophages or dendritic cells can take place. The virus might use these cells to be transported across the basement membrane towards internal organs. BoHV-4 has been demonstrated in many tissues but is often found in lymphoid tissues. Transfer to T or B lymphocytes cannot be excluded at present. In BoHV-4 persistently infected animals, BoHV-4 infection resides within macrophages. At parturition and post-partum, an inflamed uterus due to bacterial infection attracts macrophages and allows local reactivation of the virus. Endometrial stromal cells can become infected with newly replicating virus (6, 7, 15). Extracellular virus production of these infected stromal cells can contribute to BoHV-4 transmission.

In conclusion, we have visualized the mucosal behavior of BoHV-4 in a bovine genital mucosa model and shed some light on the primary replication of BoHV-4. To our knowledge, this is the first report describing an invasion route of BoHV-4. At present, both BoHV-4 and MuHV-4 are accessible, animal gammaherpesviruses and serve as experimental models for the hard-to-study human Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV). Therefore, homologous *in vivo* models are extremely important not only for pathogenesis studies but also to verify *in vitro* acquired results. We can only speculate on which mechanisms all these gammaherpesviruses imply in order to successfully establish host infection, but the observed striking parallels between BoHV-4 and MuHV-4 host colonization suggest that other lymphotropic viruses follow similar routes.

5.6. Acknowledgements

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CHAPTER 6.

MIMICKING HSV-1 AND HSV-2 MUCOSAL BEHAVIOR IN A WELL CHARACTERIZED HUMAN GENITAL ORGAN CULTURE

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6.1. Abstract

In general, mucosal invasion is poorly characterized for herpes simplex virus 1 (HSV-1) and 2 (HSV-2). We developed and morphologically characterized a human genital mucosa explant model (endocervix and ectocervix/vagina) to mimic genital herpes infections. Subsequent analysis of entry receptor expression, including nectin-1, nectin-2 and HVEM, throughout the menstrual cycle in genital tissues was performed and the evolution of HSV-1/-2 mucosal spread at different time points pi within ectocervical and endocervical mucosa was assessed. Nectin-1 and -2 were found to be expressed in both endocervix and ectocervix during all phases of the menstrual cycle. On the contrary, HVEM expression was limited to some cells, mainly present in the connective tissue. HSV-1 and HSV-2 exhibited a plaquewise mucosal spread, induced prominent epithelial syncytia in ectocervical mucosa and behaved in a similar way in replication at the genital tract. From 48h pi, epithelial plaques crossed the basement membrane in all tissues proving that the virus is able to breach a critical barrier and infiltrate the host.

6.2. Introduction

Human genital herpes is worldwide one of the most prevalent causes of genital ulcer disease and with a prevalence reaching up to 80% (developing countries), one of the most important sexually transmitted infections (STI). The principal causative agent is herpes simplex virus type 2 (HSV-2); however, the frequency of primary genital herpes infections caused by herpes simplex virus type 1 (HSV-1) is on the increase. Genital manifestations of initial infection display an array of symptoms going from asymptomatic to severe bilateral vesicular lesions. Because of extensive replication of the virus these lesions can be purulent, and in some cases lead to necrotic trauma. Systemic consequences consist of flu-like symptoms (fever, headache, myalgia) and dysuria. Congenital and perinatal herpes infections, and brainstem encephalitis in neonates, are well known complications of maternal genital herpes (1, 21). Moreover, genital herpes lesions promote the transmission of HIV (1, 24). HSV-2 has been shown to modulate the mucosal microenvironment including recruiting of submucosal DC's, influencing DC function, upregulating integrin α4β7 expression on $CD4^+$ T-cells and stimulating retinoic acid production, an immunomodulator. These mechanisms might contribute to a more prone susceptibility to HIV (20, 32). No efficacious HSV-vaccine is currently on the market, and the likelihood of one coming to market soon is low (2). When

treating herpes virus infections with antiviral compounds, resistance occurs frequently which limits the application of these products. Indeed, acyclovir-resistant HSV strains are becoming increasingly common, especially in HIV^+ individuals. Infection with acyclovir-resistant strains are 10-fold higher in HIV^+ persons than in immunocompetent patients and appears related to the degree of immunosuppression and the duration of antiretroviral therapy (31).

Rodent models are considered as golden standard for genital herpes research (9, 19). Although they play a robust role in elucidating aspects of primary genital mucosa infections, they clearly lack homology. Potential species-specific differences in cellular mediators of infection and invasion, impede extrapolation of results. For example, several entry mediators have been described for HSV, including nectins (nectin-1 and nectin-2), herpes virus entry mediator (HVEM) and specific sites in heparan sulfate (26). Apparently, both nectin-1 and HVEM mediate viral entry *in vivo* most efficiently (15). Although nectin homologues in mice resemble the human forms, mouse nectin-2 is not functional as entry receptor for HSV-1 and HSV-2 whereas human nectin-2 clearly mediates entry of HSV-2 and certain HSV-1 recombinants (28). Interestingly, until now, information on either the presence and distribution of entry mediators or on its potential *in vivo* role within the genital tract is scarce.

Previously we optimized a human genital mucosa explant model and extensively checked tissue viability and morphology during *in vitro* cultivation. This homologous model is a valuable tool to study the intriguing interplay of HSV with mucosal surfaces at the level of the genital tract. In a first part of the present study, we analyzed the expression patterns of different herpes virus entry receptors including nectin-1, nectin-2 and HVEM in both endocervix and ectocervix/vagina throughout the menstrual cycle. In a second part, we modeled the mucosal behavior in genital mucosa of both HSV-1 and HSV-2 by inoculating our established genital organ cultures and compared the HSV-1 and HSV-2 replication characteristics within the same patients. Nectin-1 and -2 showed a similar expression pattern in both endocervix and ectocervix/vagina and moreover, this was consistent at all stages of the menstrual cycle. On the contrary, HVEM was not expressed in endocervical or ectocervical epithelial cells. However, scarcely distributed cells in the epithelium and several cells in the lamina propria did express HVEM on the entire cell membrane. HSV-1 and -2 both exhibited a plaquewise mucosal spread and induced prominent epithelial syncytia. In addition, both endocervix and ectocervix were clearly susceptible to both HSV-1 and HSV-2 infection. Starting from 48h pi, few epithelial viral plaques crossed the basement membrane (BM) showing HSV-1 and HSV-2 can cause stromal invasion in the genital tract.

6.3. Materials and methods

6.3.1. Patients

Pieces of healthy genital mucosa were gathered from women undergoing either a routine hysterectomy or a reconstructive plastic surgery (Ghent University hospital). Apart from this study, pieces of the mucosa were subjected to a pathological examination in order to exclude concurrent papilloma virus infection and early stages of papilloma virus induced endocervical adenocarcinoma. From all included patients, serum was collected. First, a progesterone and estradiol determination was performed by the hormonology department of the Ghent University Hospital to determine the ovarian cycle stage. Second, HSV specific antibody titers were determined by means of a complement-dependent seroneutralization (SN)-test. This test does not allow discrimination between antibodies raised against HSV-1 and HSV-2. All persons provided written informed consent and the ethics committee of the Ghent University Hospital approved the study (EC/2010/152). Different patients of various stages of the menstrual cycle were included for analyzing both herpes virus entry receptor expression and HSV-1/-2 mucosal dissemination kinetics (Table 1).

6.3.2. Human genital mucosa model set up

A similar set up was used as for bovine respiratory and genital mucosal explants (27). Briefly, tissues were immediately placed in phosphate buffered saline (PBS), supplemented with 1 µg/mL gentamycin (Invitrogen, Paisley, UK), 1 mg/mL streptomycin (Certa, Braine l'Alleud, Belgium), 1 mg/mL kanamycin (Sigma, St. Louis, MO, USA), 1000 U/mL penicillin (Continental Pharma, Puurs, Belgium) and 5 µg/mL fungizone (Bristol-Myers Squibb, New York, USA) for transportation to the laboratory. Genital mucosa was stripped from the underlying layers. Small square tissue pieces were made and placed on fine-meshed gauze for culture. The explants were cultivated in serum-free medium (50% DMEM (Invitrogen)/50% Ham's F-12 GlutaMAX (Invitrogen)) supplemented with 0.1 mg/mL streptomycin (Certa), 100 U/mL penicillin (Continental Pharma) and 1 µg/mL gentamycin (Invitrogen) for up to 96h (37 °C, 5% CO_2).

6.3.3. Analysis of herpes virus entry receptor expression

We used immunofluorescence stainings to determine the distribution of nectin-1, nectin-2 and HVEM in both endocervical and ectocervical tissue from women in different stages of the menstrual cycle. Evaluation of relative receptor expression was performed on non-cultured tissues (time point 0h) of 10 different patients (Table 1). Cryosections of 10µm were fixed in methanol (-20°C, 100%) for 20 min. We used mouse monoclonal antibodies CK41 (18), L14 (3) and D-5 (Santa Cruz Biotechnology, Dallas, Texas, USA) directed against CD111 (nectin-1) (1:50 in PBS); CD112 (nectin-2) (1:20 in PBS) and TR2 (HVEM) (1:50 in PBS) respectively. After 1 hour incubation at 37°C, samples were thoroughly washed and incubated at 37°C for another hour with goat anti-mouse FITC®. Next, nuclei were stained by Hoechst. Finally, all samples were mounted using glycerin-DABCO.

6.3.4. Dissemination kinetics of HSV in genital mucosa

HSV strains and tissue inoculation

The strains used in this study were HSV-1 strain F (ATCC VR-733) and HSV-2 strain MS (ATCC VR-540). From each of them a second passage was produced on African green monkey kidney cells (Vero, ATCC CCL-81) and used for inoculation.

Briefly, the inoculation procedure was as follows. Transfer of explanted tissue from the gauze to a 24-well plate was followed by several washing steps. After adding 1 ml of virus-containing medium ($10^{6.5}$ $TCID_{50}$/ml), the submerged tissues were incubated for 1 hour (37°C, 5% CO_2). Afterwards, prior to transfer from the 24-well plate to the gauze, stringent rinsing was performed. Finally, samples were collected at different time points including 0h, 24h and 48h pi. All gathered explants were embedded in a cryoprotection medium (Methocel®, Fluka) and frozen at -70°C.

Plaque analysis by immunofluorescence stainings

Series of 20µm thick consecutive cryosections were made from all collected explants and fixed in methanol (-20°C, 100%) for 20 min. In order to evaluate thoroughly the dissemination characteristics of HSV within the mucosa, we used immunofluorescent double

stainings. In a first part, to visualize the BM barrier and underlying connective tissue, we used primary goat anti-collagen IV antibodies (Southern Biotech, Birmingham, USA; 1:50 in PBS); secondary biotinylated rabbit anti-goat antibodies (Sigma, 1:100 in PBS) and tertiary streptavidin-Texas Red® antibodies (Molecular Probes, Invitrogen; 1:50 in PBS). In a second part, HSV-1 or HSV-2 antibodies were stained with primary mouse monoclonal antibodies against HSV-1 gD (Santa Cruz Biotechnology, 1:100 in PBS, 10% NGS) or HSV-2 gB (Santa Cruz Biotechnology, 1:50 in PBS, 10% NGS) respectively, followed by secondary FITC®-labeled goat anti-mouse antibodies (Molecular Probes, 1:100 in PBS, 10% NGS). Analysis of all IF stainings was performed by using a DM2500(Sim) confocal microscope (Leica). Next, measurements of average HSV-1/-2 plaque latitude and plaque penetration underneath the BM were performed at 0h and 48h pi.

6.3.5. Statistical analysis

SPSS software (one way ANOVA, Post-hoc Bonferroni and Tukey's HSD) was used to evaluate the variance. The results are given as means + standard deviation of different (≥3) independent experiments. Results with *P* values of ≤ 0.05 were considered significant.

6.4. Results

6.4.1. HSV-specific neutralizing antibody and sex hormone level analysis

An overview of the serum progesterone and estradiol levels of the included patients is given in Table 1. The seroprevalence within the included patients was found to be 71.4% (Table 1).

6.4.2. Distribution of herpes virus entry receptors

By means of immunofluorescence stainings, relative expression of different herpes virus entry receptors was evaluated in ectocervical/vaginal and endocervical tissues from 10 different patients, including premenopausal (follicular and luteal phase) and postmenopausal. At least 2 different patients were included for each menstrual cycle stage. Evaluation was performed on non-cultured tissues (time point 0h). In general, for all patients, both nectins were clearly more abundantly expressed in endocervix compared to ectocervix/vagina. Regardless of the

phase of the menstrual cycle, expression pattern and localization was noticed to be consistent for both endocervix and ectocervix/vagina.

Table 1. Overview of the included donor patients. Serum progesterone and estradiol levels were analyzed to determine menstrual cycle phase. HSV specific neutralizing antibodies were determined to investigate the seroprevalence within the group. ND = Not Determined.

Patient	Age	Progesterone (ng/mL)	Estrogen (pg/mL)	Menstrual Cycle Phase	HSV SN-Titer	HSV Kinetics	Entry receptor analysis
001	67	0.36	<12.0	Menopause	ND		Determined
002	70	<0.15	<12.0	Menopause	ND		Determined
003	48	0.38	12.9	Menopause(Follicular)	ND		Determined
004	33	0.38	36.1	Follicular	<2	Determined	Determined
005	41	9.0	145	Luteal	<2		Determined
006	50	0.20	26.2	Menopause	96	Determined	
007	49	0.94	68.6	Follicular	256		Determined
008	34	1.5	49.6	Follicular	ND		Determined
009	38	21	122	Luteal	128	Determined	Determined
010	50	<0.15	13.9	Menopause	64	Determined	Determined
011	31	0.92	32.9	Follicular	24		Determined

At all times, the nectin-2 antibody staining was found to be more intense than the antibody staining for nectin-1 in the genital tract. In endocervical epithelium nectins were highly present on the luminal side of the cell, thus apicolateral. In ectocervical/vaginal epithelium, nectin-1 and -2 expression was most intense in the *stratum spinosum* and minimal in the *stratum basale*. Loss of nectin-1 and -2 expression was seen within the superficial layers of the ectocervix/vagina. Consequently, whenever epithelial thickness shrivels, nectin expression was located closer to the luminal side, as was observed in postmenopausal women and women in the luteal phase of the menstrual cycle. The majority of HVEM expressing cells was observed in the lamina propria and deeper connective tissue. Scarcely distributed single cells resident within the epithelium were also found to be HVEM positive. Nevertheless, the majority of the endocervical and ectocervical epithelium clearly did not express HVEM.

Representative pictures of nectin-1, nectin-2 and HVEM expression for both ectocervix/vagina and endocervix are given in Figure 1.

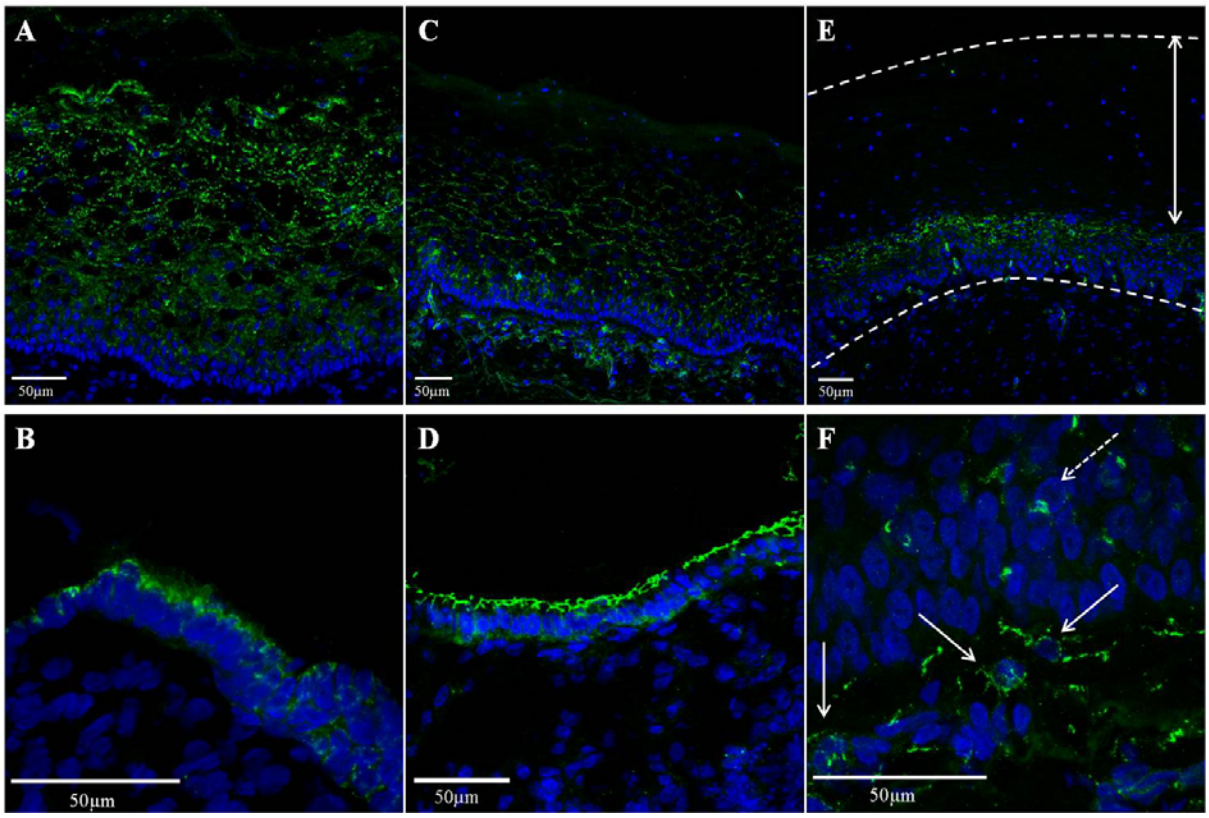

Figure 1. HSV entry receptor expression in human endocervical and ectocervical tissues. Nectin-1 (A) and nectin-2 (C) expression is mainly localized in the stratum spinosum of the ectocervix at all phases of the menstrual cycle. In the endocervix a strong nectin-1 (B) and nectin-2 (D) expression can be observed on mainly the apicolateral sides of the cell membrane throughout the menstrual cycle. (E) Especially during the follicular phase of the menstrual cycle, the ectocervical stratum superficial consists of multiple layers (double-headed arrow) of non-nectin expressing cells (dashed line marks the entire epithelium). (F) HVEM expression was observed on cells located in the lamina propria (arrows) and scarcely on individual cells residing in the epithelium (dashed arrow) regardless of the menstrual cycle stage.

6.4.3. HSV-1 and HSV-2 genital mucosal spread

HSV-1 and HSV-2 both replicated plaquewise in endocervical and ectocervical mucosa of all included patients to a similar extent (Figure 2). Importantly, we did not observe obvious differences in susceptibility to HSV and HSV dissemination due to the menstrual cycle stage. In ectocervical mucosa, HSV plaques were mainly localized at places of slight abrasion

within the superficial layers. All HSV-induced ectocervical plaques were situated within the *stratum spinosum* and some extended to the *stratum basale*. Infection within the *stratum superficialis* was never observed. Interestingly, large multinucleated giant cells were observed in HSV epithelial plaques in the ectocervix. Starting from 48h pi, few (<10%) ectocervical and endocervical epithelial viral plaques crossed the BM, albeit in a localized manner.

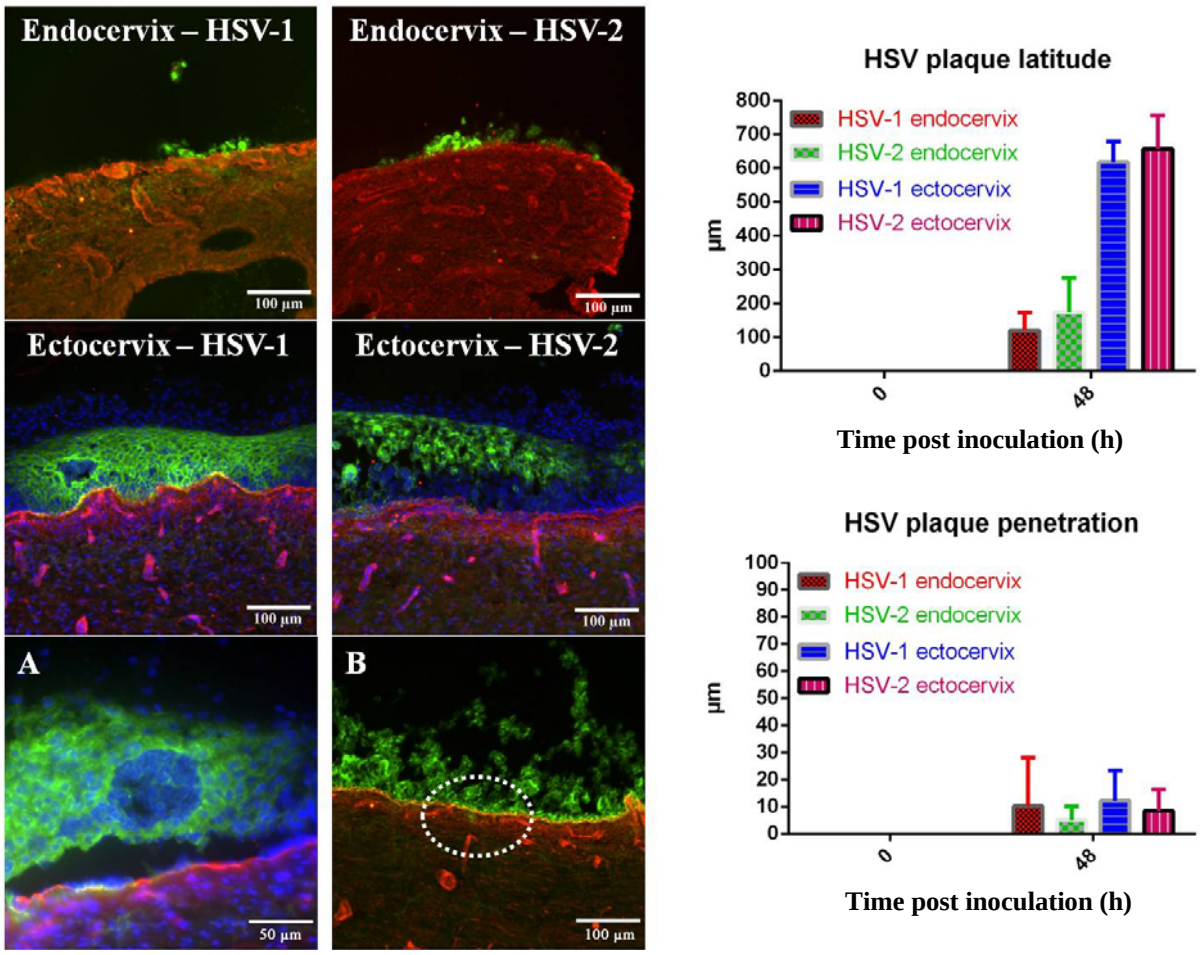

Figure 2. HSV dissemination at 0 and 48h pi in genital mucosa. Representative immunofluorescent images of HSV-1 and HSV-2 plaques in endocervical and ectocervical mucosa are shown. (A) In ectocervical mucosa HSV-1 and -2 produced multinuclear giant cells (syncytia). (B) Few HSV-1/-2 plaques in both endo- and ectocervix locally crossed the BM (indicated by white circle). The graphs show average measurements at 0h and 48h pi of plaque latitude and penetration underneath the BM for HSV-1/-2 in endo- or ectocervical mucosa. Data are represent as means + SD of three independent experiments.

6.5. Discussion

The mucosal behavior of genital herpes infection caused by HSV-1 and HSV-2 at the level of the mucosa within human tissues is poorly documented until now. Little information is known about the presence of herpesvirus entry factors at different menstrual cycle phases in both endocervical and ectocervical/vaginal epithelium. One *in vivo* comparative study describes the expression pattern of nectin-1 in the human and mouse vagina. Interestingly, little extrapolation can be made from one to another. In humans, nectin-1 expression and localization remains identical at the different stages of the menstrual cycle whereas in mice striking differences in expression levels were seen. We observed similar results when analyzing nectin-1 expression in human ectocervix/vagina for all menstrual cycle stages (19). Interestingly, we found that the localization of nectin-2 within the ectocervical epithelium was comparable with that of nectin-1. Membrane expression showed a dispersed pattern on ectocervical epithelial cells. The expression of nectin-1 and nectin-2 was found to be similar in endocervix at all times. The luminal half of these columnar cells, thus apicolateral, showed expression of nectins and this did not alter with change in menstrual cycle phase. Importantly, analysis of nectin-2 expression in endocervix revealed a more abundant presence of nectin-2 compared to ectocervix/vagina during the entire menstrual cycle. In polarized epithelia, nectins localize in adherens junctions and contribute to their formation and maintenance (11). Our study confirms the preferential lateral sorting of nectins in polarized human endocervical epithelium compared to non-polarized ectocervical epithelium (25). HVEM expression was found to be limited to multiple cells in the connective tissue and some single cells residing in the epithelium. However, no clear expression within epithelial cells could be observed. HVEM is found in multiple tissues and, as a member of the TNF receptor family, broadly expressed in hematopoietic cells. Thus, its primary natural function is to act in immune responses. However, the level of HVEM expression on immune cells varies at different times in the immune response. For example, naïve and some memory $CD4^+$ and $CD8^+$ lymphocytes constitutively express HVEM (5, 16, 17). Assumedly, the $HVEM^+$ cells we observed within the mucosa are resident immune cells strolling as sentinels in both the epithelium and underlying lamina propria. Recently, a study described that the interaction of gD and HVEM during acute infection with HSV may influence the magnitude and quality of the subsequent $CD8^+$ T cell recall response at the genital mucosa, although its role in HSV pathogenesis is still unclear (16).

When testing the human genital mucosa explants for HSV susceptibility, we observed some interesting facts. HSV-1/-2 induced prominent epithelial syncytia, the latter unlike animal alphaherpesviruses act in mucosal tissues. Indeed, we did not observe polykaryocyte formation for BoHV-1, EHV-1 and PRV in both genital and respiratory mucosa (10, 27, 29). The core membrane fusion machinery in herpesviruses consists of glycoprotein B, a type III fusion protein and the gH-gL heterodimer. It has been shown that gB modulates cell-cell fusion in several herpesviruses. For most, if not all fusion proteins a conversion from an inactive pre-fusion to an active post-fusion state occurs. Assumedly, gH/gL uses triggering mechanisms that are functionally analogous to the known fusion triggering mechanisms like low pH or protease cleavage. Interestingly, herpesvirus gB homologues can be subdivided into two groups based on whether a posttranslational proteolytic cleavage occurs or not. As such, gB of BoHV-1 and PRV is cleaved and HSV-1 gB remains uncleaved. Thus there may be some variability in how gB is activated. In addition, fusion triggering is regulated by other viral glycoproteins and their receptors as well. The question remains whether these slight differences in this exquisite mechanism of gB activation across herpesviruses have *in vivo* implications and if this might explain the different observed phenotypes of alphaherpesvirus mucosal replication (6, 14). Alternatively, the ability of human alphaherpesviruses to induce prominent syncytia in mucosal tissues might be an example of co-evolution with the host. Indeed, syncytia formation is also a hallmark of varicella-zoster virus (VZV) infection *in vivo*, another human alphaherpesvirus (6, 23).

HSV-1/-2 replication was found to be hampered in intact ectocervical epithelium. In endocervical tissues, HSV was found to replicate well. Taken into account our observations that nectin-1 and -2 as well as HVEM expression is negligible in the most outer layers of the ectocervical/vaginal epithelium, this clearly confirms the need of HSV for epithelial microlaesions to initiate invasion in ectocervix/vagina. This is also reflected in the HSV plaque analysis we performed. Indeed, ectocervical HSV plaques were only found within the *stratum spinosum* and *basale* and extended by analogy with their entry receptors. In addition, HSV plaques were more frequently found at places of slight abrasion or in explants were the epithelium was slightly scarified before inoculation (data not shown). Also, the abundant presence of nectins, especially nectin-2, in endocervix renders this anatomical site a potential target for efficient invasion. Importantly, one key phenomenon, termed cervical ectopy, exposes the endocervical epithelium to the ectocervical/vaginal cavity due to hormonal influences. This is most commonly seen in adolescents and pregnant women as among

women using hormonal contraceptives. The endocervical epithelium on this squamo-columnar junction converts into squamous epithelium over time (metaplasia). These regions of epithelial conversion are highly susceptible to HIV and other STI (30). Our results indicate that, since (i) nectins are abundantly present and (ii) endocervical epithelium sustains productive infection of HSV-1/-2, the squamo-columnar junction might be highly prone to herpesvirus infections. In line with the findings made by Mostad and co-workers, we did not see an association of HSV susceptibility and replication with phase of the menstrual cycle or with serum estradiol/progesterone levels (22). Although there are reports describing the regulation of HSV infections and immune responses to genital HSV by sex hormones, the majority is performed in mouse models (7-9). We did not evaluate the effect on HSV mucosal dissemination of more profound changes in hormone levels, e.g. pregnancy and hormonal contraceptives.

At 48h pi, proof of HSV breaching through the basement membrane barrier was observed in a minority of endocervical and ectocervical plaques. However, this number might increase at a later time point post infection. This observation reflects what is seen *in vivo*. A study of Johnston and co-workers shows that primary HSV infections of the genital tract in humans are often followed by a clear HSV-viremia (12). In light of safe transfusion medicine, several patients with signs or history of herpes infections have been submitted to a screen for HSV DNA within their blood. Interestingly, in several primary genital herpes cases, significant levels of HSV DNA within the plasma could be demonstrated (13). This indicates that HSV is able to reach blood vessels and spread within the host by a yet unknown mechanism. Hence, the in this study established homologous model is an excellent tool for performing in-depth mechanistic studies of HSV host invasion.

In summary, the results of this study document to our knowledge for the first time the distribution of nectin-1, nectin-2 and HVEM within the lower female genital tract including endocervix and ectocervix/vagina during different phases of the normal menstrual cycle and point out the anatomical relevance of nectins to act as entry receptor for HSV. In addition, we report the first comparison of the mucosal dissemination of both human herpes simplex viruses within endocervical and ectocervical/vaginal mucosa derived from the same patients. Despite their different tropism, these viruses are both able to replicate and spread to a similar extent within genital mucosa and penetrate the BM. This study puts emphasis on the observed trend of increasing HSV-1 genital infections seen in the field (4) and stresses the need to combat HSV as measure in aid of successful anti-HIV therapy.

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CHAPTER 7.

THE gE/gI COMPLEX IS A CRUCIAL VIRAL FACTOR DURING BoHV-1 STROMAL INVASION IN RESPIRATORY MUCOSA

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In preparation

7.1. Abstract

Recently, we described the mucosal behavior of alphaherpesviruses including HSV-1, PRV and BoHV-1. Stromal invasion originates from epithelial plaques, happens plaquewise mainly between 24h and 48h pi and is unrestrained by the basement membrane barrier. Movement of virus across cell-cell contacts and axonal transport of viruses both require a functional glycoprotein complex gE/gI. Hence, we investigated the role of gE/gI in BoHV-1 directional spread across the basement membrane. First, we analyzed the effect of deleting or inhibiting gE/gI by using recombinants of BoHV-1 and anti-gE/gI monoclonal antibodies respectively on viral replication, plaque latitude and penetration depth underneath the basement membrane in a bovine respiratory mucosa model at 48h pi. Knockout of either gE and/or gI resulted in strong reduction of plaque latitude (on average 41.2 ± 7.7%) and plaque depth (on average 87.8 ± 6.0%) as well as viral replication (on average 1 to 2 log). Interestingly, usage of a gE cytoplasmic tail-truncated BoHV-1 (amber mutation at aa453) resulted in a similar phenotype as gE^-. None of the anti-gE/gI antibodies affected plaque latitude or viral replication. However, a reduction in plaque depth of on average 53.0 ± 15.8% compared to the controls could be observed. Finally, the distribution of viral particles for wild-type and knockout strains within cells and on the surfaces of cells in contact with the BM was studied at 48h pi by TEM and revealed a hampered sorting towards the basement membrane in gE/gI knockouts. These data indicate that gE/gI mediates sorting towards basal domains and possibly orchestrates BoHV-1 invasion once present at basal domains.

7.2. Introduction

Upon host entry, alphaherpesviruses are capable of breaching several selective barriers such as the basement membrane (BM) underneath epithelia in order to gain access to the stroma for further spread in the host by yet unidentified mechanisms (42). We used *in vitro* models consisting of excised mucosae as tools for visualization, identification and characterization of putative invasion mechanisms. At present, a classification can be made based on mucosal behavior of alphaherpesviruses. A first group spreads easily across the BM (e.g. PRV, BoHV-1 and HSV-1) in a plaquewise manner (15, 16, 43, 44). An unidentified trypsin-like serine protease was found to be involved in PRV-BM crossing (17). A second group, hitchhikes across the BM (e.g. EHV-1) by using local immune cells as Trojan horses (50). A third group, remains locally confined to the epithelium (e.g. EHV-4) and lacks the capacity to efficiently

invade the host (51). Hence, for many alphaherpesviruses transport towards the BM is a crucial event in the pathogenesis but studies describing these mechanisms in the mucosa are limited.

Glycoprotein E (US8) forms a non-covalently bound heterodimer with glycoprotein I (US7). They are both nonessential type 1 transmembrane proteins with a size of 575aa and 382aa respectively in BoHV-1 (5). BoHV-1 gE contains two cysteine-rich domains C1 and C2, and a GLYV domain on the N-terminal extracellular (ET) domain (1-421aa), whereas BoHV-1 gI only has one cysteine-rich domain on its ET domain (1-299aa). Both ET domains are sufficient for formation of the heterodimer (48). The C-terminal cytoplasmic tail (CT) of BoHV-1 gE (445-575aa) contains 4 YXXΦ motifs. There are three predicted tyrosine phosphorylation sites including residues 467-470aa (YTSL), 513-516aa (YDLA) and 563-566aa (YTVV). In addition, two serine residues within the gE acidic domain, S493 and S499, could potentially be phosphorylated (29).

gE and gI are multivirulence genes and are present in all field isolates, suggesting an important role for these proteins in pathogenesis (1, 39). The gE/gI complex promotes viral spread in many different ways (40). Herpesviruses use a direct cell-to-cell transmission to spread laterally across cell junctions and this is critical to successfully establish latency in neuronal cells. The molecular mechanism behind this event is not fully understood but shares similarities with the mechanism of entry employed by extracellular virions. Indeed, the core fusion machinery (gD, gH/gL, gB) is required but unlike for “cell-free” transmission, gE and gI heavily influence the efficiency of cell-to-cell spread. Mutants lacking gE and/or gI generate smaller plaques in cell cultures and moreover, are severely restricted for spread to neuronal cells (6, 7, 38, 55). For several alphaherpesviruses gE was found to modulate retrograde (e.g. HSV-1) and/or anterograde (e.g. BoHV-1, PRV, HSV-1) transport in neurons (10, 29, 31, 32). Similar observations are also made in several *in vivo* studies. Deleting gE in PRV resulted in decreased virulence and reduced stromal invasion (14, 19, 24). gE knockout in HSV-1 prevented the virus from causing severe stromal disease and potential encephalitis after epithelial keratitis (6). BoHV-1 gE null or gE truncated strains are strongly attenuated *in vivo* and all current “safe” vaccine strains are in fact gE deleted (3, 23, 29, 45, 49). In general, studies on gE/gI functionality have been performed mainly within the PRV and HSV background. However, BoHV-1 gE and gI can complement the virulence functions of PRV gE and gI (25, 26). The YXXΦ motifs on the gE CT allow the glycoprotein to interact with the trans-Golgi network (TGN) resulting in accumulation at the TGN. At the TGN, gE/gI

contributes to secondary envelopment of tegument-coated capsids but in addition it also routes TGN derived vesicles carrying mature virions towards lateral cell junctions. Indeed, the TGN is the major site for basolateral versus apical sorting in polarized cells as it regulates distribution of cellular membrane proteins into basolateral and apical domains (12, 21, 33, 52). gE/gI specifically accumulates at lateral surfaces of epithelial cells in confluent monolayers and colocalizes with the adherens junction protein β-catenin (8, 34). Alterations in the gE CT reduce the number of virions at lateral cell junctions and instead virions accumulate on apical surfaces (22). Several tegument proteins including the triplex UL11-UL16-UL21 are believed to play a role in trafficking of virions by binding the gE tail (13, 18). Once present at junctions, the ET domains of the complex gE/gI probably bind to extracellular ligands such as proteins of intercellular junctions and promote spread to adjacent cells (36). Interestingly, in some monolayers of epithelial cells viruses were found to be specifically routed to cell junctions in lateral domains, rather than to basal domains. Although, in cell biology it is accepted that the plasma membrane of polarized cells can be grossly subdivided in apical and basolateral domains, separated by tight junctions (30), it appears that additional sorting mechanisms might exist beyond the broad targeting of molecules to basolateral surfaces. Indeed, some proteins of cell-cell junctions in lateral domains are not found in basal domains of the plasma membrane and vice versa, proteins such as integrins are exclusive to cell-matrix junctions in basal domains (22, 53).

Bovine herpesvirus 1 (BoHV-1) causes severe respiratory and genital lesions in ruminants that easily penetrate through both mucosa and submucosa (35, 43). We previously optimized a bovine respiratory mucosa model to study several aspects of the primary replication of BoHV-1 and observed that most BoHV-1 plaques cross the BM at 48h pi (44). In this study, we applied this homologous model to specifically investigate the role of the viral complex gE/gI in vertical spread and hence the invasion mechanism of BoHV-1 through the BM. We constructed gE-null, gI-null, gEgI-null viruses and repaired strains by using a BoHV-1 bacterial artificial chromosome (BAC) clone. In addition, a gE CT-truncated virus was included. Both usage of gE and/or gI knockout strains and gE and/or gI inhibiting monoclonal antibodies resulted in severe reduction in BoHV-1 vertical spread and invasion through the BM barrier. Moreover, gE/gI was found to mediate efficient sorting of vesicles and virus particles in basal cells towards the BM as shown by TEM. These observations put emphasis on the involvement of the gE/gI complex in BoHV-1 invasion through the BM towards the lamina propria.

7.3. Materials and methods

7.3.1. Construction of BoHV-1 gE null, gI null, gE/gI null by en passant mutagenesis

We obtained a previously established BoHV-1 Cooper BAC clone (29) and stably transformed the BAC plasmid containing BoHV-1 genome into GS1783 (DH10B λ *cI*857 Δ(*cro-bio*A)<>*araC*-P_{BAD} *I-SceI*), which is the preferred host for en passant mutagenesis. This resulted in the construction of pBoHV-1_GS1783_GP1. Subsequent, en passant mutagenesis was performed as described earlier (46). In a first step, the mutated glycoprotein allele along with an antibiotic resistance gene and adjoining *I-SceI* recognition site was produced by polymerase chain reaction (PCR). To this end, primers BoHV-1_ΔgE-for, BoHV-1_ΔgE-rev, BoHV-1_ΔgI-for, BoHV-1_ΔgI-rev were designed to delete US7 and/or US8 (Table 1). The 3' end of the forward primers were designed in this way that it overlapped with an *I-SceI* site located upstream of aphAI (kanamycin resistance), and that the 3' end of the reverse primers were complementary to the sequences downstream of aphAI of pEP-kan-S2. Second, approximately 100ng of *DpnI*-digested ΔgE PCR product was incorporated via electroporation into 50µl of recombinant- and electrocompetent pBoHV-1_GS1783_GP1. After arabinose induction, several kanamycin-sensitive clones were obtained and screened via RFLP, PCR and sequencing. This resulted in pBoHV-1_GS1783_ΔgEC6. Subsequently, a similar approach was applied to transform both pBoHV-1_GS1783_GP1 and pBoHV-1_GS1783_ΔgE_P6C6 with DpnI-digested ΔgI PCR product to obtain pBoHV-1_GS1783_ΔgI_P3C1 and pBoHV-1_GS1783_ΔgEgI_P7C1 respectively. Finally, to excise the BAC cassette, all obtained clones as well as the wild-type BAC clone were co-transfected along with pCAGGS-nls-cre into MDBK cells. At least three rounds of plaque purification were performed to obtain pure BAC-excised clones. Henceforth, reconstituted BAC-excised vBoHV-1_GS1783_GP1, vBoHV-1_GS1783_ΔgE_P6C6, vBoHV-1_GS1783_ΔgI_P3C1 and vBoHV-1_GS1783_ΔgEgI_P7C1 are named WT, gE null, gI null and gE/gI null virus.

7.3.2. Construction of BoHV-1 gE null, gI null and gE/gI null rescue BoHV-1

Construction of repaired strains was performed in two phases. A first phase, consisted of the insertion of a kanamycin resistance gene into the open reading frame coding for gE(US8) of the pBoHV-1_GS1783_GP1 (WT BAC). A similar PCR was performed as described for the construction of mutant alleles using forward and reverse primers (Table 1) and plasmid pEP-

kan-S2 to generate a PCR product for insertion in pBoHV-1_GS1783_GP1 by en passant mutagenesis. This resulted in pBoHV-1_GS1783_GP1_gEgIKan.

Table 1. Primers used for generating gE and/or gI null and repaired strains

Primers	Nucleotide sequence ([a])
Mutants	
gE deletion	
Forward	5'AACGGCGCACGCGAGAGGGTTCGAAAAGGGCATTTGGCAATG-**TAA**-CCGGCCGCACCAGAT-aggatgacgacgataagtaggg
Reverse	5'ACTTGAGTCGCGCTGCTACCACGGTGTAATCTGGTGCGGCCGG-**TTA**-CATTGCCAAATGCCCTT-caaccaattaaccaattctgattag
gI deletion	
Forward	5'ACTCAAGCCATTGCCGCGACCTTGTCCTCCGGCGCGCTCGCGATG-**TAA**-ACGCCGCGCGCGGCA-aggatgacgacgataagtaggg
Reverse	5'GAAACCAAGACACACGCGAGCGAGATCGTTTGCCGCGCGCGGCGT-**TTA**-CATCGCGAGCGCGCC-caaccaattaaccaattctgattag
Rescues	
Kan insertion gE	
Forward	5'AACGGCGCACGCGAGAGGGTTCGAAAAGGGCATTTGGCAATG-CAACCCACCGCGCCGCCC-aggatgacgacgataagtaggg
Reverse	5'GCAGCAGCAGCGGCAGCAACCGCCGCCGGGGGCGGCGCGGTGGGTTG-CATTGCCAAATGCCCTT-caaccaattaaccaattctgattag
Pull out gE/gI	
Forward	5'CGCGGTGCGGGTCCGCTGCCCAGAAAGCCAAAAAAGCTGCCG-**CCATGG**-*ccacatgtggaattcccat* (**NcoI**)
Reverse	5'CGCTCACCCGGGCAGCGCGCTGTAGTTGACGTTGCCAAAGGC-**GAATTC**-*gtcatccatatcaccacg* (**Eco-RI**)

[a] Nucleotides in lower case and italicized lower case share homology to pEP-kan-S2 and oriR6K+ampres (pGS1292) respectively. Sequences that are underlined are complementary to each other in inverse orientation. Finally, introduced stop codons in the mutant primers and introduced restriction endonuclease sites in the rescue primers are in bold.

In a second phase, a pull-out of the gE/gI region out of the WT BAC was performed. For this purpose, primers were designed (Table 1) to anneal with their 5' end to a region upstream of the gE/gI region and their 3' end to pGS1292 (oriR6K+ampres). Upstream of the 3' end homology of the primers with pGS1292 an *NcoI* RE-site and *Eco-RI* RE-site were introduced in the forward and reverse primer respectively. *NcoI* was chosen, as an endogenous *NcoI* RE site is located downstream of the gE/gI region and moreover does not cut the gE/gI region, nor the previously introduced kanamycin cassette (pEP-kan-S2), nor the oriR6K+ampres (pGS1292). *Eco-RI* was introduced to finally remove oriR6K+ampres (pGS1292). The obtained PCR product was finally employed to transform pBoHV-1_GS1783_GP1_gEgIKan

into pBoHV-1_GS1783_GP1_gEgIKanAmp. Next, a pull-out of the wild-type gE/gI region was performed using *Nco-I*. The obtained fragments were self-ligated and electroporated into GS111 (S17λpir). Correct clones were saved and subsequently *Nco-I* and *Eco-RI* were used to liberate the gE/gI region from oriR6K+ampres (pGS1292). Finally, we used the obtained pull-out gE/gI fragment, which still possessed a kanamycin marker in US8 (first phase), to transform pBoHV-1_GS1783_ΔgE_P6C6, pBoHV-1_GS1783_ΔgI_P3C1 and pBoHV-1_GS1783_ΔgEgI_P7C1. After arabinose induction, kanamycin sensitive clones were screened (RFLP, PCR and sequencing), co-transfected with pCAGGS-nls-cre into MDBK's for BAC excision, trice plaque purified and finally named vBoHV-1_GS1783_ΔgE_P6C6_RESC (gE rescue), vBoHV-1_GS1783_ΔgI_P3C1_RESC (gI rescue) and vBoHV-1_GS1783_ΔgEgI_P7C1_RESC (gE/gI rescue).

7.3.3. Virus strains and cell line

The BoHV-1 WT, gE null, gI null, gE/gI null, gE rescue, gI rescue, gE/gI rescue together with a previously constructed gE truncated CT tail gEAm453 (gE CT null) (29) were propagated (2^{nd} passage) and titrated in Madin-Darby bovine kidney (MDBK) cells as previously described (2).

7.3.4. Antibodies

In the inhibition experiment we used several monoclonal antibodies including 14B11 (anti-gE), 13E8 (anti-gE) and 2E12 (anti-gE/gI), which were a kind gift of the Veterinary and Agrochemical Research Centre Belgium. The gE/gI protein specificity of the monoclonal antibodies was determined via IPMA, IF stainings on cells inoculated with BoHV-1 WT, gE null, gI null, gE/gI null and all rescue strains; and by Western blot analysis. Isotype matched, irrelevant IgG1 (41D3) and IgG2a (XVI-11C) antibodies for the latter monoclonal antibodies against gE/gI were included in the inhibition experiment. 41D3 is directed against porcine Sialoadhesin (9) and XVI-11C/5-10F recognizes GP4 of the porcine reproductive and respiratory syndrome virus (PRRSV) (4). In addition, mouse anti-collagen VII antibodies (Sigma, St. Louis, MO, USA) and goat anti-mouse Texas Red® (Molecular Probes (Invitrogen, Paisley, UK)) were used to stain the basement membrane, and an FITC®-labeled

goat anti-IBR polyclonal antiserum (VMRD, Pullman, WA, USA) was used to stain the virus in immunofluorescence stainings.

7.3.5. Selection of animals and set up of bovine respiratory mucosa explants

Bovine respiratory mucosa explants were obtained as previously described (44). Briefly, from 4 cows (2-5 years old) tracheal respiratory mucosa was obtained in the slaughterhouse. In addition, blood was collected to determine the BoHV-1 specific neutralizing antibody level. Tracheal tissue was immediately placed in tubes containing phosphate buffered saline (PBS), supplemented with 1 µg/mL gentamycin (Invitrogen), 1 mg/mL streptomycin (Certa, Braine l'Alleud, Belgium), 1 mg/mL kanamycin (Sigma), 1000 U/mL penicillin (Continental Pharma, Puurs, Belgium) and 5 µg/mL fungizone (Bristol-Myers Squibb, New York, USA) for transportation to the laboratory. Next, small square tissue pieces were produced and placed on fine-meshed gauze for culture. The explants were cultivated in serum-free medium (50% DMEM (Invitrogen)/50% Ham's F-12 GlutaMAX (Invitrogen)) supplemented with 0.1 mg/mL streptomycin (Certa), 100 U/mL penicillin (Continental Pharma) and 1 µg/mL gentamycin (Invitrogen) for up to 72h (37 °C, 5% CO_2). Explants were kept in culture for 24h before inoculation and during the entire cultivation period, the culture medium was not replaced.

7.3.6. Inoculation and inhibition method

At 24h of cultivation, explants were taken from their gauze and placed in a 24-well plate after rinsing with warm medium. In each well, 1 mL of virus-containing medium (10^7 $TCID_{50}$/mL) was added. The submerged explants were incubated for 1h (37 °C, 5% CO2). Before the tissues were placed back again on their gauze, they were thoroughly washed.

For the gE/gI knockout experiment, we inoculated 2 tracheal explants of all the animals with all different BoHV-1 mutated and repaired strains as well as the wild-type strain and collected them at 0h and 48h pi for snap-freezing at -70°C. Mock-inoculated explants were included as controls.

For the gE/gI inhibition experiment, we inoculated 2 tracheal explants of each animal with the wild-type strain. However, from 0h pi onwards, medium was replaced by either medium

supplemented with the different monoclonal antibodies against gE and/or gI or medium supplement with irrelevant, isotype-matching antibodies. As a control, several explants were cultivated in medium without antibodies. During the 1st h pi, all explants were completely submerged. At 0h and 48h pi, explants were collected and snap-frozen.

For the analysis of subcellular gE, several explants were wild-type BoHV-1-inoculated. At 48h pi, we rinsed the tissues before submerging them in an anti-gE monoclonal antibody, 14B11, rich medium for 4h at 4°C, allowing the antibodies to bind to gE. Afterwards, explants were gathered and snap-frozen.

For the assessment on the localization of virions within basal cells by TEM, we inoculated several explants of all animals either mock or with WT, gE null, gI null, gE/gI null, gE rescue, gI rescue and gE/gI rescue strains. At 48h pi, tissues samples were taken for an overnight incubation at 4°C in Karnovsky's fixative (2% paraformaldehyde and 2.5% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7.4)). After overnight fixation, samples were washed in sodium cacodylate buffer and post-fixed for 1.5h in 2% osmium tetroxide (4°C). Subsequently, dehydration of tissue, using a graded series of alcohol (50–100%) was performed and finally samples were embedded in Spurr's resin. For localization of viral plaques within the epithelium (region of interests), semithin sections stained with haematoxylin-eosin staining were analyzed. Afterwards, ultrathin sections of 60nm thickness were made using a Leica EM UC 6 ultramicrotome (Leica Mircosystems GmbH), and mounted on formvar coated single slot copper grids (Laborimpex N.V., Brussels, Belgium). The sections were post-stained with uranyl acetate and lead citrate (Leica EM Stain, Leica Microsystems GmbH) and viewed on a Jeol 1200 EXII TEM (JEOL Ltd., Tokyo, Japan) at 80 kV accelerating voltage.

7.3.7. Analysis of virus replication and dissemination in mucosa

Growth kinetics

We first performed one-step growth kinetics of both wild-type, mutants and revertants on MDBK cells. Confluent MDBK cells in 6-well plates were inoculated with 1mL of 10^3 $TCID_{50}$/mL virus containing medium for 1h (37°C, 5% CO_2). Cell supernatant was harvested at 2h, 24h and 48h after inoculation and submitted for virus titration.

In a second step, from all explants either inoculated with wild-type, mutants and revertants (gE/gI knockout) or with monoclonal antibodies (gE/gI inhibition), explant cultivation medium was collected at 2h, 24h and 48h pi for analysis of viral titers.

Briefly, for virus titration of all samples, confluent MDBK cells were inoculated for 1h (37°C, 5% CO_2) with serial 10-fold dilutions (10^0 to 10^{-7} in quadruplicate) of virus-containing medium. Afterwards, MDBK's were overlaid with medium and observed daily for cytopathic effect (CPE) for 7-9 days. Time point 2h was included to determine residual virus titers after inoculation. As a control, BoHV-1 inactivation at 37°C, 5% CO_2 in explant medium was examined at 0h, 2h, 24h, 48h and 72h after adding either 10^7 $TCID_{50}$/mL, 10^5 $TCID_{50}$/mL or 10^3 $TCID_{50}$/mL of BoHV-1 as a start titer to control wells.

Immunofluorescence staining and plaque analysis

In general, cryosections were made from all frozen explants and fixed in methanol (-20°C, 100%) for 25 minutes.

The immunofluorescence staining aimed at visualizing the effect of gE/gI knockout and inhibition on plaque characteristics was a double staining, as previously described (43). Firstly, to stain the BM, mouse anti-collagen VII antibodies (Sigma) (1:300 in PBS) and goat anti-mouse Texas Red® (Molecular Probes (Invitrogen)) (1:50 in PBS) were used. Next, an FITC®-labeled goat anti-IBR polyclonal antiserum (VMRD, Pullman, WA, USA) (undiluted) directed against viral proteins was applied. Before mounting with glycerin-DABCO, a Hoechst staining, visualizing nuclei, was included (1:100 in PBS). In between each step, thorough washing steps were included.

When staining cryosections for the localization of gE within an BoHV-1 epithelial plaque, we first used secondary goat anti-mouse Texas Red® to detect the already bound mouse monoclonal antibodies on gE. Secondly, we used FITC®-labeled goat anti-IBR polyclonal antiserum and Hoechst. Secure washing was performed at each interval of the immunofluorescence staining before mounting with glycerin-DABCO.

Finally, all samples were analyzed by using a DM2500(Sim) confocal microscope (Leica).

7.3.8. Statistical analysis

Evaluation of variance was performed using SPSS software (ANOVA) to analyze the data statistically. Differences in results that had P values of ≤ 0.05 were considered as being significant. All data represent means + SD of ≥ 3 independent experiments.

7.4. Results

7.4.1. BoHV-1 gE null, gE CT null, gI null and gE/gI null strains are severely impaired in stromal invasion

We previously observed that the majority of BoHV-1 plaques is penetrating the basement membrane at 48 pi. Therefore, respiratory tissues from different animals, infected with different infectious clones were analyzed for the effect of gE/gI knockout on plaque latitude and plaque penetration depth underneath the basement membrane at 48h pi. We analyzed the dissemination characteristics of on average 8-10 plaques for each condition.

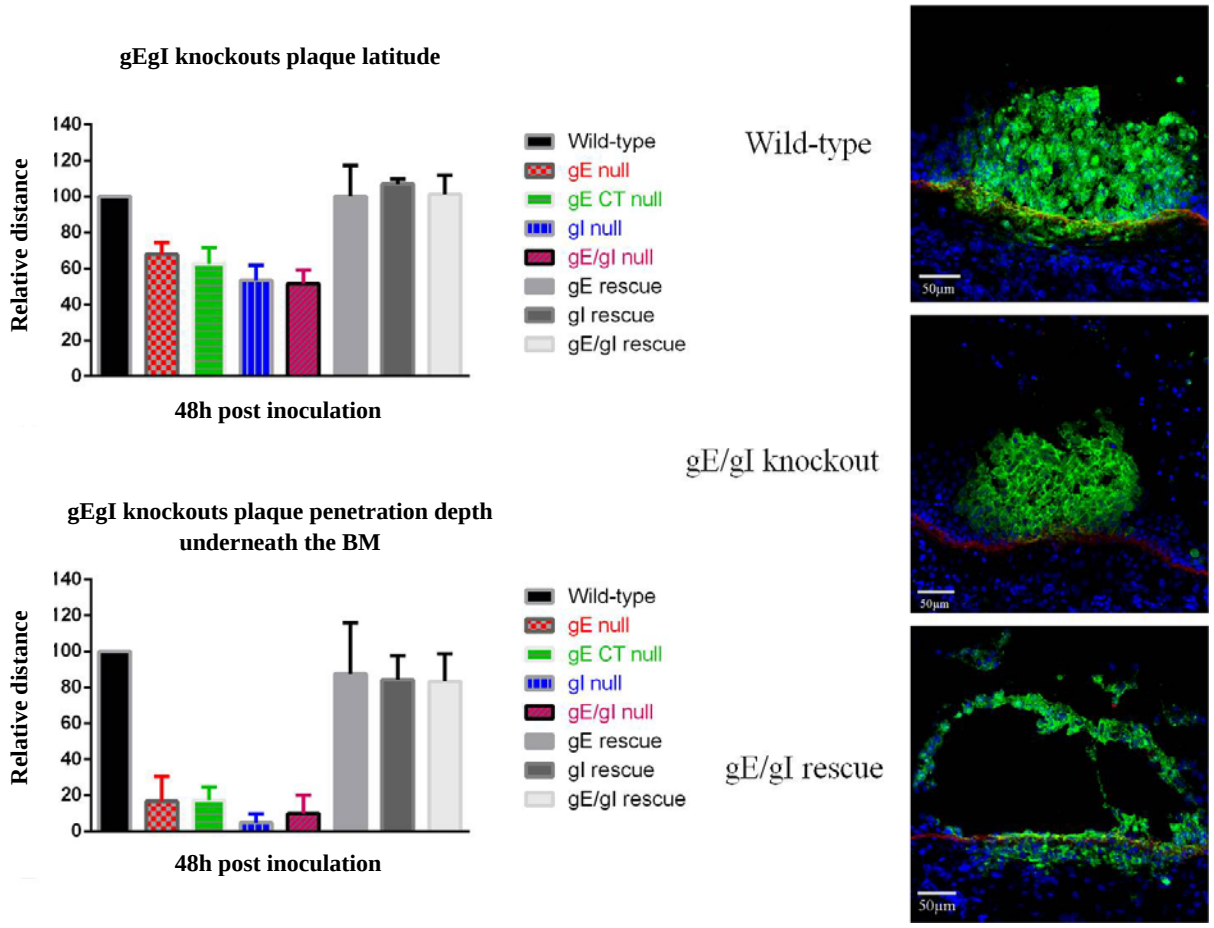


Figure 1. Dissemination characteristics of wild-type; gE and/or gI null; and repaired strains in respiratory mucosa at 48h pi. The graphs show quantitative data (relative) comparing the plaque latitude and penetration depth underneath the BM of mutant and repaired strains in relation to the wild-type. The data are given as means + SD and represent three different independent experiments. In addition, representative confocal immunofluorescence images are shown for wild-type, gE and/or gI null and gE and/or gI rescued strains. Viral antigens are stained by FITC, the BM by Texas Red and cell nuclei by Hoechst.

The gE null mutant caused a significant reduction in plaque latitude and plaque penetration depth compared to the wild-type strain (Figure 1). Knockout of gI in both WT (gI null) and gE null (gE/gI null) resulted in a strong decrease in plaque latitude and plaque penetration depth. Next, we applied a gE cytoplasmic tail-truncated BoHV-1 (amber mutation at aa453). Interestingly, a similar phenotype as gE null could be observed (Figure 1). All revertant strains (gE rescue, gI rescue and gE/gI rescue) displayed similar dissemination characteristics as the wild-type strain.

7.4.2. Anti-gE/gI monoclonal antibodies inhibit BoHV-1 invasion through the basement membrane

Several wild-type inoculated explants were submerged for 1h after inoculation within medium enriched with anti-gE/gI monoclonal antibodies or irrelevant, isotype matching antibodies, or in medium without antibodies (mock). Afterwards, they were further cultivated on gauze in medium containing or lacking antibodies until collection (48h pi).

None of the gE and/or gI-specific monoclonals affected plaque latitude. However, when analyzing BoHV-1 penetration depth underneath the basement membrane, all gE/gI-specific monoclonals did exhibit to some degree a reduction compared to the mock-treated and irrelevant antibodies-treated situation. However, the reduction did vary in between the different monoclonals tested. Both 14B11 and 13E8 showed a significantly stronger reduction compared to the mock and isotype controls. In contrast, although 2E12 shows reduction, this difference was found not to be significant. In general, the observed effect of gE/gI inhibiting antibodies on BoHV-1 stromal invasion was less dramatic than the effect seen with gE/gI knockout strains.

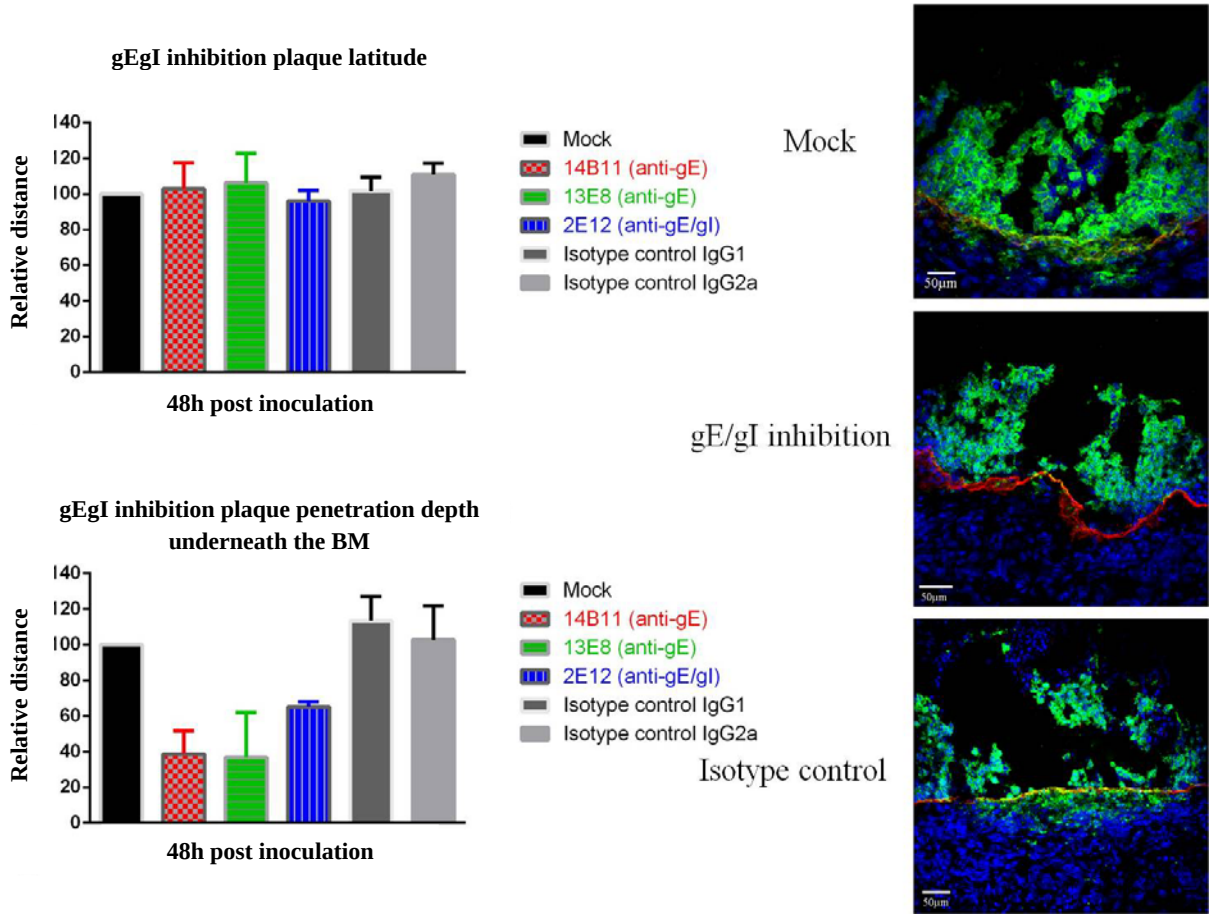
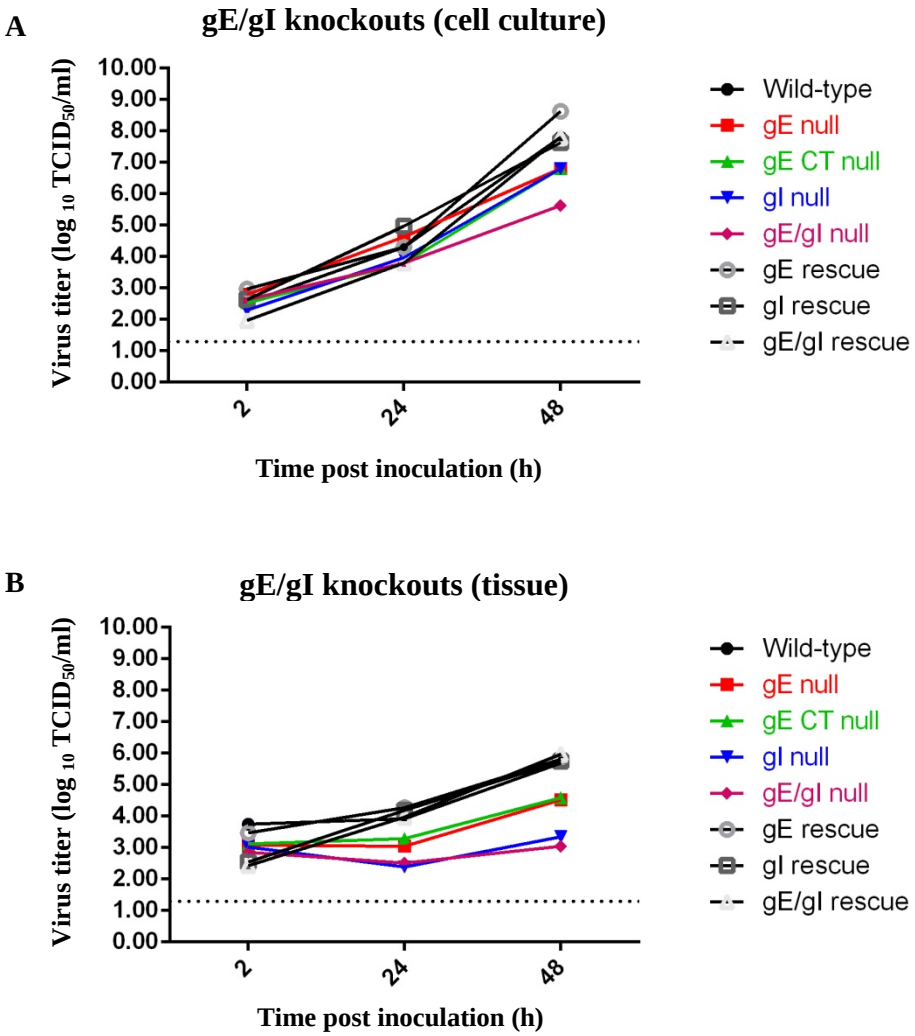


Figure 2. The effect of anti-gE/gI monoclonal antibodies on BoHV-1 stromal invasion. A comparison between the plaque latitude and penetration depth underneath the BM of wild-type virus within respiratory mucosa treated with gE/gI inhibiting antibodies or irrelevant, isotype matching control antibodies and mock-treated explants is given in the graphs (relative data). Data are given as means +SD of three different experiments. Moreover, illustrative photomicrographs taken by a confocal microscope depict BoHV-1 (FITC) invasion through the basement membrane (Texas Red) in either mock-treated, gE/gI inhibiting antibodies-treated or isotype control antibodies-treated explants. Nuclei are stained by Hoechst.

7.4.3. Knockout of BoHV-1 gE/gI results in a kinetic defect in virus production in both cell culture and tissue

We analyzed growth kinetics to determine viral replication (Figure 3). First, we looked at the replication characteristics of gE/gI knockout strains in both MDBK cells and respiratory mucosa explants. In both cell culture and tissue we could observe a kinetic defect in viral replication in both gE null, gE CT null, gI null and gE/gI null strains compared to wild-type and repaired strains. Interestingly, at 24h pi this discrepancy in replication efficiency was

already visible in tissues but not in cell culture. Although at 48h pi a clear reduction in viral titers of gE and/or gI knockouts can be observed in cell culture as well as tissue, the most dramatic effect is seen in tissue replication. In tissue, knockout of gE resulted in a 1 log reduction whereas knockout of gI resulted in a 2 log reduction in viral progeny titers. In cell culture, knock-out of gE and/or gI resulted in smaller plaques. In cell culture, much higher viral titers were reached within the same time span compared to tissues. Second, when analyzing viral titers of the gE/gI inhibition experiment using anti-gE/gI monoclonal antibodies, we could not observe any effect on virus production due to the presence of antibodies in the medium. In addition, a normal BoHV-1 inactivation course over time at 37°C is given in Figure 3D.



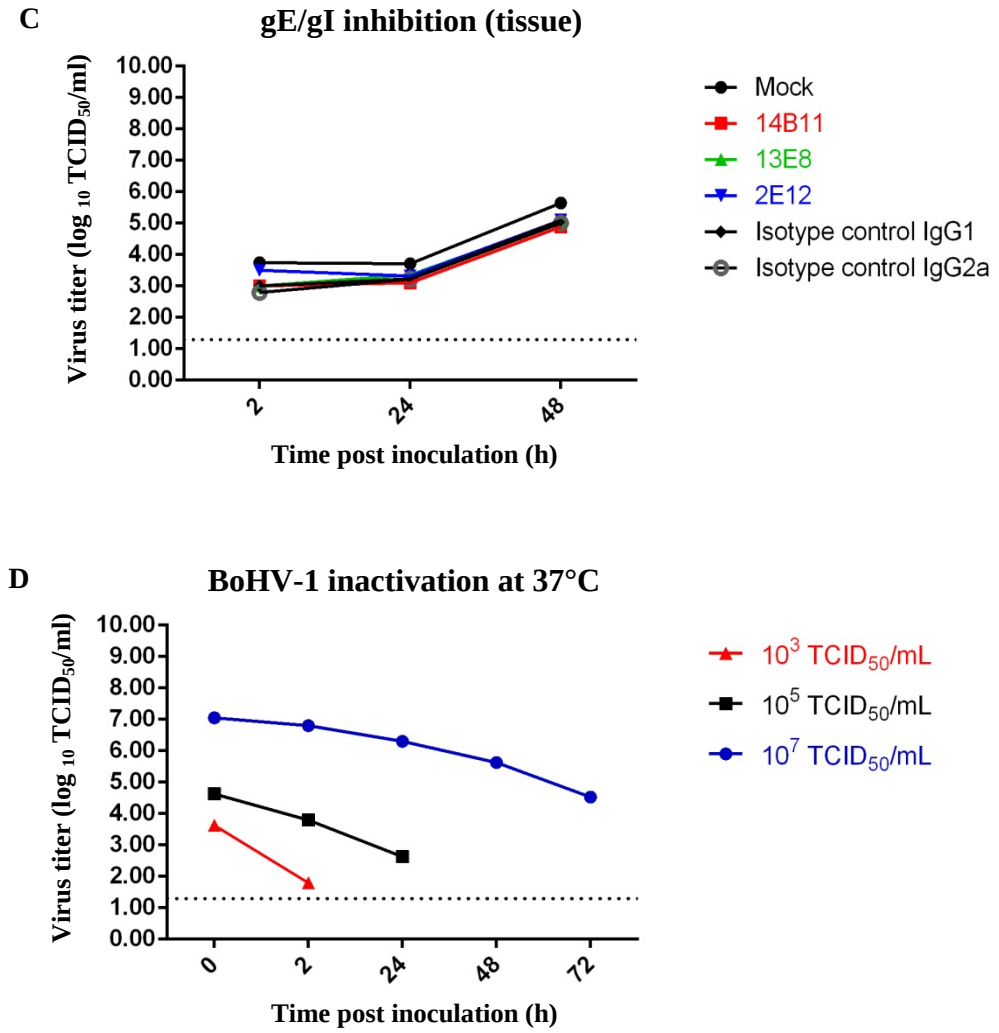


Figure 3. Representative graphs showing one-step growth curves of (A) BoHV-1 wild-type, gE and/or gI null and rescued strains in MDBK cells; (B) BoHV-1 wild-type, gE and/or gI null and rescued strains in respiratory mucosa explants and (C) BoHV-1 wild-type strain in the presence of either anti-gE/gI, irrelevant or no antibodies in respiratory mucosa explants. Samples were taken at 2h, 24h and 48h pi. As a reference, a normal BoHV-1 inactivation curve over time at 37°C for different starting titers is given in D.

7.4.4. gE predominantly localizes at basal cell sides near the basement membrane in BoHV-1 epithelial plaques

Distribution of subcellular gE was assessed by looking at wild-type inoculated explants that were submerged for 4h at 4°C within gE monoclonal antibodies before snap-freezing. Within the majority of BoHV-1 plaques, a strong expression of gE was observed near the basement

membrane at 48h pi in respiratory mucosa explants. Less expression of gE was found in between cells and was even negligible at the luminal side of epithelial plaques. On the contrary, a polyclonal antibody recognizing multiple proteins of BoHV-1 we employed in double stainings showed a dispersed pattern ranging from the apical to basal sides of BoHV-1 induced epithelial plaques. Representative pictures are provided in Figure 4.

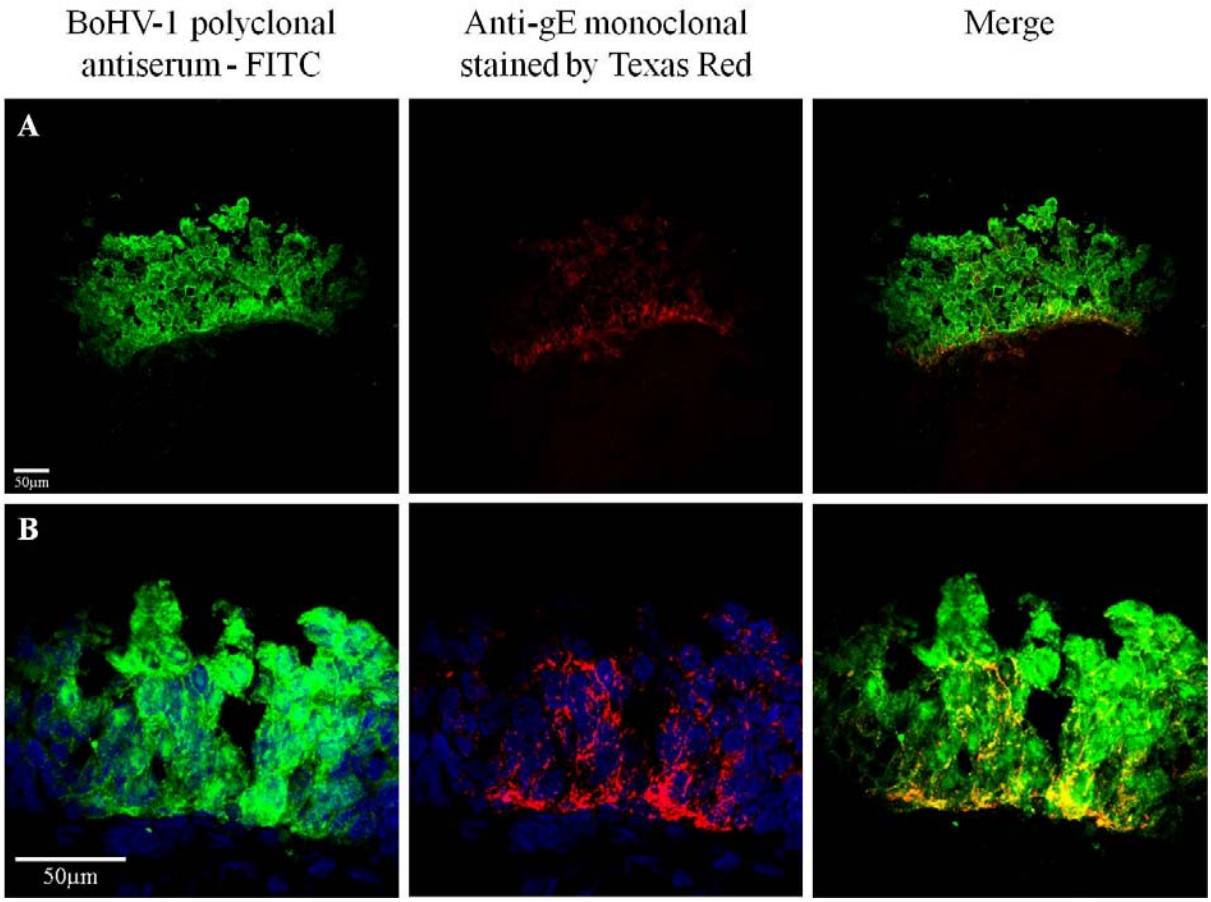


Figure 4. Double immunofluorescence stainings showing a predominant basal expression of gE within BoHV-1 epithelial plaques. First, a BoHV-1 polyclonal anti-serum, staining multiple proteins (FITC) was used. Next, a Texas Red conjugate was used to visualize the already bound gE monoclonals within the tissues. Pictures in row A and B show representative pictures for gE localization. Row B is taken at a higher magnification than row A.

7.4.5. Basal sorting in basal epithelial cells towards the basement membrane depends upon gE/gI

Analysis of virion localization within basal epithelial cells and on the surface of basal epithelial cells near the basement membrane by means of transmission electron microscopy (TEM) was performed on respiratory tissues at 48h pi inoculated with either wild-type, gE and/or gI null or gE and/or gE rescue strains. It is noteworthy that this study is technically difficult to perform as fewer epithelial cells are being infected compared to monolayers of cells. For wild type and repaired strains, we observed several virus-containing vesicles being sorted towards the basement membrane and noted several virus particles crossing the basement membrane barrier (Figure 5). In addition, the majority of virus particles accumulated near lateral and especially basal borders of cells. We did not observe many apical sorted virions.

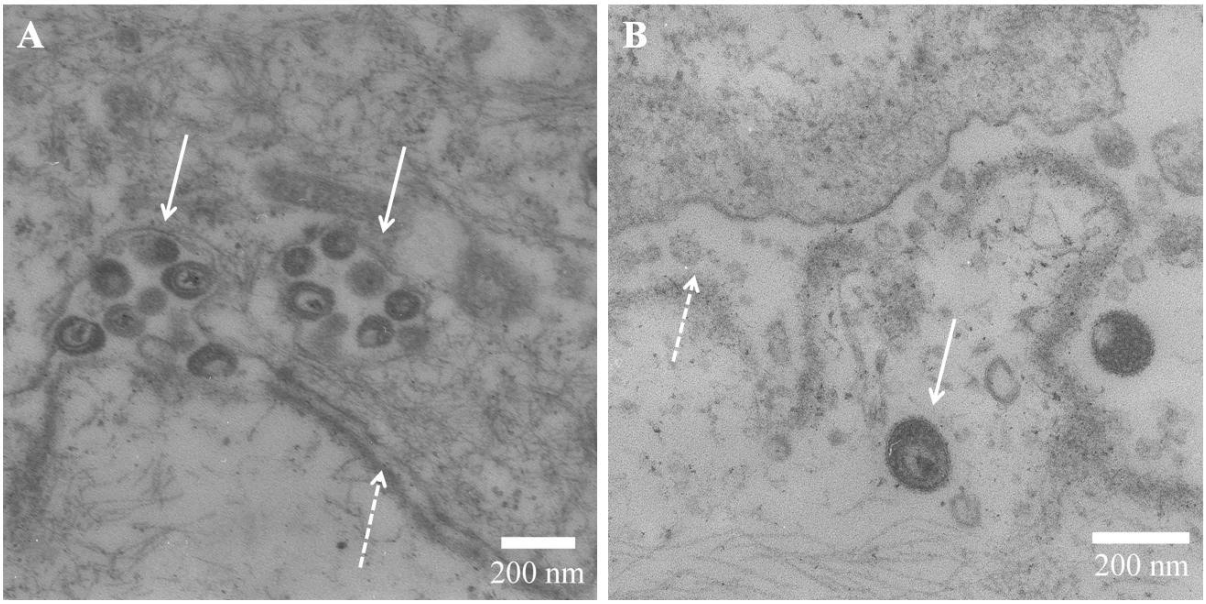


Figure 5. Images taken by TEM showing (A) sorting virus-containing endocytotic vesicles towards the basement membrane and (B) virus particle passage across the basement membrane in wild-type and rescue BoHV-1 strains inoculated respiratory mucosa explants. Vesicles carrying virions and free virions are marked by a white arrow. The basement membrane barrier is indicated by a dashed white arrow. Magnification 30.000x (A) and 40.000x (B).

On the contrary, when looking at gE null, gE CT null, gI null and gE/gI null strains, a highly reduced percentage of virions were found on basal domains compared to the WT and rescue

strains (Figure 6). The majority of virions accumulated more frequently on non-basal cell sides and showed a more dispersed pattern within cells. Importantly, the gE CT null resulted in a similar phenotype as gE null indicating that, taken into account all latter findings, gE/gI appears to have the ability to govern basal sorting of virus particles towards basal cell sides of cell in direct contact with the basement membrane.

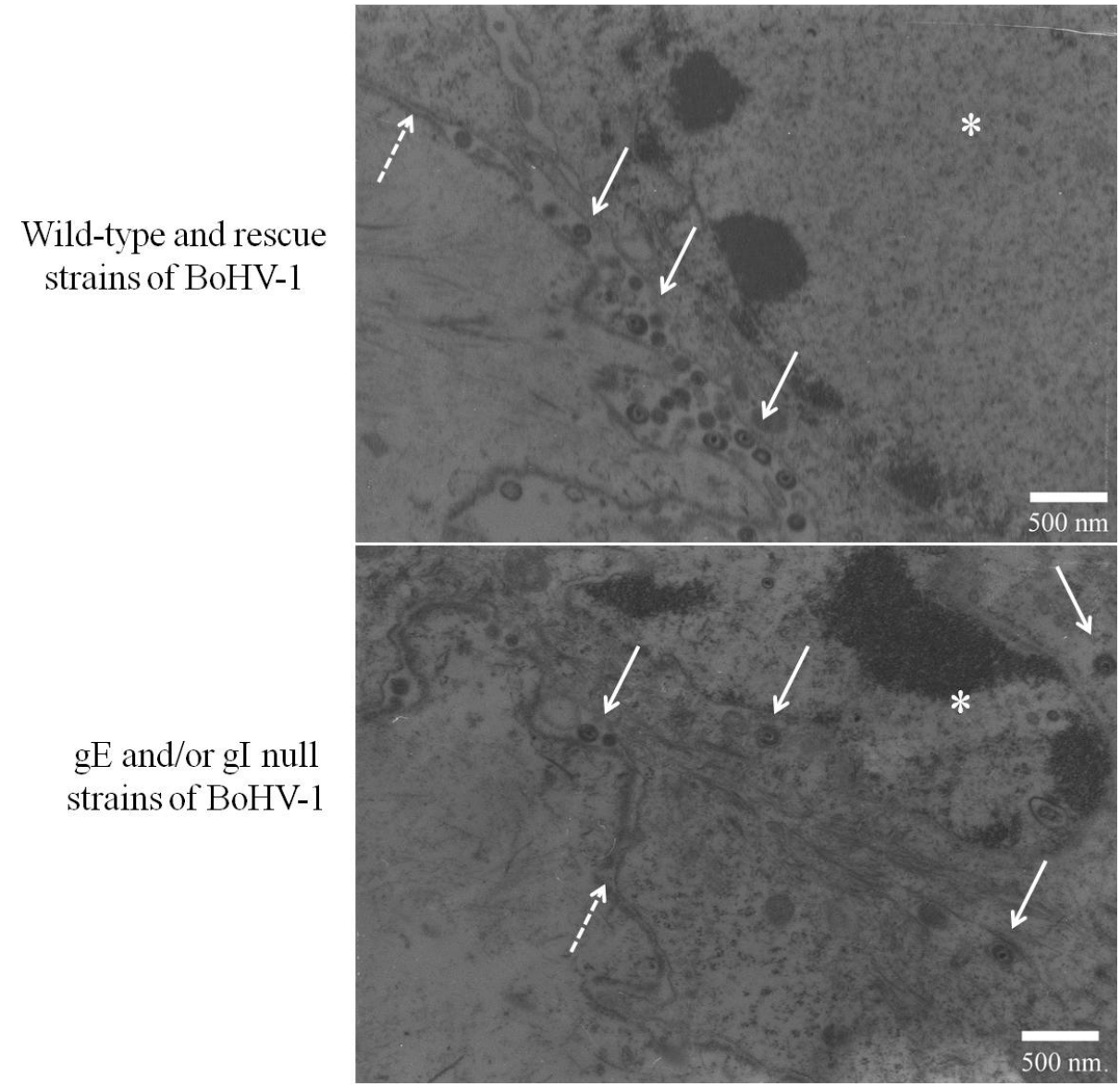


Figure 6. Representative TEM images of a basal cell infected with either wild-type (above, representative for WT and rescue strains) or gE null (below, representative for gE and/or gI null strains) of BoHV-1 at 48h pi in bovine respiratory mucosa. The majority of wild-type and rescue strain virions accumulated near the basement membrane at basal plasma membrane domains. On the contrary, gE and/or gI null strains particles were more frequently observed on the non-basal sides of basal epithelial cells. Virions are indicated by white arrows and the basement membrane barrier by a white dashed arrow. An asterisk marks the location of the basal cell nucleus. Magnification 15.000x.

7.5. Discussion

Bovine herpesvirus 1 (BoHV-1) uses the respiratory epithelium as an important portal for host entry. A hallmark for many herpesviruses is that protective barriers such as the basement membrane (BM) underneath epithelia are not able to restrain these viruses. They devised ingenious mechanisms to overcome these barriers and spread within the host (42). To date, knowledge on viral invasion mechanisms within mucosal tissues is however, largely unknown. Recently, we studied the mucosal behavior of BoHV-1 at the level of the respiratory and genital tract. BoHV-1 induces prominent epithelial plaques and penetrates in a plaquewise manner the BM starting from 24h pi. At 48h pi, nearly all plaques show a clear stromal invasion (43). Thus, a crucial step in this dissemination process is virus transport towards the BM. The main cell type in contact with and covering the BM are basal cells (11). This could involve a directed transport via basal cells by a so far, unidentified mechanism. In addition, when comparing different zones of the upper respiratory tract, including nasal septum, ventral conchae and proximal trachea; we could observe a more efficient spread of BoHV-1 in tracheal tissue compared to septum and conchae. Therefore, tracheal tissue is an interesting target tissue to unravel BoHV-1 stromal invasion (44).

In the present study, we found different indications that gE/gI mediates efficient invasion of BoHV-1 at mucosal surfaces. In a first part, we constructed gE null, gI null and gE/gI null strains and their respective rescued strains. Analysis of growth kinetics of gE and/or gI null infectious clones in both cell culture and tissue showed a kinetic defect that was most clear at 48h pi compared to wild-type and repaired strains. At 24h pi, this effect was already observed in tissue but not in cell culture. Unlike a monolayer of cells, tissues are a 3D-structure. Any defect in spread to naïve cells is exponentially more substantial within a 3D compared to a 2D model. Both gE null, gI null and gE/gI null viruses resulted in severely suppressed BoHV-1 spread within respiratory mucosa. All mutants were analyzed for plaque latitude and penetration depth underneath the BM in respiratory mucosa at 48h pi. Knockout of gI caused an even more dramatic phenotype, both in a wild-type background (gI null) as in a gE null background (gE/gI null) compared to knockout of gE. Both observations indicate that a full functional gE/gI complex is required for BoHV-1 invasion. In addition, application of a gE cytoplasmic tail-truncated BoHV-1 (amber mutation at AA 453) produced a similar phenotype as gE null. This observation suggests that sorting, mediated by previously identified cytoplasmic tail motifs, is required for basal migration of viral particles. HSV-1 and PRV gE are known to mimic intracellular sorting pathways to route viral particles towards

cell-cell junctions in lateral domains (6, 8, 55). Especially in certain polarized epithelial cells a predominant transportation towards cell-cell junctions in lateral domains is observed and less to basal domains (22). It should be noted that these observations have been made in monolayers of cells, a 2D environment. As these cells are not anchored via cell-matrix adhesions to the stroma, additional sorting pathways towards basal domains might simply not be present/functional. Reasons to believe additional basal sorting pathways exist in tissues, representing a 3D structure, are threefold. First, some proteins present in basal domains (e.g. integrins) are not present in lateral domains whereas proteins in lateral cell-cell junctions are not found basally (53). Second, HSV virions were found to accumulate in some epithelial cells mainly on lateral sides and only scarcely basal and apical (22). Third, during interkinetic nuclear migration (INM), the nuclei in many epithelial cells specifically migrate between apical and basal surfaces, coordinating with the cell cycle. INM is observed in a wide variety of tissues and species but especially in pseudostratified epithelia such as the neuroepithelium but also the respiratory epithelium. This directed transport is either actomyosin-driven or microtubule-driven. As such, basal movement of nuclei requires either myosin II or motor protein kinesin 3 KIF1A (28, 41, 47). Herpesviruses are known to misuse motor proteins in general including kinesin and dynein for anterograde and retrograde transportation respectively within neuronal cells (37, 54). Interestingly, it has been recently shown for PRV that the gE/gI complex is vital in mediating binding of US9 to motor protein KIF1A for anterograde microtubule-mediated transport in neurons (27). It is likely, that a similar anterograde mechanism, away from the nucleus towards basal domains, exists within epithelial cells using a KIF motor. In addition, another study describes the presence of a kinesin 2 family member KIF17 in polarized epithelial cells and elaborately summarize the role of KIF17 in the establishment of polarized trafficking routes to the apical pole (20). Another speculation might be that herpesviruses interact with different kinesin motors to be specifically targeted towards apical, basal or lateral domains.

In a second part, we looked at the distribution of gE in epithelial plaques as well as the localization of virions of wild-type, gE and/or gI null and repaired strains in inoculated tissues. Confocal analysis of subcellular gE presence within epithelial plaques revealed a predominant basal localization near the BM. TEM analysis showed that virus particles of wild-type and repaired strains accumulated mainly near basal sides of basal cells or on the surface of basal cells next to the BM. On the contrary, virions of gE and/or gI null strains were more frequently found on non-basal cells sides and some were randomly distributed

within cells. These observations confirm our previous findings suggesting that gE/gI mediated sorting is required for virion transport towards the BM.

In a last part of the study, we enriched the medium of WT inoculated explants from 0h pi onwards with monoclonal antibodies 14B11 (anti-gE), 13E8 (anti-gE), 2E12 (anti-gE/gI); and evaluated the effect on plaque latitude and plaque penetration depth underneath the BM at 48hpi. None of the antibodies affected plaque latitude. However, 14B11, 13E8 and 2E12 caused a severe reduction in plaque penetration depth underneath the BM compared to the controls suggesting that gE/gI exerts another function in invasion once sorted correctly. In line with this finding, the extracellular domains of the complex gE/gI are suggested to bind to unidentified extracellular ligands such as cell surface components of intercellular junctions and promote spread to adjacent cells (36). Although speculative, they might in a similar way associate with receptors present at basal cell surfaces such as integrins within cell-matrix adhesions. At the moment, we cannot exclude the fact that binding of monoclonals directed against other membrane-anchored viral proteins (glycoproteins) at the level of the BM might disturb the BoHV-1 invasion process as well.

In conclusion, we can state that the gE/gI complex plays a crucial role in BoHV-1 stromal invasion through the BM barrier. Although at this moment still speculative, it seems that gE/gI mimics a basal sorting pathway via its cytoplasmic tail motifs towards basal domains in basal cells and orchestrates invasion once present near the BM. Further work is necessary to determine all different players of this herpesvirus directed transport towards the BM for successful host invasion.

7.6. Acknowledgements

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CHAPTER 8.

GENERAL DISCUSSION

Species of both vertebrates and invertebrates host various herpesviruses and may suffer from a diverse array of pathologies induced by these pathogens (22). Many structural and functional similarities exist amongst herpesviruses: (i) they encode essential viral protein homologues for successful replication (31), (ii) they are able to establish latency (55) and (iii) they use mucosal surfaces such as respiratory and genital mucosa for host entry (72). A rational thought would be to use these common characteristics of herpesviruses as targets for the development of preventive and curative approaches to counteract these viruses. However, so far this has proven to be far from axiomatic. Current existing vaccination strategies and antiviral compounds work against some herpesviruses while they have no to little effect against other herpesviruses.

Vaccination strategies and eradication programs have been developed for pseudorabies virus (PRV) and bovine herpesvirus 1 (BoHV-1) (57, 94), although they are not perfect. In the case of BoHV-1, vaccination with the current marker vaccines does not prevent BoHV-1 infection but reduces the severity of disease and viral shedding (7, 13, 14, 92). This implies that even vaccinated and seemingly healthy animals might be virus carriers. Within the context of an eradication program, this means that eventually stamping out of seropositive animals is inevitable in the process of acquiring an official IBR-free status. So far, not a single country that entered an IBR eradication campaign succeeded. Whenever a re-introduction occurs in an official IBR-free country, the current vaccines are less ideal. In this case, culling of seropositive animals should be chosen above the application of the current vaccines (2). In addition, many herpesviruses can simply not be controlled at present via vaccination. Equine herpesvirus 1 (EHV-1) is still able to cause nervous disorders and abortion despite vaccination (67). No efficacious herpes simplex virus (HSV)-vaccine is present on the market (8). When treating herpes virus infections with the current antiviral compounds, resistance has been reported to occur, which limits the application of these products (97). Moreover, some antiviral compounds simply have a low efficacy when used against animal and/or human herpes virus infections, despite their *in vitro* potency (38, 39).

It is clear that combating herpesvirus infections is not a race we can win hands down and novel approaches are urgently needed for the design of novel antiviral strategies. In our laboratory, research is conducted to specifically target the mucosal invasion mechanism of herpesviruses, a rationale aimed at future development of novel, alternative and complementary anti-herpetic measures. Previous work revealed that PRV breaches the basement membrane plaquewise after epithelial infection, involving protease-mediated

breakdown of the extracellular matrix (42). In striking contrast, EHV-1 does not cross the basement membrane in such a way but instead infects resident immune cells within the epithelium and appears to misuse their motile nature to invade the host (95). These observations clearly underpin differences on how these otherwise closely related herpesviruses behave at the primary replication site. Many questions remain however on how other devastating respiratory and genital herpesviruses, BoHV-1/-4 and HSV-1/-2 in cattle and human respectively are causing infection and subsequent host invasion. In the current thesis we sought to establish models that allow the study of the mucosal invasion mechanism used by BoHV-1/-4 and HSV-1/-2.

- **Establishment of *ex vivo* organ cultures of bovine and human origin consisting of either respiratory or genital mucosa**

Since experiments to address specific questions about host invasion are difficult to conduct *in vivo*, development of model systems to elucidate pathogenic invasion mechanisms are essential. Typical monolayers of cells represent a poor structural approximate of the complex 3D architecture of tissues. These 2D models fail to reconstitute the complex *in vivo* cellular microenvironment. Features of living tissues that are crucial for their function include tissue–tissue interfaces (e.g. between epithelium and connective tissue), cell-ECM interaction, spatiotemporal gradients of ions and oxygen and a mechanically active microenvironment (75, 76). The establishment and maintenance of cell and tissue polarity is a prerequisite for normal function of biological processes including oriented cell division, migration, adhesion and barrier function. In addition, tissue homeostasis depends on resident mononuclear phagocytes, that is, dendritic cells and macrophages (62). Adding the third dimension to a cell's environment might generate significant differences in cellular behavior and characteristics and create a greater similarity to conditions in living organisms, which will lead in turn to more integrated data and thus relevant research (75). *Ex vivo* organ cultures provide these requirements and are an elegant, reproducible and physiologically relevant compromise between *in vivo* and *in vitro* models. They value an important ethical dogma: Refinement, Reduction and Replacement (3R principle), regarding the use of laboratory animals (84). Moreover, typical *in vivo* models in order to study aspects of herpesvirus induced diseases are rodents (21, 91). Although these models have proven to be of use to address certain questions, they clearly lack homology as the virus is not interacting with

tissues from its natural host. Again, *ex vivo* organ cultures provide an advantage in this context, as they may allow the study of a herpesvirus with tissues derived from its natural host. Furthermore, the natural route of infection can be mimicked when applying these models. Several conditions can be tested on different explants derived from the same animal and thus inter-animal variation is greatly reduced. Despite all the important advantages of organ cultures, there are also some disadvantages. First, working with mucosa explants is rather labor-intensive, especially when including various strains and/or conditions. Second, despite their very close resemblance to the *in vivo* situation, there are some important drawbacks. No blood flow is present in the mucosa model, hence mimicking a full inflammatory reaction upon stimulation by pathogens is limited. Certain aspects such as innate immune mechanisms can be studied to a certain extent though (79). Third, unlike continuous cell lines, tissue explants can only be cultivated for a limited time span.

In the first part of our study, Chapter 3, bovine respiratory and genital tissues, as well as human genital tissues were used for the *in vitro* culture of mucosa explants. Explants were maintained *in vitro* up till 96h. Different methods of organ culture exist including stationary air-liquid cultures, rocker/rotor cultures with alternating air and medium exposure and stationary immersion cultures (79). We applied metal gauze as platforms for the tissue explants to set up air-liquid interface cultures as this closely resembles the physiological conditions. Tissue viability (function) and morphology (structure) could be maintained at all times as demonstrated by TUNEL staining assays, and light microscopical, scanning electron microscopical and transmission electron microscopical analyses at different time points of tissue culture. All together, we successfully established valuable *in vitro* models that closely mimic *in vivo* tissue.

- **The established mucosa explant models are susceptible to herpesvirus infections, making them suitable to study and compare herpesvirus invasion mechanisms at mucosal surfaces.**

A next step in the investigations of this thesis was to evaluate whether the established mucosa explant model supports herpesvirus infection and replication. The use of mucosa explants to study aspects of pathogen host invasion is not new. It is widely used in the field of virology (e.g. HIV) (1, 18), bacteriology (e.g. *Staphylococcus*) (12, 90) and mycology (e.g.

Aspergillus) (74). However, the application of mucosa explant cultures in herpesvirus research was limited until now.

In Chapter 4, we showed that bovine nasal mucosa, conchal mucosa, tracheal mucosa and genital mucosa explants are susceptible to bovine herpesvirus 1 (BoHV-1) infection. Moreover, our data convincingly show that both respiratory and genital mucosa are susceptible to infection with all different BoHV-1 subtypes despite their assumed different tissue tropism. Especially at 24h pi, some interesting findings were observed about BoHV-1 dissemination in mucosal surfaces. In respiratory tissue and even more profound in tracheal mucosa, the virus is able to quickly disseminate in a plaquewise manner and invade by breaching the basement membrane barrier. This clearly shows that the virus is well adapted to disseminate within tracheal mucosa. This potent invasive capacity in the respiratory tract may explain why severe tracheitis is part of the clinical entity caused by BoHV-1 and perhaps also, why abortion is mainly seen after respiratory infection (72). In genital tissues, BoHV-1 plaques were found to have a bigger latitude compared to plaques in the respiratory tract. It is important to mention that the respiratory epithelium is a pseudostratified epithelium which gives the impression that there are multiple layers of cells (63, 96). The genital epithelium on the contrary is a true multilayered epithelium (6). Assumedly, within a similar area of epithelial surface, more cells are present in the genital epithelium compared to the respiratory epithelium. Thus, replication and dissemination might be more exponential in the genital tract considering the density of naïve cells in close proximity to each other. A reflection of this more efficient replication might be that BoHV-1 titers in nasal secretions range from 10^8 – 10^{10} Tissue Culture Infectious Dose ($TCID_{50}$)/ gram mucus whereas BoHV-1 titers in genital secretions may go as high as 10^{11} $TCID_{50}$/ gram mucus *in vivo* (28). Unfortunately, we did not perform titrations on explant supernatant in this part of the study to verify this hypothesis.

The recently established homologous model allows the study of other/additional respiratory pathogens as well. In animals, a productive BoHV-1 respiratory infection can easily be complicated by secondary bacterial pathogens which aggravate the disease image substantially (72). It would be highly interesting to investigate how a BoHV-1 infection will influence secondary bacterial colonization and invasion. Especially interactions with bacteria such as *Fusobacterium necrophorum*, a normal inhabitant of the respiratory tract and causative agent of severe necrotic laryngitis in calves, and *Arcanobacter pyogenes*, involved in clinical laryngitis, would be highly interesting (50, 73). Recently, HSV-1 infection was found to facilitate *Staphylococcus* invasion in human nasal mucosa (98). We made the

observation in this chapter that starting from 48h pi and more pronounced at 72h pi, infected epithelial cells loosened and detached from the viral plaque, thereby exposing the basement membrane. Likely, this allows efficient colonization of bacteria as the latter often adhere to ECM proteins via bacterial adhesins, as described in point 1.3.1 of this thesis.

In Chapter 5, we compared the mechanism employed by BoHV-1 to that of a related but scarcely documented bovine gammaherpesvirus namely bovine herpesvirus 4 (BoHV-4). Several respiratory and genital mucosa explants were inoculated with either BoHV-4 or BoHV-1 and analyzed for replication and dissemination parameters within mucosa. A key finding was that we did not observe signs of BoHV-4 infection in tracheal mucosa as we did see for BoHV-1. On the contrary, clear BoHV-4 and BoHV-1 infection was seen in vaginal mucosa, indicating a possible route of infection and transmission for BoHV-4. BoHV-4 displayed both an epithelial and monocyte/macrophage tropism. A scant amount of *in vivo* studies describe that BoHV-4 could be readily recovered from monocytes (9, 33, 66). Unlike BoHV-1, BoHV-4 epithelial plaques were only seen from 48h pi and plaques never crossed the basement membrane in a plaquewise manner.

Interestingly, several studies indicate that closely related gammaherpesviruses such as human Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV) as well as murine herpesvirus 4 (MuHV-4) have a tropism for the genital tract. About thirty cases of ulcerative vaginal manifestations associated with EBV have been reported with some of them being primary infections (45, 64). The presence of KSHV in the cervix of some women and in semen suggests that replication and transmission via this site is a possibility (23, 100). Genital reexcretion, in general weak and transient, is documented for BoHV-4 to occur after the establishment of latency in mice and contributes to transmission of the virus (36). An intriguing fact is that for EBV, KSHV and MuHV-4, sexual transmission has been proposed (20, 36, 77). At this moment, we must be careful with stating that BoHV-4 has a strict tropism for the genital tract. Similar as for EBV, KSHV and MuHV-4, co-existence of multiple routes of dissemination is possible and makes it relatively difficult to elucidate what the preferred mode of host invasion and transmission is (36, 61, 71, 88, 100). For example, respiratory tract-associated lymphoid tissue such as tonsils was not included in our study. Tonsillar lymphocytes are a known EBV replication site (48). As several gammaherpesviruses show such a lymphoid tropism (37, 48), it might be wise to test other topologically and structurally different respiratory areas as well in the future. Nevertheless, the finding that BoHV-4 is able to replicate and produce progeny virus in the vaginal mucosa has important implications for

the field. BoHV-4 is mainly associated with postpartum problems such as postpartum metritis and possibly mastitis (49, 99). Like most herpesviruses, BoHV-4 is able to reactivate from latency upon stress stimuli of which the final days of gestation and parturition are important examples (15, 30). Although speculative at the moment, active recurrent infection in the vaginal mucosa, as shown for MuHV-4 in mice, might be a possible route of transmission. Passage of the newborn calf through the genital tract during natural birth might result in peri-natal infection. Post-natal exposure to the virus through an active shedding dam is an additional possible route of transmission. Considering this, veterinarians that perform a vaginal exploration during parturition should carefully respect the necessary hygienic measures if they decide to perform a subsequent caesarian section and/or udder examination in order to prevent iatrogenic introduction of BoHV-4 in the uterus or udder. As for BoHV-1, true horizontal sexual transmission of BoHV-4 is probably greatly reduced through artificial insemination.

In Chapter 6, we evaluated the mucosal dissemination characteristics of HSV-1 and HSV-2 in human genital mucosa during different phases of the menstrual cycle. Somewhat similar as for the different subtypes of BoHV-1 in Chapter 4, we observed no differences between the behavior of HSV-1 and HSV-2 in genital mucosa despite their different tissue preference. In the field, there is also an increasing trend of HSV-1 induced primary genital herpes cases (16). Clearly, as we have shown in this thesis for both a veterinary and human herpesvirus, tissue tropism is a relatively abstract concept. More likely, factors such as (social) behavior of individuals as well as genetics are the true determinants of where these viruses tend to replicate. For example, especially in young adolescents HSV-1 infections of the genital tract are on the increase because of increasing oro-genital contact (4). In cattle, the incidence of genital infections with BoHV-1 has drastically diminished due to increased good-practice artificial insemination and reduced natural mating (72). We were somewhat surprised to see that the human ectocervical/vaginal mucosa is a very protective barrier against HSV-1/-2, not only because of the physical epithelial thickness measured in our study, especially under estrogen influences, but also when it comes to barrier function of the basement membrane. Indeed, we did observe only a limited number of plaques in both endo- and ectocervix that penetrated the basement membrane at 48h pi and this in a localized manner. We cannot exclude that at a later time points post inoculation the amount of BM penetrating plaques may increase. Nevertheless, it is clear that even in these few BM-penetrating plaques, the virus can easily spread further to blood vessels. Several *in vivo* studies strengthen our observation by

documenting clear episodes of HSV-viremia after primary HSV infections of the genital tract in humans (53, 56). Remarkably, we detected several syncytia or multinucleated giant cells within HSV-induced epithelial plaques. Cell-cell fusion is a key process in the formation of multinucleated giant cells. Residing macrophages in the epithelium have the ability to undergo cell-cell fusion upon specific stimuli, resulting in multinucleated giant cell formation. Hence, syncytia may play a beneficial role in the elimination of pathogens and foreign particles. However, they may also be deleterious to the host, by promoting tissue destruction and facilitating microbial survival (81). Suggestively, HSV might be subverting this mechanism of macrophages within plaques to promote prolonged persistence in the epithelium as an immune evasion strategy.

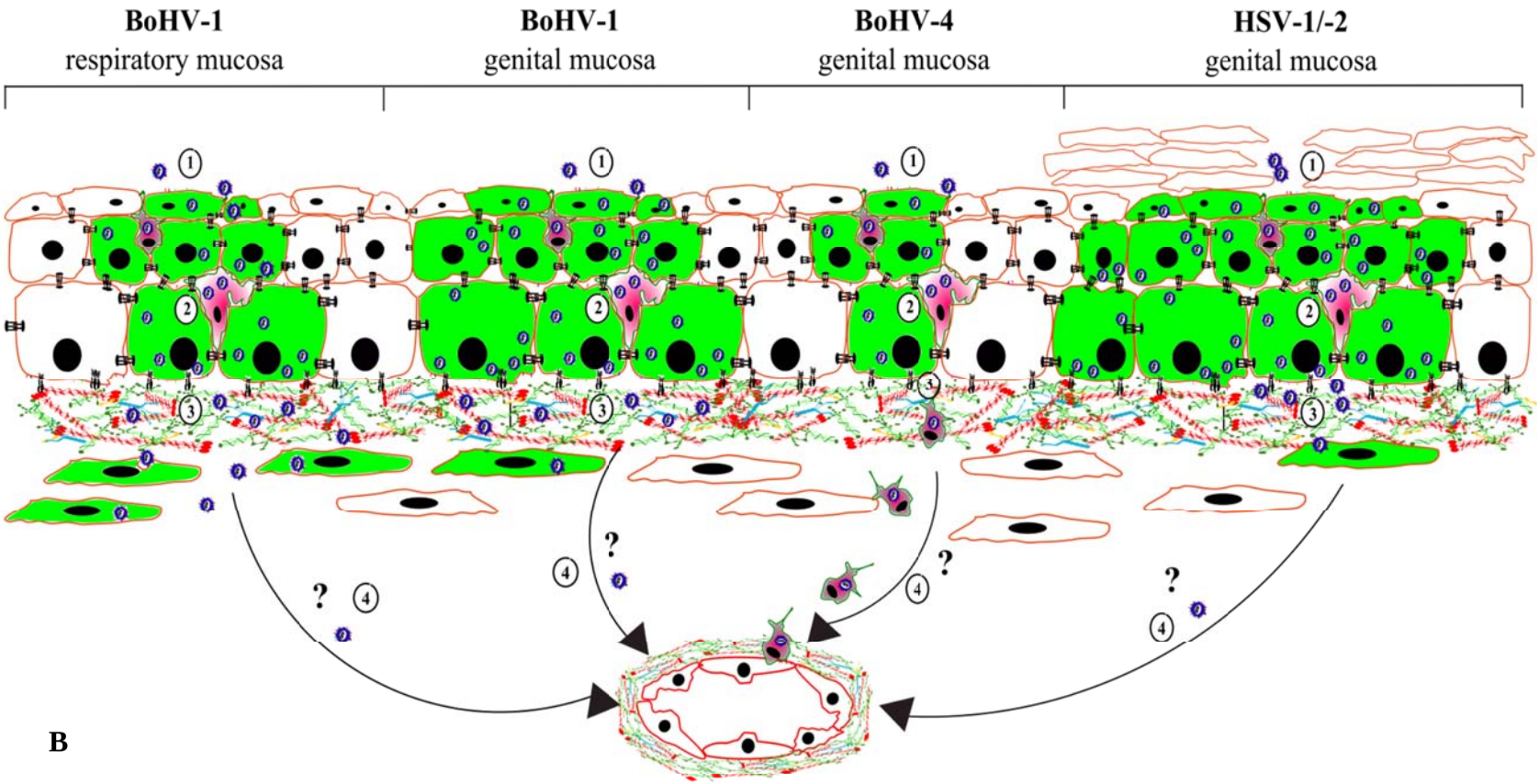
In conclusion, Figure 1. provides an overview of the observed invasion mechanisms for the viruses used in this thesis. A comparison of the mucosal behavior is made for all viruses in (pseudo)stratified epithelia at time point 48h post inoculation. Data of a previously published study of our laboratory describing the dissemination characteristics of HSV-1 in respiratory mucosa are included (41). Bovine herpesvirus 1 replicates plaquewise in both respiratory and genital mucosa to a similar extent and aggressively breaches the basement membrane barrier starting from 24h pi. However, in general at 48h pi, plaque latitude is larger in genital tissues compared to tracheal tissues. Vice versa, plaque penetration depth is more profound in respiratory tissues compared to genital tissues. We must say that this discrepancy is even more clear at 24h pi but is absent at 72h pi. In summary, BoHV-1 rapidly spreads lateral in genital tissues and more efficiently invades respiratory tissues very early after host infection. A closely related bovine gamma herpesvirus, BoHV-4, also displays an epithelial tropism but seems to be limited to infect the genital tract. BoHV-4 infects single epithelial cells starting from 12h pi. At 24h pi, small clusters of epithelial cells are visible and grow to plaques at 48h pi. We did not observe breaching of the basement membrane barrier for BoHV-4. Instead, at all time points, few single individual monocytic lineage cells can be observed in the epithelium and lamina propria. This indicates that BoHV-4 might be using these cells to be efficiently invade deeper tissues. It is known that BoHV-4 can easily infect macrophages and establish persistence within them (29).

Interestingly, if we compare the plaque latitude at 48h pi of BoHV-1 and BoHV-4, clearly BoHV-4 less efficiently paves its way through the epithelial layer. HSV-1 and HSV-2 are both able to replicate in ectocervical/vaginal epithelium. Plaque latitude increases over time and at 48h pi, few epithelial plaques show signs of basement membrane penetration, albeit in

a localized manner. Comparison of the plaque latitude and penetration depth values of HSV-1 in genital mucosa with the data of HSV-1 spread in respiratory mucosa, suggests that HSV-1 can spread more efficiently lateral in genital mucosa compared to respiratory and more efficiently in the depth in respiratory compared to genital tissues (Figure 1). Importantly, this trend is also observed for BoHV-1 in respiratory and genital tissues, especially at early time points post inoculation, and stresses out an important similarity between an animal and a human alphaherpesvirus. Two side remarks have to be made. Firstly, the data acquired by Glorieux and co-workers document HSV-1 mucosal spread only until 36h pi (41). So at this point, we compare the values of HSV-1 in the genital tract at 48h pi to values of HSV-1 in the respiratory tract at 36h pi, which might be an important bias. Nevertheless, we believe it is unlikely that the virus is able to nearly double its plaque latitude in the respiratory tract within a 12h time span. For the plaque penetration depth, the difference observed in favor of the respiratory tract might be even more profound at 48h pi compared to the genital tract at 48h pi. Second, it is important to point out we are not comparing the values for HSV-1 in tissues derived from the same individuals, as we did for BoHV-1 in the different tissues.

Although speculative, we believe that the bigger plaque latitude of BoHV-1 and HSV-1 in the genital tract compared to the respiratory tract might be a result of the presence of a different type of epithelium, as mentioned above (6, 63, 96). This may result in more quicker dissemination and replication in the genital tract considering the density of naïve cells in close proximity to each other. Considering the more efficient invasion capacity in respiratory tissues compared to genital tissues, we hypothesize that the determining factor is the BM rather than the different epithelium type and amount of epithelial layers. A reason to assume this is the fact that the invasion depth of HSV in human endocervix, which contains a one layer epithelium, was similar as in the multi-layered ectocervix. In addition, HSV-1 was found to efficiently invade across pseudostratified respiratory epithelium (41). Basement membranes barriers are not static structures, they can vary both in composition, thickness and biomechanical properties depending on the tissue/organ and age (32, 44). TEM studies have shown that the thickness of BMs can range from 100nm for average BMs to over 10µm for the lens capsule, the thickest BM in humans (44). Though we did not observe differences in *lamina reticularis* thickness of the genital and respiratory mucosa, we have neither information about the thickness of another part of the BM called *lamina densa* nor about the composition of the entire BM scaffold. If BMs of genital mucosa are structurally more complex and thick, this might require more effort of particles and time to penetrate.

A



B

Average plaque…	BoHV-1 respiratory (48h pi)	BoHV-1 genital (48h pi)	BoHV-4 genital (48h pi)	HSV-1 respiratory (36h pi)	HSV-1/-2 genital (48h pi)
Latitude	310 µm	380 µm	190 µm	310 µm	600 µm
Penetration depth underneath the BM	40 µm	20 µm	0 µm	40 µm	10 µm

Figure 1. (A) Overview of the mucosal dissemination characteristics at 48h pi or 36h pi of the herpesviruses used in this thesis including BoHV-1, BoHV-4, HSV-1 and HSV-2 in respiratory and genital mucosa. (1) prior to viral entry in cells, viruses attach to cell surface receptors. Within the human ectocervix/vagina, HSV-1 and -2 have to find a way through the stratum superficiale before reaching cells that express entry receptors. (2) viral replication and local dissemination. Local immune cells may be infected (e.g. BoHV-4). (3) viruses gain access to the stroma by breaching the BM. This may happen via a plaquewise breakdown of ECM. This is most clear for BoHV-1 in the respiratory tract followed by BoHV-1 in the genital tract. A small localized penetration can be observed for HSV-1 and -2 in the genital tract. Another possible mechanism consists of hijacking immune cells to transverse the BM and may be employed by BoHV-4. (4) finally, viruses may spread in the host by reaching blood or lymph vessels. (B) Overview of the average plaque latitude and penetration depth for the different viruses on different tissues at 48h pi. As a reference, data concerning plaque latitude and penetration depth at 36h pi from a study using HSV-1 on respiratory tissues was included (41).

❖ **The heterodimer gE/gI plays a crucial role in plaquewise crossing of the basement membrane by alphaherpesviruses like BoHV-1.**

In Chapter 4 we found that BoHV-1 is able to cross the basement membrane in a plaquewise manner, quite similar as what is previously observed for pseudorabies virus (PRV) in respiratory mucosa. Therefore, we were interested in which viral effectors are required for the viral invasion mechanism of this group of alphaherpesviruses and applied the established model of BoHV-1 on respiratory mucosa for investigation. As we found before that at 48h pi most, if not all, BoHV-1 plaques cross the basement membrane barrier, invasion of different mutant BoHV-1 strains was analyzed at 48h pi.

In Chapter 7, the role of two virulence genes, US7 (gI) and US8 (gE), in BoHV-1 invasion through the basement membrane was assessed. Glycoprotein E and glycoprotein I seem ideal candidates to be involved in efficient host invasion. First, they are present in all field isolates, pointing out their important role in pathogenesis (3, 82). BoHV-1 gE null or gE truncated strains are strongly attenuated *in vivo* and all current "safe" vaccine strains are in fact gE deleted (17, 57, 65, 87, 93). HSV-1 gE null viruses are severely impaired in establishing stromal disease and potential encephalitis after epithelial keratitis (26). Second, these glycoproteins, present in a complex, govern directional movement in different cell types for efficient spread to naïve cells and tissues. As such, movement of virus across cell-cell contacts and axonal transport of viruses both require a functional gE/gI complex (26, 27, 80, 103). Third, Glorieux and colleagues previously reported a possible role of gE in the PRV mucosal invasion mechanism towards the lamina propria (40). Taken into account these data, the gE/gI complex is of paramount importance for virus spread and invasion.

We used different approaches to investigate whether this complex plays a role in BoHV-1 invasion. First, we used either gE/gI knockout virus strains or we inhibited the gE/gI function by applying blocking monoclonal antibodies and looked at the effect on different dissemination parameters. Second, we analyzed the distribution of gE within epithelial plaques and in addition we also assessed whether gE/gI is required for efficient sorting of virus particles towards the basement membrane by using TEM. Both knocking out and inhibiting gE/gI resulted in a highly reduced penetration depth underneath the basement membrane. In addition, knock out of gE/gI resulted in a reduced plaque latitude and reduction in progeny virus production. Next, glycoprotein E was found to be highly present near the basement membrane in BoHV-1 epithelial plaques. TEM analysis revealed that gE/gI is

required for routing viral particles to basal sides of epithelial cells. In basal cells, adjacent to the basement membrane, many particles were found to sort near the basement membrane if a functional complex was present whereas knockout of gE/gI resulted in reduced amount of virions near the basement membrane. The observation that a gE CT null virus and thus a knockout of the gE sorting motifs resulted in a similar phenotype as gE null in our analysis supports the idea that gE/gI sorts virus particles towards the basement membrane.

Virus sorting towards integrins near the basement membrane, which are present in cell-matrix adhesions, and mimicking or inducing integrin signaling would represent an efficient way of transport through the basement membrane. Preliminary experiments have been performed in this context by adding a function-inhibiting monoclonal antibody directed against either the β1 or β4 subunit of integrins to the medium of wild type BoHV-1 inoculated explants from 0h pi onwards. Nine different integrins are expressed in epithelial cells of which two, α3β1 and α4β6, recognize basement membrane components and are abundantly present in anchoring cell-matrix junctions (11, 58). We observed a relative reduction of ±80% in plaque penetration depth underneath the basement membrane compared to the mock and isotype control condition when blocking both subunits. This observation suggests a direct role of integrins, as cell-matrix anchors, in the BoHV-1 invasion mechanism but more data have to be acquired in this context.

In conclusion, we want to speculate on the role of gE/gI and other potential proteins and pathways in the invasion mechanism of BoHV-1 via basal epithelial cells through the basement membrane barrier (Figure 2).

Soon after synthesis, gE and gI quickly form heterodimeric complexes. The gE CT domain promotes extensive accumulation of gE/gI in the trans-Golgi network (TGN) in infected cells at early times of infection. We suggest that the gE/gI complex functions as a chaperone to orchestrate trafficking, sorting and distribution of many other viral proteins including gD, UL11, UL16 and VP22 (19, 35, 46). Selective partitioning, with gE/gI at the steering wheel, topologically targets essential proteins such as gD, gB and gH/gL to the virion envelope and proteins required for efficient sorting such as US9 and UL56 to the transport vesicle (10, 60). Indeed, gE/gI is a central figure in a complex network of interacting tegument proteins including UL11, UL16, VP22 and UL21 that link the complex to other major proteins such as gD (35, 46, 59). From the TGN, the major sorting station for newly synthesized proteins and lipids, several trafficking routes originate towards apical, lateral and basal plasma membrane domains (34).

In monolayers of epithelial cells, the gE/gI complex accumulates specifically on those lateral surfaces of epithelial cells that are forming cell junctions whereas they are not observed on lateral surfaces of epithelial cells that are not in contact with other cells (52). We observed in this thesis that gE/gI sorts virus towards basal cell sides of basal epithelial cells that form adhesive junctions with the extracellular matrix (3D), which are absent in monolayers (2D). In addition, subcellular gE localization showed a predominant presence of gE on basal cell sides adjacent to the basement membrane within BoHV-1 epithelial plaques.

Cellular cargo including proteins and lipids are transferred between compartments by the formation, movement and subsequent specific fusion of transport intermediates. These vesicles must be coupled to the cytoskeleton via motor proteins that drive motility (83). Ample data describe the interaction of herpesviruses with microtubule-associated motor proteins and thus microtubule-based transport of virions, mainly in neuronal cells (25, 43, 47, 52, 60, 101, 102). As such, during anterograde transport of herpesviruses, it is described that US9 interacts with KIF1A, a microtubule interacting motor protein; an interaction which is dependent upon lipid raft association and stabilization by gE/gI (60). HSV-1 tegument protein US11 was found to bind KIF5B (24). VP1/2, an inner tegument protein, is also implicated in recruiting a kinesin motor to cytoplasmic vesicles filled with viral particles late in infection (85). Hence, we believe that cellular motor proteins are misused within epithelial cells quite reminiscent to herpesvirus axonal transport, to specifically route virions towards adhesive structures either in contact with other cells or with the extracellular matrix. We suggest that coat-mediated targeting to either apical, lateral or basal domains is dependent upon several limiting factors: (i) formation of adhesive junctions and thus establishment of directional microtubule transport, (ii) the kinesin motor, (iii) the adaptor protein and (iii) the viral protein interacting with the motor/adaptor complex. Epithelial cells contain different kinesin motors for anterograde transport or “away from the nucleus”, including KIF5B, KIF13A, KIF16A and KIF17 (51, 63, 86, 89). During interkinetic nuclear migration (INM), a common event in (pseudo)stratified epithelia, KIF1A traffics to basal cell sides (63). On the other hand, KIF17 is involved in sorting towards the apical pole (51). In addition to US9, US11 and VP1/2 mentioned above, there is increasing evidence that other motor engaging viral proteins such as UL56 might contribute to directional transport (59). Therefore, it is likely that different viral proteins engaging different cellular motors might determine the employed trafficking route. Because of the selective partitioning ability of gE/gI, the complex probably orchestrates the destination of vesicles by incorporating different viral effectors of cell transport such as US9 and/or UL56.

Second, gE/gI accumulates at cell junctions late in infection, apparently tethered there in a manner similar to that of various cell adhesion molecules (78). Although speculative, we believe that the gE/gI complex may be targeted to integrins within lipid rafts and activates and/or mimics integrin-mediated signaling. We do not observe common acidic peptide integrin-binding motifs such as RGD, RSD, LDV or DGE in the extracellular parts of either gE or gI of BoHV-1, suggesting no direct role for gE/gI but instead an indirect recruiting role of other proteins that can cause integrin activation (via the Eukaryotic Linear Motif resource for functional sites in proteins (ELM)-software http://elm.eu.org/). Such a candidate might be BoHV-1 gC as an RGD motif is located on its extracellular domain. On the other hand, another mechanism might be mimicking of integrin-signaling through the cytoplasmic tail of both gE and gI. Both BoHV-1 gE and gI have three predicted SH3-domains in their cytoplasmic tail. In addition, the CT of gE possesses a predicted MAPK docking motif and 2 predicted SH2 domains (ELM-software). SH3, SH2 and MAPK domains are often found in signal transduction proteins such as integrins and are important for various cellular processes including cytoskeleton remodeling, which on its turn can induce inside-out signaling allowing extracellular matrix remodeling (69, 70). Importantly, for example BoHV-1 gB, gD and gC do not possess such motifs in the cytoplasmic domain. Moreover, glycoprotein E and I of EHV-1 also lacks such predicted motifs, which makes this interesting as we do not observe a similar mechanism of invasion for EHV-1. EHV-1 is not able to mediate an aggressive breakdown of ECM for successful basement membrane passage.

We have to emphasize that the current hypothetical pathways are at present highly speculative and future research will be needed to explore all different possibilities that allow efficient invasion of herpesviruses through the basement membrane barrier for host invasion.

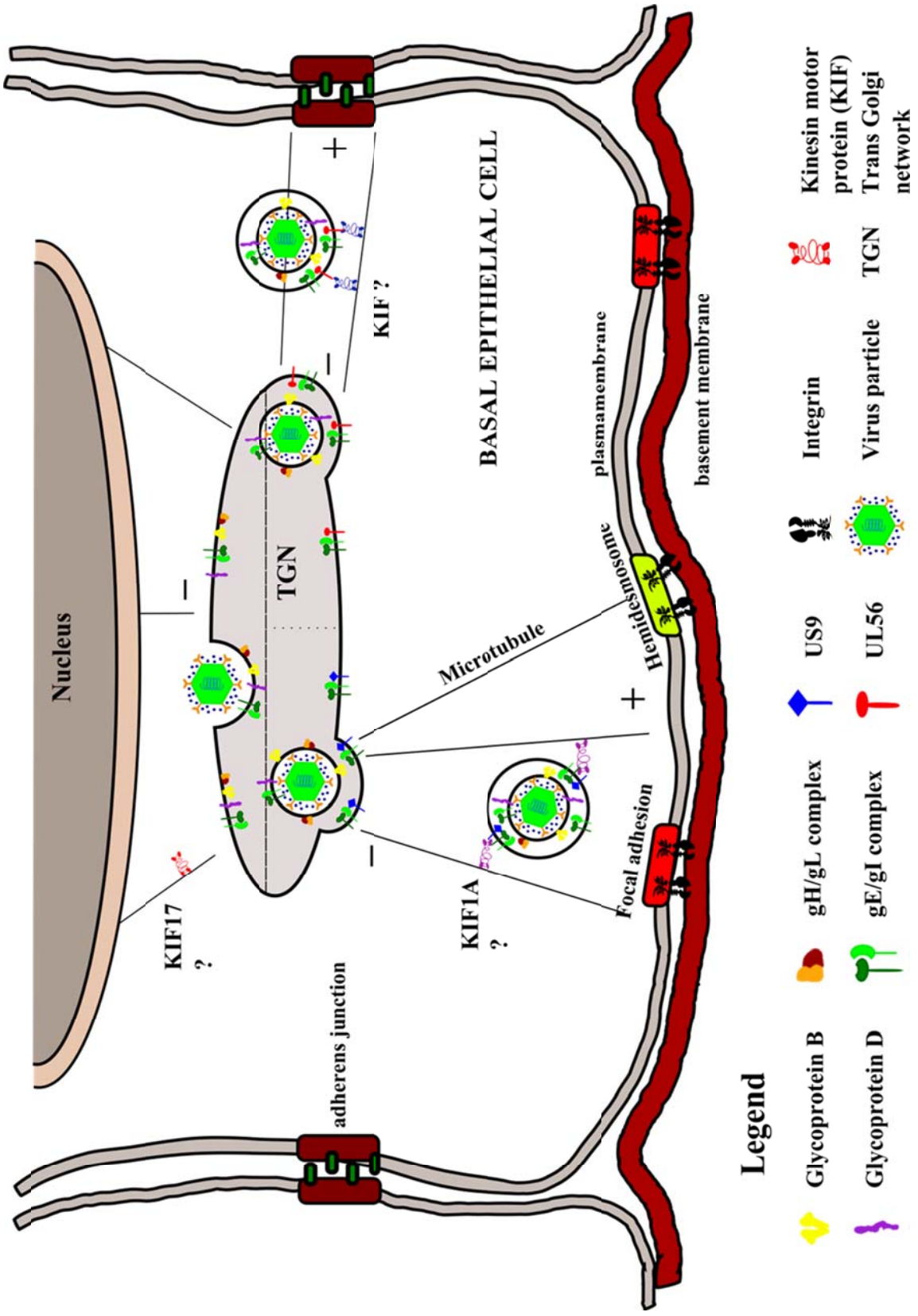

Nucleus
KIF17
?
adherens junction
TGN
KIF ?
BASAL EPITHELIAL CELL
plasmamembrane
basement membrane
Microtubule
Hemidesmosome
Focal adhesion
KIF1A
?
Legend
Glycoprotein B
Glycoprotein D
gH/gL complex
gE/gI complex
US9
UL56
Integrin
Virus particle
Kinesin motor protein (KIF)
Trans Golgi network
TGN

Figure 2. Hypothetical model. The viral heterodimer gE/gI extensively accumulates at the Trans Golgi network (TGN), the major station for protein and lipid sorting. gE/gI selectively recruits other viral proteins and virions to the TGN and increasing evidence indicates that gE/gI determines towards which topologically distinct membranes of the TGN, e.g. future viral envelope or future transport vesicle, proteins are being target, a phenomenon termed selective membrane partitioning (dashed line within TGN). We suggest an additional selective mechanism within the TGN, governed by the gE/gI complex by specifically inserting certain viral effectors for transport into different subcompartments for exquisite routing to either basal, lateral or apical plasma membrane domains (dotted line within TGN). An event which will only be observed in polarized basal cells (cell-cell adhesions) within a 3D environment (cell-matrix adhesions). In addition, the use of different cellular motor proteins e.g. kinesins for microtubule-based transport, might be another determining factor towards which domain vesicles are being routed. Once present at the plasma membrane, we suggest that the gE/gI complex is targeted to integrins within lipid rafts and activates and/or mimics integrin-mediated signaling via a yet unknown mechanism.

❖ Future directions – vaccination and antiviral therapy

Interfering with alphaherpesvirus invasion

We found BoHV-1 and HSV-1/-2 to cross the basement membrane in a plaquewise manner similar as previously described for PRV. In addition, for PRV, a trypsin-like serine protease was found to be involved in successful viral passage of the BM (42). Likely, BoHV-1 and HSV-1/-2 might apply a similar mechanism. In this context, the topical use of antiviral compounds that specifically inhibit the activity of involved proteolytic enzymes will be an interesting strategy. Moreover, we showed in this thesis that the heterodimeric complex gE/gI is crucial for efficient sorting of virus particles towards the basement membrane in basal epithelial cells and subsequent invasion. Antivirals that specifically inhibit gE/gI-mediated viral transport might be an additional interesting approach. These proposed invasion-inhibiting products can potentially be used complementary to current existing compounds (cocktail therapy) and might provide necessary alternatives when dealing with resistant strains. In humans, such products could be beneficial during clinical episodes or as preventive measures around the moment of child birth. In bovines, the application of such products may be economically justified in light of an outbreak during re-introduction in IBR-free countries, both for protecting contact animals as for ring-block measures. Indeed, because of the drawbacks of the current vaccines, using marker vaccines should not be the preferred antiviral strategy at that time. We want to point out that the established explant models are ideal tools to screen for novel potent antiviral compounds.

Induction of a specific cellular and humoral immunity via vaccination is another antimicrobial (preventive) measure. Deletion of gE/gI will result in replication-competent, live attenuated strains that might be good candidates for vaccine strains. If our hypothesis holds truth for viral effectors of virus transport in epithelial cells, then additional deletion of US9 and UL56 may make sense. Our results suggest that these strains will be severely impaired in spreading beyond the basement membrane barrier. In addition, as it is known that gE/gI and US9 play a role in viral transport in neurons, these strains will also be hampered in their neuroinvasive capacity (60). In line with our results, a recent publication shows that gE null HSV-2 strains are impaired in efficient spread from epithelial cells to axons and in anterograde transport from the neuronal cell body to axon termini. The authors propose the use of a gE null HSV-2 strain as MLV (5). Moreover, gE null and US9 null strains were found to be safe in calves as they are attenuated in spread and are not able to reactivate from latency (54). The best results

will be obtained after inducing a strong local immunity at the level of the mucosa and hence, MLV should be applied either intranasally or intravaginally.

Vaccination against gammaherpesviruses?

In this thesis we identified the vaginal mucosa as a possible primary (and recurrent) site for BoHV-4 replication. Because of the ubiquitous nature of the virus and its association with economical losses due to postpartum diseases, vaccination might be a considerable option. The established homologous model in this thesis can be applied to test several mutant BoHV-4 strains. Since glycoproteins are important in essential virus-cell interactions like adherence, penetration and spread, they might be interesting candidates. Machiels and co-workers have recently shown that the function of one of the main envelope glycoproteins of BoHV-4, gp180, is quite reminiscent to that of the MuHV-4 gp150 and EBV gp350 homologues as they all seem to play a role in cell type tropism (68). Mutants that are able to replicate in the epithelium but are locally confined, thus incapable of hijacking immune cells, can be selected for further study as potential MLV candidates. However, we first have to acquire an idea about the seroprevalence and also the incidence of postpartum problems caused by this virus in Belgian cattle farms. At farms with a high seroprevalence and a high incidence of postpartum diseases, intravaginal vaccination with an MLV might be economically justified. This valuable knowledge might also contribute to the development of antiviral measures for other gammaherpesviruses such as EBV and KSHV in humans.

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CHAPTER 9.

SUMMARY – SAMENVATTING

Summary

Alphaherpesviruses, like bovine herpesvirus 1 (BoHV-1) in cattle and herpes simplex virus 1 (HSV-1) and 2 (HSV-2) in humans, preferentially initiate host invasion at mucosal surfaces such as the upper respiratory and lower genital tract. This local dissemination often leads to destruction of mucosal and submucosal layers, resulting in ulcerative disease. To limit free pathogen invasion, the host possesses several selective barriers. Such a barrier is the basement membrane (BM) barrier underneath epithelia. In order to successfully establish viremia and neuronal infection, pathogens need to develop various mechanisms to overcome the BM. However, data describing these mechanisms of stromal invasion for viral infections, particularly, herpesviruses, are rather scarce. Nevertheless, insights in these putative viral invasion mechanisms will provide us new incentives for antiviral intervention strategies. The major aim of this thesis was to thoroughly study mucosal invasion mechanisms of both veterinary herpesviruses BoHV-1 and BoHV-4 as well as human herpesviruses HSV-1 and HSV-2 to: (i) point out differences and similarities at the level of mucosal invasion and (ii) provide insights for the development of alternative preventive and curative approaches representative for animal and human viruses.

Chapter 1 provides an introduction on herpesviruses and an overview on the characteristics of respiratory and genital mucosa. In addition, different microbial interactions with the basement membrane are reviewed.

The first section consists of a brief introduction on herpesvirus biology in general. As the major players within this thesis are bovine herpesvirus 1 (BoHV-1) and 4 (BoHV-4), as well as human herpesvirus 1 (HSV-1) and 2 (HSV-2), more detailed information is provided about their respective pathogenesis and symptomatology.

The second section focuses on structural and functional aspects of upper respiratory and lower genital tract tissues with emphasis on several barriers including mucus, junctional complexes and the basement membrane.

The third section of this chapter describes the current knowledge on existing microbial mechanisms to circumvent the basement membrane barrier for host invasion.

In Chapter 2, the aims of this thesis are formulated.

Chapter 3 describes the development of *ex vivo* organ cultures of respiratory and genital mucosa with the purpose to create a homologous model for the study of herpesvirus invasion.

A first part describes the establishment of explant models for both bovine upper respiratory tissues, including nasal septum, ventral conchae and proximal trachea; and genital tissues including *vestibulum vaginae*.

A second part tackles the optimization of a human genital organ culture consisting of either ectocervix/vagina or endocervix.

All explant models could be maintained for at least 96h of *in vitro* culture. During cultivation, no significant changes in tissue viability or tissue morphology were observed as shown by TUNEL assay and light, scanning electron and transmission electron microscopy. Several parameters such as apoptosis, epithelial integrity, basement membrane continuity and connective tissue morphology were evaluated at different time points of cultivation. The use of the present modeling tools is in line with the 3R principle of Russell and Burch (Refinement, Reduction and Replacement). The major advantage of these 3D models is the maintenance of normal cell-cell contacts and tissue structure compared to 2D cell monolayers.

Chapter 4 describes BoHV-1 mucosal dissemination and invasion at host entry ports.

In a first part, several mucosal explants derived from upper respiratory tissues were inoculated with the Cooper BoHV-1 strain. The salient findings were that BoHV-1 displays a plaquewise spread during its dissemination in respiratory mucosa explants. In addition, from 24h pi onwards crossing of the BM was observed and the majority of plaques crossed the BM at 48h pi. Furthermore, BoHV-1 invaded more efficiently across the BM in proximal tracheal tissue. Hence, proximal trachea appears to be an interesting target tissue to unravel BoHV-1 invasion mechanisms in respiratory mucosa.

In a second part, to address whether different BoHV-1 subtypes display a different tissue tropism, we inoculated several respiratory and genital mucosa explants derived from the same

animals with the different existing BoHV-1 subtypes 1.1 (respiratory tropism), 1.2a (genital tropism) and 1.2b (genital tropism). A quantitative analysis of viral invasion in the mucosa, performed at 0h, 24h, 48h and 72h post inoculation (pi) revealed that all subtypes disseminated quite alike. All BoHV-1 subtypes exhibited a more profound invasion capacity in respiratory tissue compared to that in genital tissue at 24h pi. However, at 24h pi plaque latitude was found to be larger in genital tissue compared to respiratory tissue and this for all subtypes. These similar findings among the different subtypes take the edge off the belief of the existence of specific mucosa tropisms of different BoHV-1 subtypes.

In Chapter 5, the invasion mechanism of a bovine gammaherpesvirus, BoHV-4, is compared to invasion mechanisms employed by alpha- and gammaherpesviruses.

The pathogenesis of bovine herpesvirus 4 (BoHV-4) is not well characterized. We sought to investigate the early steps in the pathogenesis of this pathogen known for its contribution to cases of post-partum metritis. To this end, both bovine respiratory and genital explants were inoculated with BoHV-4. First, we looked whether these tissues support replication and may act as primary host entry portals. In addition, we compared the dissemination kinetics of BoHV-4 with results obtained before with BoHV-1. In a last step, we studied the cell tropism of BoHV-4 at the level of the mucosa early upon host entry. Interestingly, only genital mucosa proved to be susceptible to BoHV-4 infection shown by multiple single epithelial (cytokeratin$^+$) cell infection at 12h pi. These infectious centers grew steadily over time and became plaques as soon as 48h pi. In addition, scarcely distributed single infected cells in both epithelium and lamina propria could be seen mainly at 24h pi, and were found to be of monocytic/macrophage (CD172a$^+$) origin. Unlike BoHV-1, plaques were not observed at 24h pi and plaques never breached the basement membrane. This is the first report describing aspects of the early steps of the BoHV-4 pathogenesis. Moreover, the established system might be an accessible, animal gammaherpesvirus homologous model system for the hard-to-study human gammaherpesviruses Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV).

Chapter 6 explores the mucosal behavior of human herpes simplex virus 1 (HSV-1) and 2 (HSV-2) in a human genital organ culture

Human genital herpes is worldwide one of the most prevalent causes of genital ulcer disease and with a prevalence reaching up to 80% (developing countries), one of the most important sexually transmitted infections (STI). Firstly, we looked at the distribution of several HSV entry receptors including nectin-1, nectin-2 and herpesvirus entry mediator (HVEM) within endocervical and ectocervical/vaginal non-cultured tissues by means of immunofluorescence stainings. This was performed on tissues in various stages of the menstrual cycle. In a second part, we modeled the dissemination in genital mucosa of both HSV-1 and HSV-2 by inoculating our previously established human genital organ culture and compared the HSV-1 and HSV-2 replication characteristics for different cycle stages within the same patients. Nectin-1 and -2 showed a similar expression pattern in both endocervix and ectocervix/vagina and moreover, this was consistent at all stages of the menstrual cycle. On the contrary, HVEM was found not to be expressed in endocervical or ectocervical epithelial cells. However, we noticed scarcely distributed cells in the epithelium and several cells in the lamina propria to be HVEM positive, presumably immune cells. Both endocervix and ectocervix were found susceptible to both HSV-1 and HSV-2 infection. HSV-1 and -2 both exhibited a plaquewise mucosal spread in all tissues and induced prominent epithelial syncytia in ectocervical tissues, all independent of hormonal cycle stage. Starting from 48h pi, few epithelial viral plaques crossed the basement membrane (BM) showing that HSV-1 and HSV-2 can cause stromal invasion in the genital tract and subsequent HSV viremia.

Chapter 7 investigates the role of the glycoprotein gE/gI complex in the plaquewise crossing of the basement membrane displayed by alphaherpesviruses such as BoHV-1.

To assess the role of gE/gI in the plaquewise penetration of the BM displayed by some alphaherpesviruses, we used the previously established bovine respiratory model and BoHV-1. Movement of virus across cell-cell contacts and axonal transport of viruses both require a functional gE/gI complex. Hence, we investigated the role of gE/gI in BoHV-1 directional spread across the basement membrane. We analyzed the effect of deleting gE/gI using virus recombinants or inhibiting gE/gI by monoclonal antibodies on viral replication, plaque latitude and penetration depth underneath the basement membrane in a bovine respiratory

mucosa model at 48h pi. In addition, virion distribution at the basal cell surface of wild-type and gE and/or gI knockout strains and gE membrane expression was analyzed in inoculated explants at 48h pi by means of transmission electron and confocal microscopy respectively. Strains deleted for either gE and/or gI resulted in a strong reduction in viral replication, plaque latitude and penetration depth underneath the basement membrane. Importantly, a gE CT deleted virus showed an identical phenotype as a complete gE null virus. Addition of monoclonal antibodies directed against gE/gI to the cultivation medium of wild-type inoculated explants from 0h pi onwards, suppressed plaque penetration depth underneath the basement membrane without affecting plaque latitude and viral progeny titers. Subcellular distribution of gE within BoHV-1 epithelial plaques revealed a predominant localization of gE alongside the basement membrane. Next, TEM analysis showed that the majority of wild-type virus particles are located near the basal cell side within basal epithelial cells in contrast to knockouts for gE and/or gI which were mainly found on non-basal cell sides of basal cells and more randomly spread within basal cells. In conclusion, we provided various indications that the gE/gI complex plays a crucial role in BoHV-1 stromal invasion through the basement membrane barrier. Although at this moment still speculative, it seems that gE/gI mimics a basal sorting pathway via its cytoplasmic tail motifs by interacting with specific motor proteins towards basal domains in basal epithelial cells and orchestrates invasion once present near the basement membrane.

Chapter 8 summarizes the obtained results in this thesis and presents a general discussion on the acquired knowledge.

The main conclusions drawn from this thesis are threefold:

- ***Ex vivo* organ cultures were successfully established for bovines and humans consisting of either respiratory or genital mucosa. These alternative experimental models allow mechanistic studies of mucosal invasion of bovine and human pathogens.**

- **The established mucosa explant models are susceptible to herpesvirus infections, which allows to study and compare herpesvirus invasion mechanisms at mucosal surfaces. All herpesviruses used in this study clearly exhibited an epithelial tropism, irrespective of their taxonomy or host tropism. Each of these viruses appears to apply finely tuned mechanisms for efficient invasion within the host.**

- **The viral heterodimer gE/gI plays a crucial role in the invasive pathway of BoHV-1 through the basement membrane in respiratory mucosa. We suggest that in epithelial cells gE/gI selectively and topologically distributes other viral proteins in membranes, stabilizes the (mis)usage of cellular motor proteins quite reminiscent to herpesvirus axonal transport, to specifically route virions towards adhesive structures either in contact with other cells or with the extracellular matrix and orchestrates invasion at the basement membrane once sorted correctly.**

Samenvatting

Voor alfaherpesvirussen zoals het bovien herpesvirus 1 (BoHV-1) bij runderen en het humaan herpes simplex virus 1 (HSV-1) en 2 (HSV-2) bij mensen dienen mucosale membranen van zowel het respiratoir als genitaal stelsel vaak als voorkeursplaatsen voor primaire vermeerdering. Deze lokale spreiding in het epitheel resulteert vaak in destructie van zowel mucosa als submucosa met ulcererende ziektesymptomen tot gevolg. De gastheer probeert deze vrije passage van pathogenen te verhinderen door de aanwezigheid van enkele barrières zoals de basaalmembraan onder epithelia. Om succesvol te zijn in het infiltreren van de gastheer moeten pathogenen dus beschikken over een aantal mechanismen om deze barrières te omzeilen. Het aantal studies rond deze mechanismen van stromale invasie gebruikt door virussen is echter zeer beperkt. Niettemin zouden nieuwe inzichten in deze specifieke mechanismen ons toelaten om op een rationele basis nieuwe antivirale toepassingen te ontwikkelen. Het hoofddoel van deze thesis was om de invasiemechanismen van zowel een animaal (BoHV-1) als een humaan (HSV) alfaherpesvirus te onderzoeken en ook met elkaar te vergelijken. Dit zou enkele gelijkaardigheden maar ook tegenstellingen aan het licht kunnen brengen welke van belang zouden kunnen zijn bij het ontwikkelen van alternatieve preventieve en curatieve behandelingen voor herpesvirussen in de diergeneeskundige en geneeskundige sector.

Hoofdstuk 1 leidt herpesvirussen in en geeft daarnaast een overzicht van de karakteristieken van respiratoire en genitale mucosa. Verder worden er verschillende gekende microbiële interacties met de basaalmembraan beschreven.

Het eerste deel geeft een introductie in de algemene herpesvirus biologie. Daarnaast wordt er uitvoerig gedocumenteerd over pathogenese en symptomatologie van de hoofdspelers in deze thesis namelijk bovien herpesvirus 1 (BoHV-1) en 4 (BoHV-4) alsook humaan herpes simplex virus 1 (HSV-1) en 2 (HSV-2).

Het tweede deel focust op structurele en functionele aspecten van zowel de bovenste ademhalingswegen als het genitaal stelsel met bijzondere aandacht voor verschillende barrières zoals de mucus laag, het epitheel en de basaalmembraan.

Het derde deel beschrijft de huidig gekende mechanismen die pathogenen gebruiken voor interactie met de basaalmembraan.

In Hoofdstuk 2 worden de algemene doelstellingen van deze thesis uiteengezet.

Hoofdstuk 3 handelt over het opzetten en ontwikkelen van *ex vivo* orgaan culturen bestaande uit zowel respiratoire als genitale mucosa als tools voor de studie van herpesvirus invasie.

Een eerste deel beschrijft het opzetten van explant culturen voor boviene respiratoire mucosa, meer specifiek nasaal septum, ventrale conchae en proximale trachea maar ook voor boviene genitale mucosa zoals het *vestibulum vaginae*.

Het tweede deel behandelt de optimalisatie van een humane genitale orgaan cultuur bestaande uit enerzijds ectocervix/vagina en anderzijds uit endocervix.

Alle opgezette orgaanculturen konden gecultiveerd worden *in vitro* voor minstens 96h na opzet. Tijdens deze cultivatie werden er geen significante verschillen geobserveerd in weefsel viabiliteit of morfologie wat werd aangetoond met behulp van lichtmicroscopie, scanning elektronenmicroscopie en transmissie elektronenmicroscopie. Verschillende parameters zoals het aantal apoptotische cellen, epitheliale integriteit, basaalmembraan continuïteit en bindweefsel structuur werden zo geëvalueerd op behoud tijdens cultivatie. Het gebruik van deze modellen is in lijn met het 3 V principe van Russell en Burch (Verfijning, Vermindering en Vervanging). Het grote voordeel van deze 3D modellen is dat ze perfect de noodzakelijke normale weefsel structuur en celcontacten behouden in tegenstelling tot de huidige 2D celculturen.

Hoofdstuk 4 gaat over de spreiding van BoHV-1 in mucosale weefsels en welke mechanismen er gebruikt worden door dit virus om efficiënt doorheen de basaalmembraan te dringen.

In een eerste deel, werden verschillende respiratoire mucosale explanten geïnoculeerd met de Cooper stam van BoHV-1. Vervolgens werd de kinetiek van virusspreiding nagegaan op verschillende tijdstippen na inoculatie. BoHV-1 bleek zich op een plaque-gewijze manier te verspreiden in het epitheel. Daarenboven braken enkele plaques vanaf 24h pi doorheen de basaalmembraan. Dit werd duidelijker op 48h pi aangezien het merendeel van de plaques zich

op dat moment een weg hadden gebaand doorheen de basaalmembraan. Vooral in tracheale explanten werd een zeer efficiënte spreiding doorheen de basaalmembraan geobserveerd waardoor we konden concluderen dat deze regio uit de bovenste ademhalingswegen een interessant weefsel is voor gebruik in verdere studies.

In een tweede deel, om specifiek de vraag te beantwoorden of er effectief verschillende subtypes bestaan binnen BoHV-1 met een verschillend weefsel tropisme, werden er verschillende respiratoire en genitale explanten afkomstig van dezelfde dieren geïnoculeerd met de verschillende gekende BoHV-1 subtypes namelijk 1.1 (respiratoir tropisme), 1.2a (genitaal tropisme) en 1.2b (genitaal tropisme). Opnieuw werd er met behulp van een kwantitatief analyse systeem gekeken naar virus spreiding in de mucosa op 0h, 24h, 48h en 72h pi. Alle subtypes spreidden gelijkaardig, zowel in respiratoir als in genitaal weefsel. Voor alle subtypen werd een meer efficiëntere invasie capaciteit doorheen de basaalmembraan vanuit epitheliale plaques geobserveerd in respiratoir weefsel dan in genitaal weefsel op 24h pi. Daarnaast, op 24h pi, was de plaque breedte groter in genitale weefsels in vergelijking met respiratoire weefsels, opnieuw voor alle subtypes. Deze toch wel frappante gelijkenissen in spreiding van de verschillende subtypes, ondanks de vermeende verschillende weefsel voorkeur, stellen de huidige subtype verdeling erg in vraag.

In Hoofdstuk 5 wordt uitvoerig de studie, wat betreft het invasiemechanisme van een ander boviene herpesvirus, bovien gammaherpesvirus BoHV-4, beschreven voor vergelijking van de invasiemechanismen gebruikt door alfa- en gammaherpesvirussen.

De pathogenese van BoHV-4 is niet goed gekend. Nochtans speelt dit pathogeen een belangrijke rol in post-partum baarmoederontsteking bij runderen. De ontwikkelde respiratoire en genitale explant modellen werden zodoende aangewend om de vroege aspecten van de BoHV-4 pathogenese te visualiseren. Eerst werd er nagegaan welke weefsels BoHV-4 replicatie ondersteunen. Vervolgens werd er gekeken naar de verschillen en gelijkenissen met BoHV-1. Finaal, werd er ook getracht de BoHV-4 geïnfecteerde cellen te identificeren. BoHV-4 bleek enkel in staat de genitale mucosa explanten te infecteren. Op 12h pi, werden er afzonderlijk geïnfecteerd cellen geobserveerd in het epitheel. Deze bleken hoofdzakelijk van epitheliale oorsprong (cytokeratine^{+}) te zijn. Epitheliale infectieuze centra groeiden uit tot echte plaques op 48h pi. Daarnaast werden er voornamelijk op 24h pi ook individuele geïnfecteerde cellen gekarakteriseerd als cellen van de monocyt/macrofaag lijn (CD172a^{+}), dit

zowel in het epitheel als lamina propria. In tegenstelling tot BoHV-1, werden er geen BoHV-4 epitheliale plaques geobserveerd op 24h pi en meer nog, BoHV-4 epitheliale plaques penetreerden op geen enkel tijdstip de basaalmembraan. Dit is de eerste studie die de vroege aspecten van de pathogenese van BoHV-4 beschrijft. Het gebruikte homologe systeem kan in de toekomst dienen als een goed alternatief diermodel voor de moeilijk te bestuderen humane gammaherpesvirussen Epstein-Barr virus (EBV) en Kaposi's sarcoma-associated herpesvirus (KSHV).

Hoofdstuk 6 geeft weer op welke manier er onderzocht werd hoe het humane herpes simplex virus 1 (HSV-1) en 2 (HSV-2) zich gedragen in humane genitale mucosa, wat toelaat om invasiemechanismen van animale en humane alfaherpesvirussen te vergelijken.

Humane genitale herpes is wereldwijd de meest voorkomende oorzaak van het ulceratieve urogenitale ziektebeeld en met een prevalentie tot 80% bij volwassenen (ontwikkelingslanden), één van de belangrijkste seksueel overdraagbare aandoeningen (SOA). Eerst werd er een analyse van de aanwezigheid en lokalisatie van gekende HSV entry receptoren, met name nectine-1 en -2 alsook herpesvirus entry mediator (HVEM), uitgevoerd en dit op ongecultiveerde ectocervicale/vaginale en endocervicale weefsels afkomstig van patiënten uit verschillende stadia van de menstruele cyclus. Vervolgens, werd er nagegaan hoe HSV-1 en HSV-2 spreiden in humane genitale mucosa op verschillende tijdstippen van de menstruele cyclus. Nectine-1 en -2 vertoonden een gelijkaardig distributie patroon, zowel in endocervix als ectocervix/vagina en bleken ook consistent te zijn tijdens de gehele menstruele cyclus. HVEM daarentegen kwam niet tot expressie in endocervicale en ectocervicale/vaginale epitheelcellen, maar werd geobserveerd in zeldzame cellen in het epitheel en in meerdere cellen in het bindweefsel welke naar alle waarschijnlijkheid immuun cellen waren. Zowel endocervix als ectocervix/vagina bleken gevoelig te zijn aan een infectie met HSV-1 en HSV-2. Beide virussen vertoonden een plaque-gewijze spreiding in alle weefsels en induceerden de vorming van celfusies (syncytia) in ectocervicale/vaginale mucosa. Vanaf 48h pi werd geobserveerd dat een aantal plaques doorheen de basaalmembraan penetreerden, zij het subtiel. Dit gaf een duidelijke indicatie dat HSV het lichaam kan invaderen via de genitale mucosa en zo mogelijks een viremie kan veroorzaken.

In Hoofdstuk 7 onderzochten we de rol van het virale glycoproteïnen gE/gI complex in de plaque-gewijze spreiding doorheen de basaalmembraan van alfaherpesvirussen zoals BoHV-1.

De bijdrage van een multivirulentie glycoproteïnen complex gE/gI in het invasiemechanisme van alfaherpesvirussen werd onderzocht met behulp van het geoptimaliseerde respiratoire explant model en BoHV-1. Zowel het cel tot cel spreiden van herpesvirussen als het transport in neuronale axonen heeft een functioneel gE/gI complex nodig om optimaal te functioneren. Vandaar onderzochten we specifiek de rol van dit complex in spreiding doorheen de basaalmembraan. Zo analyseerden we op 48h pi het effect op virale replicatie, plaque breedte en plaque penetratie diepte onder de basaalmembraan van enerzijds het volledig uitschakelen van de genen coderend voor gE/gI in het virus zelf en van anderzijds het inhiberen van hun functie door het toevoegen van monoklonale antistoffen die specifiek binden aan deze proteïnen. Daarnaast werd er ook gekeken door middel van transmissie elektronenmicroscopie naar de lokalisatie van zowel wild-type virus partikels als dat van gE en/of gI gedeleteerde virus partikels in basale epitheliale cellen. Meer nog, ook de subcellulaire lokalisatie van gE in BoHV-1 epitheliale plaques werd nagegaan met behulp van confocale microscopie. Virussen die gedeleteerd werden voor gE en/of gI bleken erg gehinderd te zijn in virale replicatie en in spreiding, zowel lateraal (plaque breedte) als in de diepte (plaque penetratie diepte). Belangrijk was de observatie dat een mutant waar enkel de cytoplasmatische staart van gE werd gedeleteerd hetzelfde fenotype vertoonde als een mutant met een volledig gedeleteerd gE. Het toedienen van monoklonale antistoffen, specifiek gericht tegen gE en/of gI, in het explant medium vanaf 0h pi resulteerde enkel in een sterk gereduceerde plaque penetratie diepte zonder effect op plaque breedte of virale replicatie. De distributie van gE bleek overduidelijk zich basaal te bevinden ter hoogte van de basaalmembraan. Er werd ook waargenomen dat wild-type virions zich eerder basaal en soms lateraal in basale epitheliale cellen afzetten waar gE en/of gI gedeleteerde virions zich eerder op niet-basale zijden bevinden en vaak ook verspreid doorheen de ganse cel. In conclusie kunnen we stellen dat deze studie op verschillende manieren aantoonde dat het gE/gI complex een cruciale rol speelt in het BoHV-1 stromale invasie mechanisme doorheen de basaalmembraan. Na analyse van de resultaten, kwamen we tot de volgende speculatie: gE/gI medieert via zijn cytoplasmatische staart motieven een basale sortering van virions richting basale plasmamembraan domeinen en stabiliseert hierbij mogelijks de interactie met cellulaire motor

proteïnen. Eenmaal aanwezig ter hoogte van de basaalmembraan, dirigeert het complex vervolgens invasie doorheen de basaalmembraan.

In Hoofdstuk 8 wordt een algemene discussie opgebouwd rond de bekomen resultaten in deze thesis.

De volgende algemene conclusies kunnen naar voren geschoven worden:

- **Er werd succesvol een *ex vivo* mucosaal explant model voor gebruik bij runderen en mensen op punt gezet bestaande uit respiratoire of genitale mucosa. Deze broodnodige, alternatieve experimentele modellen correleren zeer goed met de natuurlijke situatie en laten studies toe van mucosale invasiemechanismen gebruikt door verschillende ziekteverwekkers.**

- **De ontwikkelde modellen ondersteunen productieve replicatie van herpesvirussen en zijn bijgevolg zeer geschikte 'tools' om de invasiemechanismen van verschillende herpesvirussen te vergelijken maar ook in detail te bestuderen. Alle herpesvirussen gebruikt in deze studie vertoonden een duidelijke voorkeur voor epitheliale cellen, ongeacht hun fylogenetische classificatie of gastheer specificiteit. Niettegenstaande, werd er ook aangetoond dat ze allen hun eigen manier ontwikkelden om gastheer invasie te veroorzaken.**

- **Mechanistisch toonden we aan dat het heterodimeer gE/gI een cruciale rol speelt in het invasiemechanisme van BoHV-1 doorheen de basaalmembraan in respiratoire mucosa. Met de bekomen resultaten brengen we de volgende, hoewel speculatieve, hypothese aan: in epitheliale cellen, drijft gE/gI heel selectief andere virale proteïnen naar topologisch verschillende membraancompartimenten, stabiliseert het vervolgens het misbruik van cellulaire motor proteïnen om getransporteerd te worden richting naburige cellen of de basaalmembraan, heel gelijkaardig aan wat al gekend is voor axonaal transport van herpesvirussen in neuronen, en zal het tenslotte, eenmaal aanwezig ter hoogte van de basale zijde van cellen naast de basaalmembraan ook verdere invasie bewerkstelligen.**

CURRICULUM VITAE

Personalia

Lennert Steukers werd geboren op 14 september 1984 te Herentals. In 2002 behaalde hij het getuigschrift van hoger secundair onderwijs aan het Kardinaal van Roey-Instituut (Vorselaar) in de richting Moderne Talen – Wetenschappen. Datzelfde jaar startte hij zijn universitaire studies Diergeneeskunde aan de Universiteit Gent. Na het verwerven van het diploma Kandidaat-dierenarts met grote onderscheiding in 2005, behaalde hij vervolgens in 2008 het diploma van Dierenarts (optie Herkauwers) met grote onderscheiding. Door zijn laatstejaarsscriptie “Prevalentie van oviene herpesvirus type 2 (BKK) in de Belgische schapenpopulatie”, raakte hij geboeid door virale ziekten. Aansluitend startte hij dan ook in 2008 zijn doctoraat aan het laboratorium voor Virologie, Vakgroep Virologie, Parasitologie en Immunologie, Faculteit Diergeneeskunde. Zijn onderzoek handelde over de vroege pathogenese van infecties met zowel het bovien herpesvirus 1 en 4 als het humaan herpes simplex virus 1 en 2 ter hoogte van respiratoire en genitale mucosa. Daarnaast stond hij ook in voor het onderwijs van laatstejaarsstudenten optie herkauwers en was hij betrokken bij de diagnostiek van virale ziekten bij de grote huisdieren. Zijn studie werd eerst gefinancierd door een Geconcentreerde Onderzoeksactie (GOA) van de Universiteit Gent. Sinds 2009 beschikte hij over een specialisatiebeurs toegekend door het Agentschap voor Innovatie door Wetenschap en Technologie (IWT). Verder verbleef hij in het kader van zijn onderzoek vanaf 30 mei tot en met 2 december 2011 aan de Feinberg School of Medicine, Northwestern University in Chicago (USA) als international research scholar met behulp van een beurs van het Fonds Wetenschappelijk Onderzoek – Vlaanderen (FWO). Tenslotte voltooide hij in 2013 met succes de doctoraatsopleiding van de Doctoral School of Life Sciences and Medicine.

Publicaties

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- **L. Steukers** (*Invited Speaker*) "The use of mucosal explants to study herpesvirus host invasion" (November 2011), Diagnostic Center for Population and Animal Health, College of Veterinary Medicine, Michigan State University, USA.

- **L. Steukers**, S. Glorieux, S. Weyers, A.P. Vandekerckhove, M. van de Water, M. Cornelissen, W. Van den Broeck, M. Temmerman, H.J. Nauwynck. Elucidation of HSV-1 and HSV-2 mucosal behavior in a new human genital organ culture for a better understanding and control of Genital Herpes. Proceedings of 36th annual International Herpesvirus Workshop, Gdansk, Poland (2011).

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Wetenschappelijke beurzen en prijzen

jan. ’10 – jan. ’14:	Specialisatiebeurs van het Agentschap voor Innovatie door Wetenschap en Technologie (IWT) (SB 091197 & 093197).
jul. ’13:	Beurs van het Fonds Wetenschappelijk Onderzoek – Vlaanderen (FWO) voor deelname aan een congres in het buitenland (grant K193713N)
aug. ’12:	International Herpesvirus Workshop Travel Award
aug. ’12:	Beurs van het Fond Wetenschappelijk Onderzoek – Vlaanderen (FWO) voor deelname aan een congres in het buitenland (grant K197212N)
mei ’11 – dec. ’11:	Fonds Wetenschappelijk Onderzoek – Vlaanderen (FWO) International Mobility grant, Feinberg School of Medicine, Northwestern University, Chicago, Illinois, USA (grant V427011N)
nov. ’10:	Excellent Poster Prize award, Belgium Society for Microbiology Meeting, Brussel, België
aug. ’10:	Reiskrediet van de Facultaire Onderzoekscommissie, Faculteit Diergeneeskunde, Universiteit Gent voor deelname aan een congres
sep. ’08 – dec. ’09:	Doctoraatsbeurs in het kader van een Geconcentreerde Onderzoeksactie (GOA), Universiteit Gent

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