



Infection of the Ovine herpesvirus 2 in the Reservoir Host, Sheep, and the Susceptible Host, Cattle

Thesis submitted in accordance with the requirements of the University
of Liverpool for the degree of Doctor in Philosophy

by

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Author's Declaration

Apart from the help and advice acknowledged, this thesis represents the unaided work of the author

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This research was carried out in the Department of Infection Biology and School of Veterinary Science, University of Liverpool, and Institute of Veterinary Pathology, Vetsuisse Faculty, University of Zurich, Switzerland.

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Abstract

Ovine Herpesvirus-2 (OvHV-2) is a gamma-herpesvirus that belongs to genus *macavirus*, is endemic in sheep worldwide. The virus infects sheep subclinically but when it is transmitted to cattle; it induces malignant catarrhal fever (MCF), a frequently fatal lymphoproliferative disease. The pathogenesis and site of OvHV-2 is unknown in both species. In this study, we tried to: first detect the virus presence and measure its DNA loads; secondly, localise the precise cellular location of the virus in the tissues of sheep and domestic cattle. For the first purpose, we optimised and validated a sensitive quantitative polymerase chain reaction (qPCR) technique using Taqman® probe system that can detect and measure the virus's DNA as low as one viral DNA copy in a qPCR reaction. Secondly, we applied RNA *in situ* hybridisation (RNA-ISH) technique to detect viral transcripts (Ov2.5 a latent gene and ORF65 a lytic gene), and in addition we used immunohistology to stain the viral Ov8 antigen (glycoprotein) by specific polyclonal antibodies. For these purposes we have used a variety of organs and tissues, namely: respiratory tract, tongue, muzzle, lymphoid and reproductive organs as well as nasal swabs and peripheral blood leukocytes from randomly selected sheep (n=28), cows without MCF (n=50) and cattle with MCF (n=12). The results in sheep have shown that 88 % of them harbour viral DNA in most of their organs at very low amounts. The viral mRNA and antigen were also detected in a wide range of organs including epithelial cells of respiratory tract, tongue and muzzle, macrophages and lymphocytes (B cells) in bronchial associated lymphoid associated lymphocytes (BALT), lymph nodes in spleen as well as vascular endothelial cells of many of these tissues. Interestingly in cattle without MCF, results were very similar as in sheep i.e.; viral DNA was found in a large population of cattle (67 %); and viral transcripts and antigen detected in a large proportion of tested organs, similar as seen in sheep. In the MCF-affected cattle, similar types of cells were found infected as in cattle without MCF, but with significantly higher viral loads (more than three logs). This study shows for the first time OvHV-2 location and cell types they infect in sheep, and in cattle that do not show any evidence of MCF. The new question is what triggers inducing of MCF in the subclinically infected healthy cattle?. That can be addressed by further investigations.

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List of abbreviations

ABC	Avidin biotin complex
APC	Antigen presenting cells
BALT	Bronchus associated lymphoid tissue
Bcl2	B cell lymphoma 2
BEC	Bronchial epithelial cells
BL	Burkitt's lymphoma
BME	β -mercaptoethanol
BSA	Bovine serum antigen
CD	Cluster of differentiation
DAB	Diaminobencidin tetrahydrochloride
DC	Dendritic cells
ddH ₂ O	Distilled de-ionized water
DECP H ₂ O	Diethyl pyrocarbonate treated distilled water
DNA	Deoxyribonucleic acid
DPX	Distyrene plasticizer and xylene
ds	Double stranded
EBV	Epstein-Barr virus
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FDC	Follicular dendritic cells
g	Gram
h	Hour
HCl	Hydrochloric acid
HE	Haematoxylin-eosin
HPF	High power field
IF	Immunofluorescence
IFN	Interferon
Ig	Immunoglobulin
IH	Immunohistology

IL	interleukin
IM	Infectious mononucleosis
ISH	<i>in situ</i> hybridisation
Kb	Kilobase
KS	Kaposi's sarcoma
KSHV	Kaposi's sarcoma-associated herpesvirus
L	Litre
mAb	Monoclonal antibody
MCF	Malignant catarrhal fever
mg	Milligram
Mg	Microgram
MHC	Major histocompatibility complex
min	Minute
μl	Microliter
ml	Millilitre
mM	Millimole
Mm	Micrometre
mmol/L	Millimole per litre
mo	Month
mRNA	Messenger ribonucleic acid
NaCl	Sodium chloride
nt	Nucleotide
ORF	Open reading frame
pAb	Polyclonal antibody
PAP	Peroxidase anti-peroxidase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
REC	Respiratory endothelial cells
RNA	Ribonucleic acid
RNA-ISH	RNA- <i>in situ</i> hybridisation

RT	Room temperature (25 °C)
SPF	Specific pathogen free
TBST	Tris-buffered sulphate tween
TEM	Transmission electron microscopy
U	Unit
v/v	Volume/volume
VEC	Vascular endothelial cells
w/v	Weight/volume
we	Week
x g	x gravity
y	Year

CHAPTER ONE: INTRODUCTION

1.1 The order *Herpesvirales*

Herpesviruses belong to the main order *Herpesvirales* that comprises a large number of viruses and three main families; *Alloherpesviridae*, *Herpesviridae* and *Malacoherpesviridae* (Davison, 2010; Davison *et al.*, 2009) (Figure1-1). The family *Alloherpesviridae* includes the viruses of fishes and frogs. The family *Herpesviridae*, which has recently been incorporated into the order, includes the viruses of mammals, birds and reptiles. The family *Malacoherpesviridae* includes the viruses of Oyster bivalves. In this thesis, the family *Herpesviridae* is the focus of interest and is discussed in more detail.

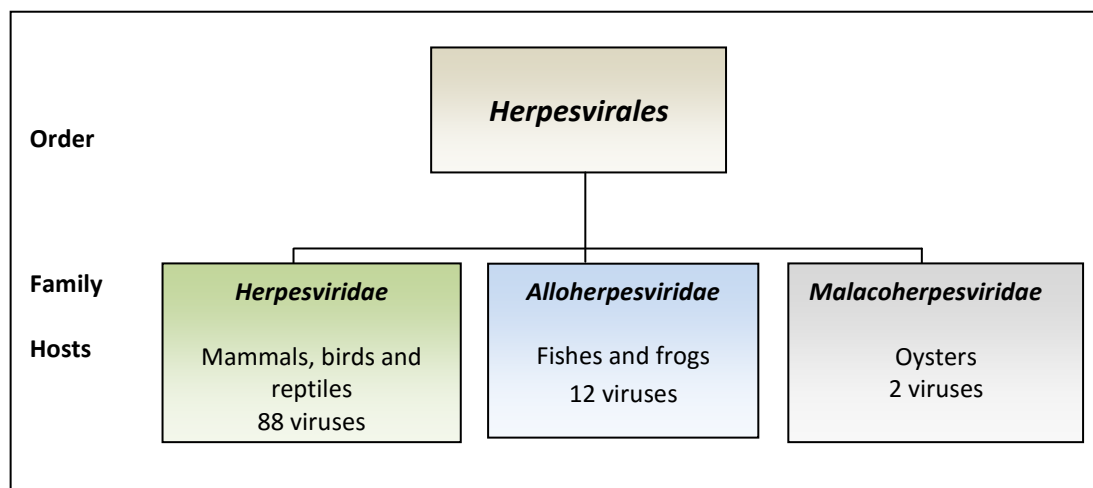


Figure 1-1. Taxonomy of Herpesviridae within the order Herpesvirales, and their natural hosts. Adopted from (King *et al.*, 2012) and (<http://ictvonline.org/virusTaxonomy.asp>).

1.1.2 The family *Herpesviridae*

This family includes the viruses of mammals, birds and reptiles; the family members were updated according to International Committee on Taxonomy of Viruses 9th report in 2011 and are shown in Table 1-1.

1.1.2.1 Biological Properties of *Herpesviridae*

Herpesviridae have a distinct morphology, they have an icosahedral symmetrical capsid of 100-110 nm in diameter that includes 162 capsomers; this is surrounded by an amorphous proteintious layer called the tegument that is further surrounded

by a lipid bilayer envelope containing the viral glycoprotein spikes (Figure 1-2). Altogether, form a virion of 120-300 nm in diameter. The genome is a linear double stranded DNA, one segment of 125-295 kb with 32-75% CG (King *et al.*, 2012; Pellett & Roizman, 2013). This genome is in a liquid crystalline array that occupies the nucleocapsid (King *et al.*, 2012).

Herpesviridae members share four main characteristics. First, they have a wide range of enzymes for nucleic acid metabolism; DNA and protein synthesis, that vary between different members. Secondly, virus gene transcription, synthesis of DNA and nucleocapsid assembly takes place in the nucleus of the host cell. Thirdly, they have a virus production infection phase that is usually characterised by lysis of the infected host cell (Pellett & Roizman, 2013). Finally, all known Herpesviruses are able to establish a long term latent host infection to maintain the viral genomic DNA for a future production cycle (Pellett & Roizman, 2013), to do this the virus DNA is maintained within the host cell as episomal DNA (Decker *et al.*, 1996).

Herpesviridae are well distributed in nature in a way that most animals have at least one herpesvirus and some viruses have more than one definitive host. To date more than 200 herpesviruses have been identified, nine of which are human viruses (Pellett & Roizman, 2013). Viruses of veterinary importance are found in mammals, birds and fish.

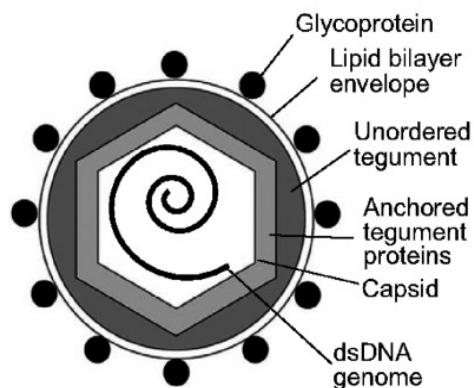


Figure 1-2. Schematic diagram of an herpesvirus. Illustrating the multilayer organisation of a human herpesviruses virion. Taken from (Arvin *et al.*, 2007).

1.1.2.2 *Herpesviridae* classifications

Herpesviridae family can be classified either according to their genome sequence arrangement or according to their phylogenetic relationships. Based on the former, they can be allocated to six main groups (A - F) on the basis of the tandem repeats as illustrated with genome structure diagrammes in Figure 1-3. The advantage of these inverted repeats within the genome is not known (Pellet and Rizmon, 2013), but the different termini repeats serve in viral DNA cleavage and packaging in to the capsid (Deiss *et al.*, 1986).

Phylogenetically, *Herpesviridae* can be further divided in to three main subfamilies and one unassigned subfamily (Pellett & Roizman, 2013). The main subfamilies are *Alphaherpesvirinae*, *Betaherpesvirinae* and *Gammaherpesvirinae*. These were early classified according to their biological features before identification of their DNA sequences (Davison *et al.*, 2009; Roizman *et al.*, 1981), which was then supported when polymerase chain reaction (PCR) amplified sequences were analysed (Davison *et al.*, 2005). The most recent taxonomy of herpesviruses can be found in latest releases of international committee on viral taxonomy (<http://www.ictvonline.org/virusTaxonomy.asp>) and it updates continually. Herpesviruses are evolutionarily well adapted to their hosts and thus are host specific and able to stay in their host latently for their lifespan (Davison, 2002).







Group	Genome	Example
A	 <p data-bbox="411 488 1053 521">Identical terminal repeats (TR) at both ends of the genome</p>	Equid Herpesvirus 2 (EHV2)
B	 <p data-bbox="411 723 1037 757">Frequent terminal repeats at both termini of the genome</p>	Murid Herpesvirus 4
C	 <p data-bbox="411 947 1165 1014">Several terminal repeats at each termini plus other additional repeats(R1, R2, R3 and R4) within the core</p>	Human Herpesvirus 4 (EBV)
D	 <p data-bbox="411 1205 1165 1294">The genome has terminal repeats next to a short unique sequence (US), then invert repeat (IR) within the genome followed by a long unique component (LU)</p>	Human Herpesvirus 3 (VZV)
E	 <p data-bbox="411 1451 1165 1574">The genome has frequent several short repeats (A) followed by a larger sequence (B), next to a long unique sequence. On the other terminus a copy of (A) is followed by a larger sequence (C) then a short unique sequence followed by inverts of B and C.</p>	Human Herpesvirus 1 (HSV)
F	 <p data-bbox="411 1776 861 1809">No terminal repeats have been identified</p>	Tupaiid herpesvirus 1 (TSHV)

Figure 1-3. Grouping of Herpesviridae according to repeated sequences at termini of the genome. Adopted after (Pellet and Rizmon, 2013).

Nowadays there are more than 90 viruses and this number seems to increase further with the discovery of new species. In terms of medical and veterinary importance, *Herpesviridae* has at least nine human viruses and 28 viruses of veterinary importance. The three main subfamilies have weak phylogenetic relationship (Figure 1-4).

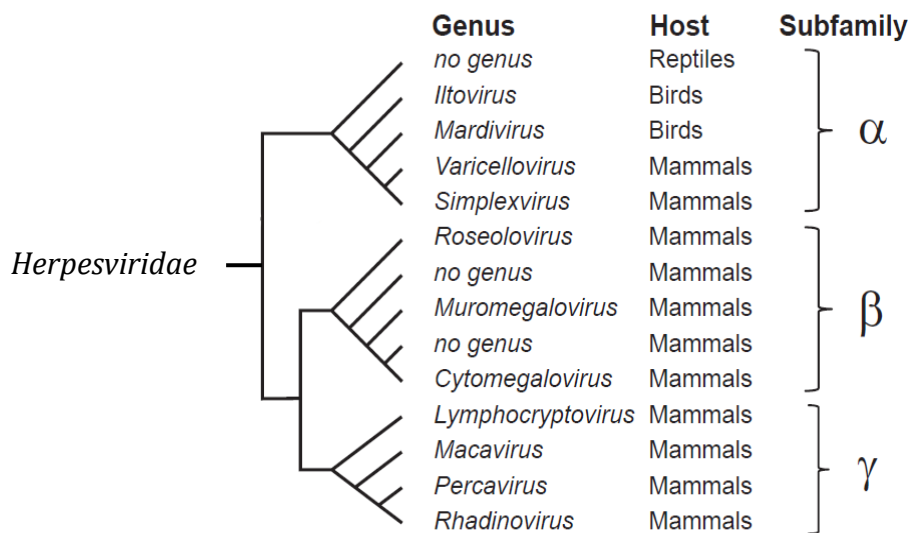


Figure 1-4. Phylogenetic relationship between genera of *Herpesviridae*. Adopted from (Davison, *et al.*, 2013).

Table 1-1. Species of the family Herpesviridae. Assembled after (Davison et al., 2009; King et al., 2012) and updates from International Committee on Taxonomy of Viruses (ICTV) Version 2, June, 2014 (<http://ictvonline.org/virusTaxonomy.asp>).

<i>Herpesviridae</i>	Species	Acronym	Common name(s)
Subfamily <i>Alphaherpesvirinae</i>			
Genus <i>Iltovirus</i>	Gallid herpesvirus 1	GaHV-1	Infectious laryngotracheitis virus
	Psittacid herpesvirus 1	PsHV-1	Pacheco's disease virus
Genus <i>Mardivirus</i>	Anatid herpesvirus 1		
	Columbid herpesvirus 1	CoHV-1	Columbid herpesvirus 1
	Gallid herpesvirus 2	GaHV-2	Marek's disease virus type 1
	Gallid herpesvirus 3	GaHV-3	Marek's disease virus type 2
	Meleagrid herpesvirus 1	MeHV-1	Turkey herpesvirus
Genus <i>Scutavirus</i>	Chelonid herpesvirus 5	ChHV-5	Chelonid fibropapilloma-associated herpesvirus
Genus <i>Simplexvirus</i>	Ateline herpesvirus 1	AtHV-1	Spider monkey herpesvirus
	Bovine herpesvirus 2	BoHV-2	Bovine mammillitis virus
	Cercopithecine herpesvirus 2	CeHV-2	Simian agent 8 (SA8))
	Human herpesvirus 1	HHV-1	Herpes simplex virus 1
	Human herpesvirus 2	HHV-2	Herpes simplex virus 2
	Leporid herpesvirus 4	LeHV-4	
	Macacine herpesvirus 1	McHV-1	Cercopithecine herpesvirus 1 B-virus, herpesvirus simiae
	Macropodid herpesvirus 1	MaHV-1	Parma wallaby herpesvirus
	Macropodid herpesvirus 2	MaHV-2	Dorcopsis wallaby herpesvirus
	Papiine herpesvirus 2	PaHV-2	Cercopithecine herpesvirus 16
	Saimiriine herpesvirus 1	SaHV-1	Herpesvirus tamarinus (Marmoset herpesvirus)
Genus Unassigned	Chelonid herpesvirus 6	ChHV-6	Lung-eye-trachea disease-associated virus
Genus <i>Varicello-virus</i>	Bovine herpesvirus 1	BoHV-1	Infectious bovine rhinotracheitis virus
	Bovine herpesvirus 5	BoHV-5	Bovine encephalitis herpesvirus
	Bubaline herpesvirus 1	BuHV-1	Water buffalo herpesvirus
	Canid herpesvirus 1	CaHV-1	Canine herpesvirus
	Caprine herpesvirus 1	CpHV-1	Goat herpesvirus
	Cercopithecine herpesvirus 9	CeHV-9	Simian varicella virus, Liverpool vervet herpesvirus, Patas monkey herpesvirus
	Cervid herpesvirus 1	CvHV-1	Red deer herpesvirus
	Cervid herpesvirus 2	CvHV-2	Reindeer herpesvirus
	Equid herpesvirus 1	EHV-1	Equine abortion virus
	Equid herpesvirus 3	EHV-3	Equine coital exanthema virus
	Equid herpesvirus 4	EHV-4	Equine rhinopneumonitis virus
	Equid herpesvirus 8	EHV-8	Asinine herpesvirus 3
	Equid herpesvirus 9	EHV-9	Zebra herpesvirus Gazelle herpesvirus
	Felid herpesvirus 1	FeHV-1	Feline rhinotracheitis virus
	Human herpesvirus 3	HHV-3	Varicella-zoster virus
	Phocid herpesvirus 1	PhoHV-1	Harbour seal herpesvirus
	Suid herpesvirus 1	SuHV-1	Pseudorabies virus

Subfamily <i>Betaherpesvirinae</i>			
Genus <i>Cytomegalovirus</i>	Aotine herpesvirus 1	AoHV-1	Herpesvirus aotus type 1 Owl monkey cytomegalovirus
	Cebine herpesvirus 1	CeHV-1	
	Cercopithecine herpesvirus 5	CeHV-5	African green monkey cytomegalovirus Simian cytomegalovirus
	Human herpesvirus 5	HHV-5	Human cytomegalovirus
	Macacine herpesvirus 3	McHV-3	Cercopithecine herpesvirus 8 Rhesus monkey cytomegalovirus
	Panine herpesvirus 2	PnHV-2	Pongine herpesvirus 4
	Papiine herpesvirus 3	PaHV-3	Baboon cytomegalovirus
Saimiriine herpesvirus 4	SaHV-4		
Genus <i>Muromegalovirus</i>	Murid herpesvirus 1	MuHV-1	Mouse cytomegalovirus
	Murid herpesvirus 2	MuHV-2	Rat cytomegalovirus, Maastricht strain
	Murid herpesvirus 8	MuHV-8	Rat cytomegalovirus, England strain
Genus <i>Proboscivirus</i>	Elephantid herpesvirus 1	EiHV-1	Elephant endotheliotropic herpesvirus
	Human herpesvirus 6A	HHV-6A	Human herpesvirus 6, variant B
	Human herpesvirus 6B	HHV-6B	Human herpesvirus 6, variant A
Genus <i>Roseolovirus</i>	Human herpesvirus 7	HHV-7	
Genus Unassigned	Caviid herpesvirus 2	CavHV-2	Guinea pig cytomegalovirus
	Suid herpesvirus 2	SuHV-2	Pig cytomegalovirus
	Tupaïid herpesvirus 1	TuHV-1	Tree shrew herpesvirus
Subfamily <i>Gammaherpesvirinae</i>			
Genus <i>Lymphocryptovirus</i>	Callitrichine herpesvirus 3	CalHV-3	Marmoset lymphocryptovirus
	Cercopithecine herpesvirus 14	CeHV-14	African green monkey EBV-like virus
	Gorilline herpesvirus 1	GoHV-1	Pongine herpesvirus 3 Gorilla herpesvirus
	Human herpesvirus 4	HHV-4	Epstein–Barr virus (EBV)
	Macacine herpesvirus 4	McHV-4	Cercopithecine herpesvirus 1 Rhesus EBV-like herpesvirus
	Panine herpesvirus 1	PnHV-1	Pongine herpesvirus 1 (Herpesvirus pan)
	Papiine herpesvirus 1	PaHV-1	Cercopithecine herpesvirus 12
	Pongine herpesvirus 2	PoHV-2	Orangutan herpesvirus
Genus <i>Macavirus</i>	Alcelaphine herpesvirus 1	AlHV-1	Malignant catarrhal fever virus
	Alcelaphine herpesvirus 2	AlHV-2	Hartebeest malignant catarrhal fever virus
	Bovine herpesvirus 6	BoHV-6	Bovine lymphotropic herpesvirus
	Caprine herpesvirus 2	CpHV-2	
	Hippotragine herpesvirus 1	HiHV-1	Roan antelope herpesvirus
	Ovine herpesvirus 2	OvHV-2	Sheep-associated malignant catarrhal fever virus
	Suid herpesvirus 3	SuHV-3	Porcine lymphotropic herpesvirus 1
	Suid herpesvirus 4	SuHV-4	Porcine lymphotropic herpesvirus 2
	Suid herpesvirus 5	SuHV-5	Porcine lymphotropic herpesvirus 3

Genus <i>Percavirus</i>	Equid herpesvirus 2	EHV-2	Equine herpesvirus 2
	Equid herpesvirus 5	EHV-	Equine herpesvirus 5
	Mustelid herpesvirus 1	MusHV-1	Badger herpesvirus
Genus <i>Rhadinovirus</i>	Ateline herpesvirus 2	AtHV-2	Herpesvirus ateles strain 810
	Ateline herpesvirus 3	AtHV-3	Herpesvirus ateles strain 73
	Bovine herpesvirus 4	BoHV-4	Movar virus
	Cricetid herpesvirus 2		
	Human herpesvirus 8	HHV-8	Kaposi's sarcoma-associated herpesvirus
	Macacine herpesvirus 5	McHV-5	Cercopithecine herpesvirus 17 Rhesus rhadinovirus
	Murid herpesvirus 4	MuHV-4	Murine gammaherpesvirus 68 (MHV-68)
	Murid herpesvirus 7	MuHV-7	Wood mouse herpesvirus
	Saimiriine herpesvirus 2	SaHV-2	Herpesvirus saimiri
Genus Unassigned	Equid herpesvirus 7	EHV-7	Asinine herpesvirus 2
	Phocid herpesvirus 2	PhoHV-2	
	Saguinine herpesvirus 1	SgHV-1	Callitrichine herpesvirus 1 Herpesvirus saguinus
Subfamily Unassigned	Iguanid herpesvirus 2	IgHV-2	Iguana herpesvirus
	Ovine herpesvirus 1	OvHV-1	Sheep pulmonary adenomatosis-associated herpesvirus

1.1.2.2.1 *Alphaherpesvirinae*

Alphaherpesviruses are known for their wide and variable host range, from mammals, birds to reptiles, rapid spread, quick reproductive cycle of about 17 hours (Rajcani & Durmanova, 2001). They have efficient lysis of the infected cells such as fibroblasts in culture and epithelial cells in hosts (King *et al.*, 2012), and maintaining latency in sensory nerve ganglia (Pellett & Roizman, 2013). This subfamily is well presented by herpes simplex virus 1 (HSV-1) that cause oral blisters in human. Those of veterinary medicine importance such as bovine herpesvirus 2 (BoHV-2) that causes lumpy skin disease, bovine herpesvirus 1 (BoHV-1) that cause infectious bovine rhinotracheitis in cattle, gallid herpesvirus 2 that cause Marek's disease in poultry, and many other viruses. Species of *Alphaherpesvirinae* listed in Table 1-1.

1.1.2.2.2 *Betaherpesvirinae*

Betaherpesviruses have a narrower host range than alphaherpesviruses, slower replication cycle in cell culture that can be two to seven days long and virus remain cell associated. Distinguishing features of betaherpesviruses are that they can produce cytomegaly (cell enlargement) in infected cells and the ability of latent

infection in secretory glands, lymphoid cells and kidney (Pellett & Roizman, 2013). Infection with this subfamily tends to be subclinical in immune competent hosts (King *et al.*, 2012). Diseases caused by this subfamily members such as Human cytomegalovirus (HHV-5), suid herpesvirus 2 (porcine cytomegalovirus virus) that causes rhinitis in swine. All species of *Betaherpesvirinae* are listed in Table 1-1.

1.1.2.2.3 *Gammaherpesvirinae*

Gammaherpesviruses are the most host-restricted viruses among *Herpesviridae* and viruses are usually associated within a family or order of a natural host. Most members can replicate in lymphoblastoid cells, either B or T lymphocytes, and other members can replicate in certain epithelial cells and cause cell lysis (Pellett & Roizman, 2013; Roizmann *et al.*, 1992). A feature of *Gammaherpesvirinae* is that they infect hosts often without evoking clinical signs. The subfamily have four main genera. *Lymphocryptovirus* is currently only found in primates, examples are human herpesvirus 4 (HHV-4) that is also known as Epstein–Barr virus (EBV), which was first found in a lymphoblastoid cell line from a B-cell lymphoma in 1964 (Epstein *et al.*, 1964). *Rhadinovirus* is found in a wider range of mammals such as human herpesvirus 8 (Kaposi's sarcoma associated herpesvirus - KSHV) that was first found in a Kaposi's sarcoma in an AIDS patient (Chang *et al.*, 1994). *Percavirus* such as equid herpesvirus 2 (EHV-2) establishes latency in B-cells in respiratory tract in horses is transmitted via respiratory tract, is associated with upper respiratory tract diseases (Brault *et al.*, 2011). *Macavirus* members are of veterinary importance; and contain of malignant catarrhal fever viruses (MCFVs), which are reviewed in more detail in the section 1-4. Important gammaherpesviruses are listed in Table 1-1.

1.1.3 Infection with herpesviruses

Literature describing the detailed life cycle of herpesviruses is reviewed in many virology textbooks. Herpesvirus infection is only briefly reviewed here. A complete infection cycle of herpesviruses include attachment to the cell surface, fusion and entry into cytoplasm and then injection of viral capsid to cell nucleus, lytic (or latent) infection, virus components assembly and then new virion egress out of the cell. The lifespan of herpesviruses is simplified in (Figure 1-5).

1.1.3.1 Virus attachment and entry

Viruses should first attach and react with the cell membrane in order to enter the cell. For the attachment, herpesviruses are known for having their specific glycoproteins and receptor binding glycoproteins (Campadelli-Fiume *et al.*, 2000). Extracellular virions glycoproteins interact and fuse with different cell membrane surface protein receptors such as heparan sulphate (Shukla & Spear, 2001) or nectins (Krummenacher *et al.*, 2004). These receptors determine the virus's tropism to different cells. Herpesvirus enter the cell by two different pathways, either by endocytosis as with most cases (Murphy *et al.*, 1999; Zilliox *et al.*, 2007) or by fusion with the plasma membrane as in case of primary neurons (Pellett & Roizman, 2013). With varicella zoster virus (VZV), entry pathway depends on the type of cell infected, entry in epithelium is by endocytosis, but in fibroblast is by membrane fusion. (Compton *et al.*, 1992; Ryckman *et al.*, 2006). Herpesviruses have three different glycoproteins (gB, gH and gL), and a receptor binding glycoprotein (gD) that determines fusion pathways (Gillet *et al.*, 2006; Wu *et al.*, 2005). In EBV, another glycoproteins (gP42) has been discovered that plays role in virus cell tropism and pathogenies (Borza & Hutt-Fletcher, 2002).

Following fusion with the cell surface, next step is entry of the virus in to the cell. During the fusion, the virus loses its envelope; the tegument components will be exposed and liberated. In case of endosome, the virus undergoes uncoating by viral cellular enzymes. In uncoating, the virus loses its tegument then the capsid will translocate into the nucleus via microtubules (Döhner *et al.*, 2005; Smith & Enquist, 2002). Then the virus initiates the replication mechanism by altering cellular machinery, either by using host cell to synthesize viral components such as by VP16 in HSV-1, or by host shut off such as by SOX in KSHV, or by immediate early genes activation (Kalejta, 2008; Mettenleiter *et al.*, 2006). The virus enters the nucleus through the nuclear pores and eventually it either induces a lytic (productive) infection or it stays latently with expression of certain genes until further activation (Estes *et al.*, 2007). Herpesviruses usually can switch between the two infections by different biological determinants or viral genes for example the immediate-early (IE) BZLF1 trans activator gene in EBV (Countryman & Miller, 1985).

1.1.3.2 Lytic infection, virus assembly and egress

The purpose of lytic infection is for virus replication to produce new virions that can spread and infect new cells. In lytic infection, there are regulated viral genes expression and genome replication, virion assembly, egress, and transmission. Early in this cycle, sequential sets of genes are transcribed including α (immediate early [IE]) that are involved in initiation of virus DNA replication, β (early [E]) genes that act to DNA replication and control cellular activities such as RNA polymerase II (Estes *et al.*, 2007). In later stages, the transcription program switches to γ (late) genes expressing γ proteins. Most of α and β proteins are enzymes and most of γ proteins are structural ones for virus maturation and generally herpesviruses encode for over 70 proteins (Dubovi, 2011). Virus proteins also alter cellular functions; in KSHV, E protein such as viral IL-6 that induces B cell proliferation in a similar way of human IL-6, or Kaposin (an α gene) that has been identified to cause host cell transformation (Kliche *et al.*, 2001; Muralidhar *et al.*, 1998). The newly replicated viral DNA is integrated into immature capsids and tegument proteins in the nucleus (Mettenleiter & Minson, 2006). The virus also gets its primary envelope in the nucleus, nuclear membranes play role in virus capsid nuclear egress (Peng *et al.*, 2010). The virus gain its secondary membrane when buds through Golgi apparatus derived vesicles (Mettenleiter *et al.*, 2006). Eventually, mature virion fuse with the cell membrane and buds to the extracellular spaces (Mettenleiter, 2006).

1.1.3.3 Herpesvirus latency

A distinct feature of herpesviruses is their latent or dormant infection, and those herpesviruses studied so far can establish latency in specific cell types. In this cycle, the viral DNA is harboured in a covalently closed circular form and only limited genes stay transcribed (Pellett & Roizman, 2013). This can possibly be due to increase in density and stability of viral nucleosomes (Nevels *et al.*, 2011; Paulus *et al.*, 2010). Herpesviruses establish latency in different mechanisms with varying degrees of ORFs expression. In certain viruses, unique ORFs are expressed such as EBNA1 (Epstein-Barr nuclear antigen) (Sivachandran *et al.*, 2012) in EBV, or LANA (latency associated nuclear antigen) in KSHV (Hu *et al.*, 2002). These genes products act to maintain latency by blocking virus genes BRLF1 or ORF50 respectively, which

help in viral episomes latent replication to tether its copies to host chromosomes during mitosis in dividing cells (Hu *et al.*, 2002). They also help ubiquitin-dependant degradation for immune evasion (Cotter & Robertson, 1999; Garber *et al.*, 2002; Leight & Sugden, 2000). In addition, LANA interferes with the cellular pathways that inhibit tumour inductions and cell transformation (Dittmer *et al.*, 1998; Verma *et al.*, 2007; Zhong *et al.*, 1996).

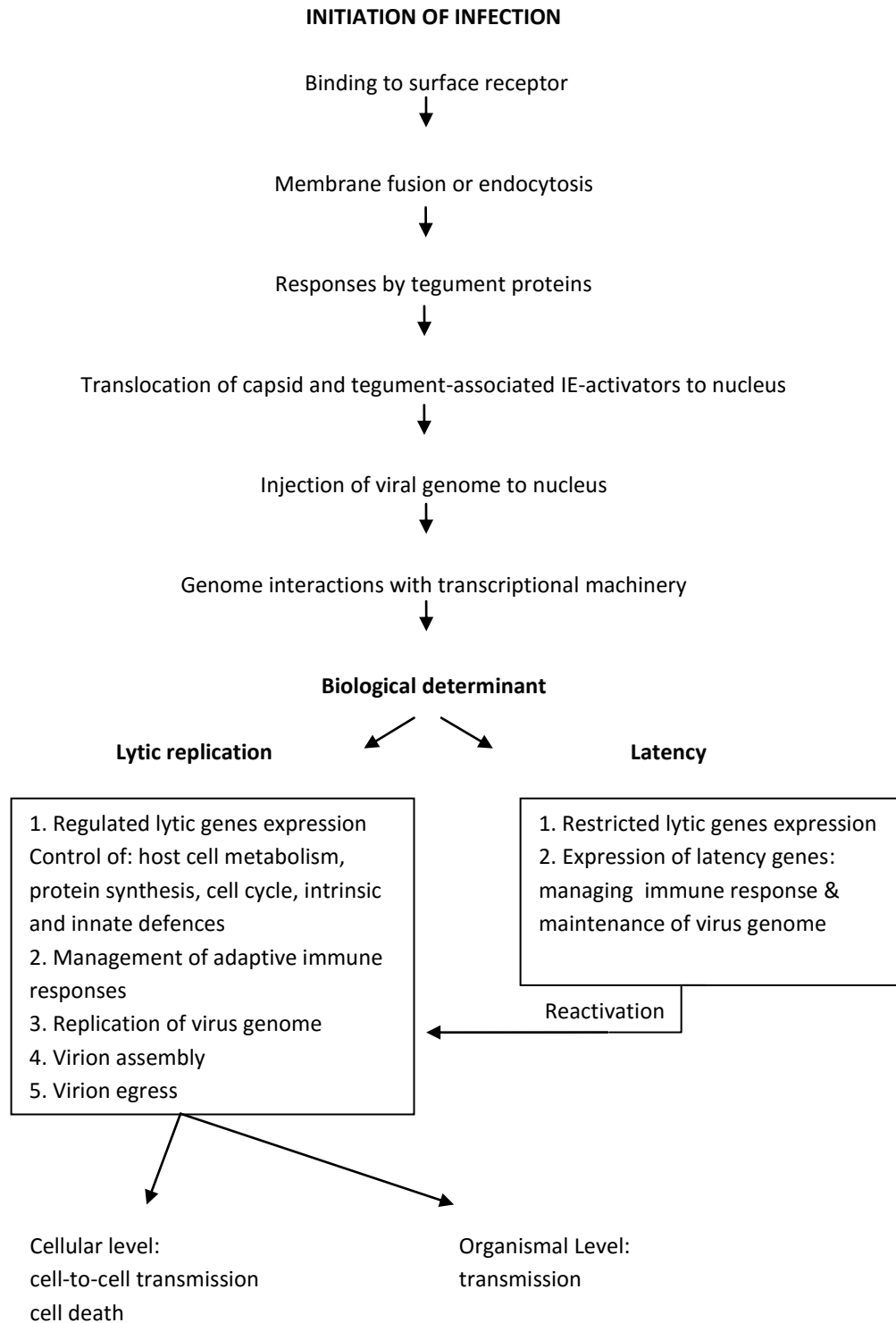


Figure 1-5. Schematic diagram of herpesviruses infection and biological cycles. Adopted after (Pellett & Roizman, 2013).

1.2 Examples gammaherpesviruses

1.2.1 Epstein-Barr virus

One of the best studied gammaherpesviruses is Epstein–Barr virus (EBV) or human herpesvirus 4 (HHV-4), that belongs to the *Lymphocryptovirus* genus (Davison *et al.*, 2009). The virus particles was first noticed by electron microscopy in a malignant Burkitt’s lymphoma (BL) in 1964 (Epstein *et al.*, 1964), which is a B cell neoplasm that occur unusual locations such as mandible, nasopharynx, orbit, kidney, adrenal glands and ovaries. The virus was the candidate causative agent and many research groups having extensively studied it since. EBV is worldwide spread in human and as high as 90% of population are being exposed to and produce antibodies to the virus’s lytic and latent proteins (Longnecker *et al.*, 2013). Virus transmission is by oral rout at teenage period, then individuals stay as carriers for their lifespan. The virus causes infectious mononucleosis (IM) which is also known as kissing disease (Henle *et al.*, 1968). The disease has 4 - 6 weeks incubation period, commonly characterized by fever, pharyngitis and lymphadenopathy, and more serious complications especially in AIDS patients. EBV is also associated with other diseases such as nasopharyngeal carcinomas (Wolf *et al.*, 1973), post-transplant lymphoproliferative disease (Tanner & Alfieri, 2001), Hodgkin lymphoma (Lange *et al.*, 1978), oral hairy leukoplakia which mainly occur in AIDS patients (Greenspan *et al.*, 1984), oral dysplasia and squamous cell carcinomas (Jiang *et al.*, 2012), and also nasopharyngeal, gastric and other lymphomas in non-immunocompromised individuals (Longnecker *et al.*, 2013). Recently, EBV is reported to be also associated with T-cell lymphoproliferative disorder in adolescents and young adults with clinical signs of fever, mass in neck, hepatosplenomegaly, and pathologically patients reveal haemophagocytic lymphohistiocytosis (a haematological disorder) (Wang *et al.*, 2014).

EBV genome comprises of 172 kb with 60% G:C ratio and has reiterated terminal and internal repeats (Figure 1-3). These repeats stay the same as the parental genome during latent replication, a feature can be used in tracking latently infected cells to see if they originate from a clonal virus expansion (Raab-Traub & Flynn, 1986). Latency of EBV in infected cells is a complex process and involves different virus and cellular factors. EBV has a homologue of cellular IL-10 encoded

by *BCRF1* (Moore *et al.*, 1990), and two homologues of *bcl-2* encoded by genes *BHRF1* and *BALF1* that play role in mechanism of cell immortalisation by inhibiting apoptosis (Bellows *et al.*, 2002). Other proteins are expressed in latently infected B cell include LMP1 that activates cellular NF-KB and JAK/STAT pathways to inhibit apoptosis. With these mechanisms the virus may facilitate B cell or other lymphomas (Stricker & Kumar, 2010). The EBV DNA is highly methylated during latency in infected B cells (Falk & Ernberg, 1993), this contributes in inhibiting lytic genes expression. Treating with substances that reduce DNA methylation such as 5-aza-cytidine in latently infected human lymphoid cell lines result in reactivation of the virus by removing the suppression on lytic genes (Ben-Sasson & Klein, 1981).

With EBV, infection initially occurs in those B cells within the oral epithelium, which happens by binding of viral envelope gp350 and gp220 with cellular CD21 (Fingeroth *et al.*, 1984; Shannon-Lowe *et al.*, 2006). CD21 binding leads to either lytic infection and virus spread, or establishment of latent infection. To ensure maintaining latent infection longest possible, EBV infected B cells have to be activated in a similar way to the physiological conditions, i.e.: to producing specific immunoglobulin and become memory B cell to avoid apoptosis (Longnecker *et al.*, 2013). It is also thought that the virus induces neoplasm in BL by translocations in *c-MYC* gene to promote initiation a cell cycle and activating the anti-apoptotic pathways (Longnecker *et al.*, 2013).

1.2.2 Bovine herpesvirus 6

Bovine herpesvirus 6 (BoHV-6), previously was known as bovine lymphotropic herpesvirus (BLHV). It was first isolated from leukocytes of a cattle lymphosarcoma in the USA (Van der Maaten & Boothe, 1972). The isolate was able to induce syncytia formation in bovine embryonic spleen cell cultures. Later, the virus was first sequenced from product of the pan-herpesvirus PCR for DNA isolated from peripheral blood mononuclear cells (PBMCs) and from B-lymphoma cells. Then when the predicted amino acid sequence was aligned with other known herpesviruses, it showed similarities as 70% to (OvHV2), 69% to (AIHV1), 65% to (BoHV4) and 42% to (BoHV1). From this phylogenetic analysis, the virus was considered as a new gammaherpesvirus and named as BLHV (Rovnak *et al.*, 1998),

which was later classified within *Macavirus* genus (Carstens & Ball, 2009). BoHV-6 also has been detected in cattle in Canada (Gagnon *et al.*, 2010) and Europe (Cobb *et al.*, 2006; Garigliany *et al.*, 2013). The genome of BoHV-6 is similar in structure (such as L-DNA coding and repetitive H-DNA sequences) to other *Macavirus*. The virus has 77 predicted genes and the genes blocks arrangements are similar to those in gammaherpesviruses (Jia *et al.*, 2014). Similar to AIHV-1 and OvHV-2, BoHV-6 has *Bov4.5* (homologue to EBV BALF1) and *Bov9* genes, coding for homologues of bcl-2 (Bellows *et al.*, 2002; Jia *et al.*, 2014). In addition, the virus has a sequence partially similar to exons of OvHV-2 and cellular IL-10. Interestingly, BoHV-6 encodes for Bov2.b2 similar to the cellular ornithine decarboxylase (ODC) that catalyse polyamines biosynthesis which affects cellular functions such as proliferation (Jia *et al.*, 2014).

BoHV-6 seems to be ubiquitous in cattle since the viral DNA was detected in samples of PBMCs from more than 70% of adult health cattle and from 30% of calves (Kubiś *et al.*, 2013). A link between the virus and a pathological condition is not supported, however the virus is highly prevalent in dairy cattle, and also was isolated from vaginal exudates of a cow with post-partum metritis irresponsive to antibiotics (Banks *et al.*, 2008). In addition, in an abortion case study of 26 cattle, BoHV-6 DNA polymerase (DPOL) sequence was found in placenta and foetal tissues. Beside this, infectious bovine rhinotracheitis virus (an alphaherpesvirus) was found in foetuses (Gagnon *et al.*, 2010). In another case study, a cow with depression, anorexia, weight loss and purulent metritis irresponsive to antibiotics, severe emaciation, chronic peritonitis and multiple abscesses in the uterine wall, was tested positive for BoHV-6, suggesting a pathological role of the virus (Garigliany *et al.*, 2013). Recently, BoHV-6 was also isolated from buffalo with lymphoproliferative diseases, which was tested negative for bovine leukaemia virus (BLV) (de Oliveira *et al.*, 2014). To know relation between herpesviruses in cattle, a PCR survey of dairy cattle farms has shown a variable BoHV-6 prevalence, in different herds as 52% to 78.7% for BoHV-6 and 2% to 51% for BHV-4 (Collins *et al.*, 2000). Also when SA-MCF cases were tested, those two viruses were found, which are normally not associated with MCF (Collins *et al.*, 2000). Despite presence of BoHV-6 in cattle, not much is

known about virus's infection impact, biological effects and association with bovine conditions including MCF.

1.3 Malignant catarrhal fever

Malignant catarrhal fever (MCF) is also known as malignant head catarrh, malignant catarrh, wildebeest disease and snotziekte or snotting sickness (Maxie, 2007). MCF is a usually fatal disease of many ungulate species of the families *Bovidae* and *Cervidae*, mainly affecting cattle, bison, deer, and occasionally swine. The disease occurs as a consequence of infection by one of the MCF herpesviruses (Buxton *et al.*, 1984; Løken *et al.*, 1998; Plowright, 1990). MCF-causing viruses are present in nature and are harboured by those animals known as reservoir hosts. In the latter, the viruses are well adapted to the host by co evolution and exist in a balance without harming the host. When these viruses are transmitted to those animals known as susceptible hosts to which the virus is poorly adapted to, they induce the clinical disease known as MCF (Plowright, 1990). MCF was early described in domestic cattle that were associated with domestic sheep in Europe in 1700s (Ackermann, 2005; Werner & Hugh, 2008).

1.3.1 Importance of MCF

The importance of MCF varies according to the causative virus, the type of animals that become infected, and the circumstances under which different animals are kept. In Africa, AIHV-1 causes MCF outbreaks and significant economic loss, the incidence rate is estimated to be between 5-10 % in domestic cattle (Bedelian *et al.*, 2007; Cleaveland *et al.*, 2001; Plowright *et al.*, 1975). This ratio can vary according to the size of the wildebeest population and the time of year when the wildebeest are giving birth (Rossiter *et al.*, 1983). Cattle are affected by sheep associated MCF (SA-MCF) in all parts of the world and this is the most prevalent form of MCF as the disease occurs wherever sheep are found. European cattle are relatively resistant to the disease; however, outbreaks with a considerable mortality in domestic cattle herds can occur (Collery & Foley, 1996; Otter *et al.*, 2002; Pierson *et al.*, 1973; Schultheiss *et al.*, 2000; Taneichi *et al.*, 1986; Twomey *et al.*, 2002). Other cattle species like American bison, buffalo and Bali cattle are more susceptible to MCF and

are recommended to be kept well separated from sheep (Pierson *et al.*, 1979; Taneichi *et al.*, 1986). In bison, severe outbreaks have been reported (O'Toole *et al.*, 2002) and that SA-MCF even acquired from locations distant from where lambs were farmed (Li *et al.*, 2008b). In one outbreak 8000 bison were died which caused an approximately one million US dollar loss (Li *et al.*, 2006). Buffalos are a highly susceptible, in which MCF is considered an emerging disease. It is recently discovered that buffalo can be affected by more than one type of MCFV and cases have been reported in different parts of the world, such as South Africa (Pfitzer *et al.*, 2013; 2015) and Switzerland (Dettwiler *et al.*, 2011; Stahel *et al.*, 2013).

SA-MCF has also been reported in free ranging deer (Neimanis *et al.*, 2009; Schultheiss *et al.*, 2007; Vikøren *et al.*, 2006). MCF is a problem of commercially farmed deer in New Zealand (Mackintosh, 1993), outbreaks can affect up to 50% of the herd (Reid *et al.*, 1979). MCF with CpHV-2 as the causative virus has recently been reported in free ranging and farmed deer in Europe and Asia (Chen *et al.*, 2007; Vikøren *et al.*, 2006). In white tailed deer, a number of outbreaks caused by white-tailed deer MCF virus (WTD-MCFV) have been reported (Kleiboeker *et al.*, 2002; Li *et al.*, 2000a), but symptoms were less severe than those seen with other MCFVs.

MCF has been frequently been reported in petting zoos or zoologic parks where sheep were kept together with susceptible ruminants (Li *et al.*, 1999b). In Pigs MCF was first reported in Scandinavian countries (Løken *et al.*, 1998), later in countries of Europe (Albini *et al.*, 2003a; Syrjälä *et al.*, 2006) and North America (Alcaraz *et al.*, 2009). It is thought that the real incidence of MCF is higher than what is known, as many cases are not reported or misdiagnosed (Li *et al.*, 2014).

1.4 Malignant catarrhal fever viruses and their transmission

MCF is caused by several gammaherpesviruses of the genus *Macavirus*; six of which are pathogenic and cause disease under natural conditions (Li *et al.*, 2005a), as listed in Table 1-2. The disease is mainly caused by either alcelaphine herpesvirus 1 (AIHV-1) which is harboured by wildebeest (*Connochaetes* species) as the natural host, or ovine herpesvirus 2 (OvHV-2) for which sheep (*Ovis aries*) are the natural host. Wildebeest associated MCF (WA-MCF) is only prevalent in Africa where the

natural hosts live and spread the virus that causes outbreaks (Plowright, 1960). While the SA-MCF occurs worldwide and is the major form of the disease. In the following sections other MCF viruses of minor relevance are discussed first, then AIHV-1, and then the most relevant virus in this study, the OvHV-2, is discussed in more detail.

Table 1-2. List of important naturally occurring MCF viruses, and susceptible hosts in which MCF has been reported. Adopted after (O'Toole & Li, 2014).

MCFV	Reservoir Host	Susceptible Host	Economic Importance
Ovine herpesvirus 2 (OvHV-2)	Sheep	Cattle, bison, deer, pig, giraffe	Moderate
Alcelaphine herpesvirus 1 (AIHV-1)	Wildebeest	Cattle, deer	Moderate
Caprine herpesvirus 2 (CpHV-2)	Goat	Sika deer, white-tailed deer	Minimal
White-tailed deer- MCFV (Caprine herpesvirus 3)	Goat	White-tailed deer, red brocket deer, reindeer	Minimal
Ibex –MFCV	Ibex	Bongo, anoa, pronghorn	Minimal
Alcelaphine herpesvirus 2 (AIHV-2)	Hartebeest, tpoi	Barbary red deer, bison	Minimal

1.4.1 Caprine herpesvirus 2 and other MCFVs

Goats are another source of MCF since they carry a MCF virus (Li *et al.*, 2001b). The virus can spread between goats when housed together, it is highly similar to OvHV-2 and AIHV-1 and has been named as caprine herpesvirus 2 (CpHV-2) (Chmielewicz *et al.*, 2001). Goat associated MCF (GA-MCF) is naturally occurring in cervid species such as white-tailed deer, sika deer, roe deer and moose (Chen *et al.*, 2007; Crawford *et al.*, 2002; Keel *et al.*, 2003; Li *et al.*, 2003b; Vikøren *et al.*, 2006). GA-MCF symptoms are different from that induced by OvHV-2 or AIHV-1, as the disease tends to be more chronic and dominated by skin conditions such as dermatitis and

alopecia. Although, the histopathological examination reveals a systemic lymphoproliferative vasculitis also in these cases (Crawford *et al.*, 2002).

MCF affecting white-tailed deer (WTD-MCF) was first described in 2000 (Li *et al.*, 2000a) when PCR testing of a deer with MCF was negative for OvHV-2 or AIHV-1 specific primers. Degenerative primers for a conserved DNA region (herpesvirus polymerase gene) were used and yielded a PCR product which was then sequenced and found to be identical to OvHV-2 by 82% and to AIHV-1 by 71% (Kleiboeker *et al.*, 2002; Li *et al.*, 2000a). From this finding, the virus was recognised as a distinct new pathogenic MCFV (Li *et al.*, 2000a). The disease occurs during periods of stress around late fall, but the transmission route of the virus is not yet known because the reservoir host is unknown. However, species such as sheep or goat are suspected (Kleiboeker *et al.*, 2002; Li *et al.*, 2000a; O'Toole & Li, 2008). In a recent study, it was proposed that goats are a possible source of WTD-MCF because deer developed MCF after exposure to goats (Li *et al.*, 2013a). Deer affected with WTD-MCF show the classical symptoms of MCF like that in cattle, but without corneal involvement (Li *et al.*, 2000a).

Hippotragine herpesvirus 1 (HipHV-1) was first recovered from cell cultures of samples from a roan antelope in Africa. It is not reported to induce MCF naturally. The virus DNA was shown to be more closely related to AIHV-1 than to OvHV-2, based on the hybridisation intensity (Reid & Bridgen, 1991), and when inoculated into rabbits it induced classical MCF lesions (Reid & Bridgen, 1991; Schock & Reid, 1996).

Muskox (*Ovibos moschatus*), Nubian ibex (*Capra nubiana*) and gemsbok (South African oryx, *Oryx gazella*) herpesviruses are grouped with MCFVs based on the sequence of their herpesviral DNA polymerase (DPOL) gene which is similar to that of other MCFVs. So far, there is no evidence that these viruses cause disease (Li *et al.*, 2003a). Muskox-MCFV was recently identified outside America, in Norway, in free ranging muskox, the study suspect that calves are infected in an early age (Vikøren *et al.*, 2013).

1.4.2 Alcelaphine herpesvirus 1

The AIHV-1 causes the wildebeest associated MCF (WA-MCF) in cattle in Africa. It is carried by African wildebeest (*Connochaetes taurinus*) (Plowright, 1960). WA-MCF is mainly a problem in east and south Africa where wildebeest are found (Bedelian *et al.*, 2007; Cleaveland, 2001), and in zoological parks (Meteyer *et al.*, 1989; Whitaker *et al.*, 2007). Calves are infected at as early as three months of age and they shed the virus through their nasal and ocular secretions as cell free virus (Mushi *et al.*, 1980; Mushi *et al.*, 1981), and by the age of four months all calves are infected (Barnard *et al.*, 1989). After six months of age, animals tend to shed less virus except when under stress, like during parturition (Barnard *et al.*, 1989; Rweyemamu *et al.*, 1974). The virus was first recovered from a splenic cell culture of a wildebeest foetus (Plowright, 1965), which showed that AIHV-1 can also be transmitted vertically. In cattle the disease has been shown to occur after exposure to blue or black wildebeest (Daubney & Hudson, 1936; Mettam, 1923). A study showed that pasturelands where wildebeest gave birth and left foetal afterbirth, were highly contaminated from ocular-nasal discharges of the mother, but not from foetal membranes. The virus is inactivated by sunlight within one hour, so it was recommended that cattle could graze such pastures after a few hours to minimise the risk of MCF (Rossiter *et al.*, 1983). Under natural conditions, AIHV-1 is not horizontally transmitted from MCF affected animals to naïve animals, because diseased animals do not produce cell free virus (Mushi & Rurangirwa, 1981). Vertical transmission appears possible, by transmission of the virus from pregnant MCF affected dams to calves (Plowright *et al.*, 1972).

1.4.3 Ovine herpesvirus 2

Historically, sheep were always thought to be another source of MCF, but attempts to isolate the causative agent were elusive (Selman *et al.*, 1974). Based on cross-hybridisation of a DNA clone of AIHV-1 with another DNA from a lymphoblastoid cell line derived from a cattle with SA-MCF, it was postulated that the new agent is closely related (Bridgen & Reid, 1991). The virus later designated as OvHV-2 (Roizmann *et al.*, 1992). Based on those postulated sequences in clones, primers were designed and PCR was established to amplify OvHV-2 DNA in clinical MCF

cases and in peripheral blood leukocytes of sheep (Baxter *et al.*, 1993). Now, OvHV-2 is believed to be the most important cause of MCF in cattle worldwide, especially where sheep and other MCF-susceptible species are kept close to each other. SA-MCF is reported in almost all parts of the world, in Europe (Benazzi *et al.*, 2004; Collery & Foley, 1996; Decaro *et al.*, 2003; Frölich *et al.*, 1998; Løken *et al.*, 1998; Mateusen *et al.*, 2009; Pardon *et al.*, 2009), north and south America (Alcaraz *et al.*, 2009; Bratanich *et al.*, 2012; Cunha *et al.*, 2012), Canada (Neimanis *et al.*, 2009; Zarnke *et al.*, 2002), Africa (Bremer, 2010; Gelaye *et al.*, 2013; Iman M. Bastawecy and Abd El-Samee, 2012), the Middle East (Brenner *et al.*, 2002; Kirbas *et al.*, 2013; Yazici *et al.*, 2006) and Asia (Taneichi *et al.*, 1986; Vinod Kumar *et al.*, 2014; Wani *et al.*, 2004; Wani *et al.*, 2006; Wiyono *et al.*, 1994).

1.4.3.1 OvHV-2 in sheep

All sheep breeds are thought to become infected with OvHV-2 (Baxter *et al.*, 1997; Li *et al.*, 1995). OvHV-2 is believed to be transferred horizontally by close contact, aerosols from respiratory secretions (Li *et al.*, 2004) or via the sexual route (Hüssy *et al.*, 2002). In most lambs over three months and under one year of age (especially 5.5 - 7.5 months), viral DNA can be found in peripheral blood leukocytes and in nasal secretions (Ackermann, 2005; Li *et al.*, 1998). The OvHV-2 DNA have a much higher concentrations in nasal secretions than in peripheral blood leukocytes in the same animal, and the load starts to decline before one year of age (Li *et al.*, 2001a). At the age of 6-9 months, viral shedding occurs as multiple episodes with a dramatic rise in shedding in a 24-36 hour period which may represent a single viral replication cycle, but in older sheep shedding is less frequent (Li *et al.*, 2001a; Li *et al.*, 2004). Sheep remain infected for their lifespan (Li *et al.*, 2001a) and are liable to infection at any age (Li *et al.*, 2000b). In sheep, different organs and body secretions have been tested positive by PCR for OvHV-2 detection (Hüssy *et al.*, 2002). Sites of excretion and organs harbouring OvHV-2 are illustrated in figure 1-5. In a study using laser microdissection technique to identify the host cells positive for OvHV-2, respiratory epithelial cells were found to carry high virus copy numbers (Kim & Oh, 2003).

In more recent studies, it has been shown that when naive sheep are experimentally infected with OvHV-2 via nebulisation, the initial site targeted by the virus is the lung. Infection was not established by intravenous or intraperitoneal injection; this is possibly because cells that support initial virus replication are not available in the two later locations (Li *et al.*, 2008a). Furthermore, by using immunofluorescence techniques, it was found that mainly type II pneumocytes support the initial lytic replication in either experimentally or naturally infected sheep during episodes of virus shedding (Taus *et al.*, 2010). Newborn lambs do not show significant OvHV-2 DNA levels and are not considered as an important source of infection (Li *et al.*, 1998). However, detection of OvHV-2 antibodies in SPF lambs suggests the possibility of vertical virus transmission (Rossiter, 1981a). Supporting this, when pregnant ewes were experimentally infected, the foetuses developed MCF-like lesions (Buxton *et al.*, 1985). Virus shedding by adult sheep is different from AIHV-1 shedding by wildebeest, i.e. is not associated with stress or lambing, and is rather stable (Li *et al.*, 2001a). Infected sheep at any age remain asymptomatic, but when aerosolised with large amounts of OvHV-2 (3×10^9 virus copies), they show slight MCF-like signs, such as fever and nasal discharges and also develop lesions typical for MCF in the respiratory and alimentary tract (Li *et al.*, 2005b). The necropsy findings revealed multifocal erosions and ulcers in the mucosa of the cheeks, tongue, pharynx and oesophagus. Histologically, moderate superficial lymphohistiocytic rhinitis, degeneration of keratinocytes in the oral mucosa and multifocal histiocytic broncho-interstitial pneumonia with lymphocytic vasculitis were observed. Some infected sheep in the experiment recovered clinically within two weeks after the onset of clinical signs (Li *et al.*, 2005b).

OvHV-2 can be also found in species other than sheep. Pigs can harbour OvHV-2. The semen of boars was tested positive for OvHV-2 DNA by PCR and the virus was shown to be transmitted sexually to sows (Costa *et al.*, 2010). Also goats have tested positive for OvHV-2 (Løken *et al.*, 2009).

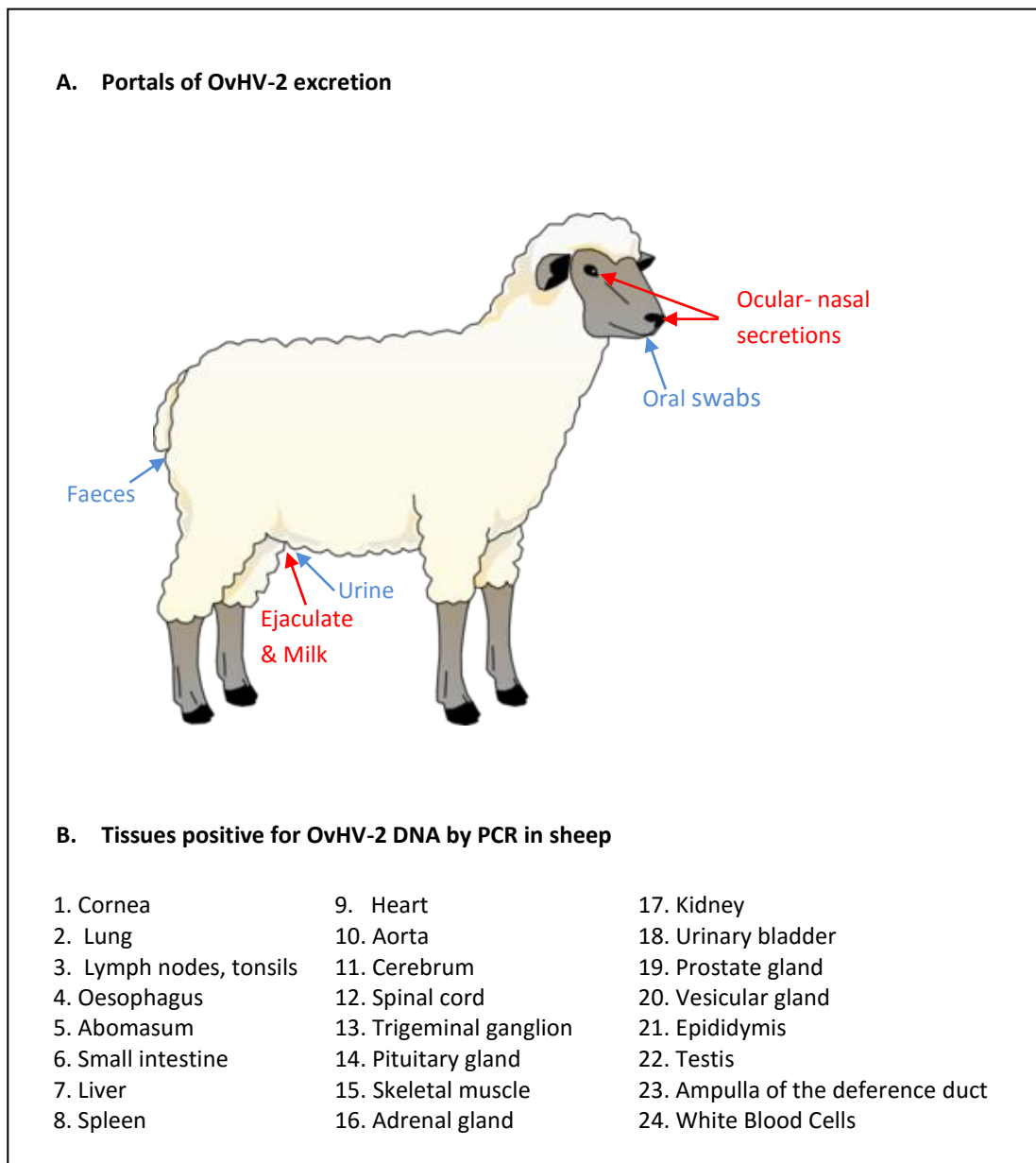


Figure 1- 6. Portals of OvHV-2 excretion and tissues harbouring viral DNA in sheep.

- A.** Excretion of OvHV-2 by sheep from different sites, those written in red are positive for viral episodic shedding, those written in blue have been PCR-negative for the virus. Adopted from (Ackermann, 2005).
- B.** Organs that tested positive for OvHV-2 DNA by PCR. Adopted after (Hüssy *et al.*, 2002; Hüssy *et al.*, 2001).

1.4.3.2 Genome and biology of OvHV-2

OvHV-2 has never been isolated, which has limited its study to a great extent. There is no permissive cell culture system to support the propagation of the virus. The only way to culture OvHV-2 was by culturing lymphoblastoid cell lines derived from cattle, rabbit and deer affected with MCF (Reid *et al.*, 1989; Reid *et al.*, 1983; Schuller *et al.*, 1990). OvHV-2 and other MCFVs have DNA sequence similarity in conserved regions (Li *et al.*, 2005a). The increasing availability of herpesvirus sequences and the use of PCR, made the phylogenetic analysis and assessment of the relation between *Macavirus* or other herpesviruses much easier (McGeoch *et al.*, 2006). The full genome of OvHV-2 was sequenced from a lymphoblastoid cell line generated from a MCF affected cow, this was fundamental to understanding the virus (Hart *et al.*, 2007).

The unique portion of the genome consists of 130,930 bp with 52% G:C ratio, that is bounded by GC-rich terminal repeats. The genome was found to have 73 open reading frames (ORFs) most of which (62) show similarity with other gammaherpesviruses. Nine ORFs show similarity with AIHV-1 only. The composition of the conserved regions was similar to those of other gammaherpesviruses (Hart *et al.*, 2007). Four genes are completely unique to OvHV-2, namely Ov2.5, Ov3.5, Ov4.5 and Ov8.5 (Russell *et al.*, 2009). The Ov2.5 encodes for an interleukin-10 (IL-10) homologue. This viral spliced gene that retains the exon structures (five) similar to the cellular gene, but intron sizes are significantly reduced (Hart *et al.*, 2007; Russell *et al.*, 2009). The viral IL-10 protein functions like ovine IL-10, stimulating proliferation of mast cells and inhibiting macrophage inflammatory chemokine production (Jayawardane *et al.*, 2008). The Ov3.5 is predicted to encode a protein of 163 amino acid with a signal peptide (Hart *et al.*, 2007). The Ov4.5 encodes a protein of 212 amino acid, which is predicted to function as vBcl-2 (viral B cell Lymphoma-2) homologue of the EBV BALF1 anti-apoptosis protein that regulates cell death. The Ov8.5 codes for a 390 amino acid proline-rich protein of unknown function (Hart *et al.*, 2007; Russell *et al.*, 2009). It has been shown that the OvHV-2 genome in domestic sheep (*Ovis aries*) collected during virus shedding episodes differs from that in MCF affected cattle (*Bos bovis*). The amino acid similarity between three ORFs (ORF17, a predicted maturation protein or a capsid protein;

Ov3, a semaphorin; and Ov10 a putative nuclear protein) of the two viruses were between 94-100% while the similarity between ORF73s was 83% (Taus *et al.*, 2007). The ORF73s were highly variable, except for 32 N-terminus and 136 C-terminus amino acids where they were highly conserved (Taus *et al.*, 2007) between different isolates. Further sequencing studies have indicated that the variability in ORF73 could be a useful tool for informing epidemiological studies (Russell *et al.*, 2009). In sheep, the viral DNA is in a circular form in polymorphonuclear leukocytes (PMNC) indicating latent infection with ORF73 transcription (Thonur *et al.*, 2006). In the same study, a mixture of circular and linear DNA was seen in cell lines derived from rabbits with MCF indicating latent and productive infection at the same time.

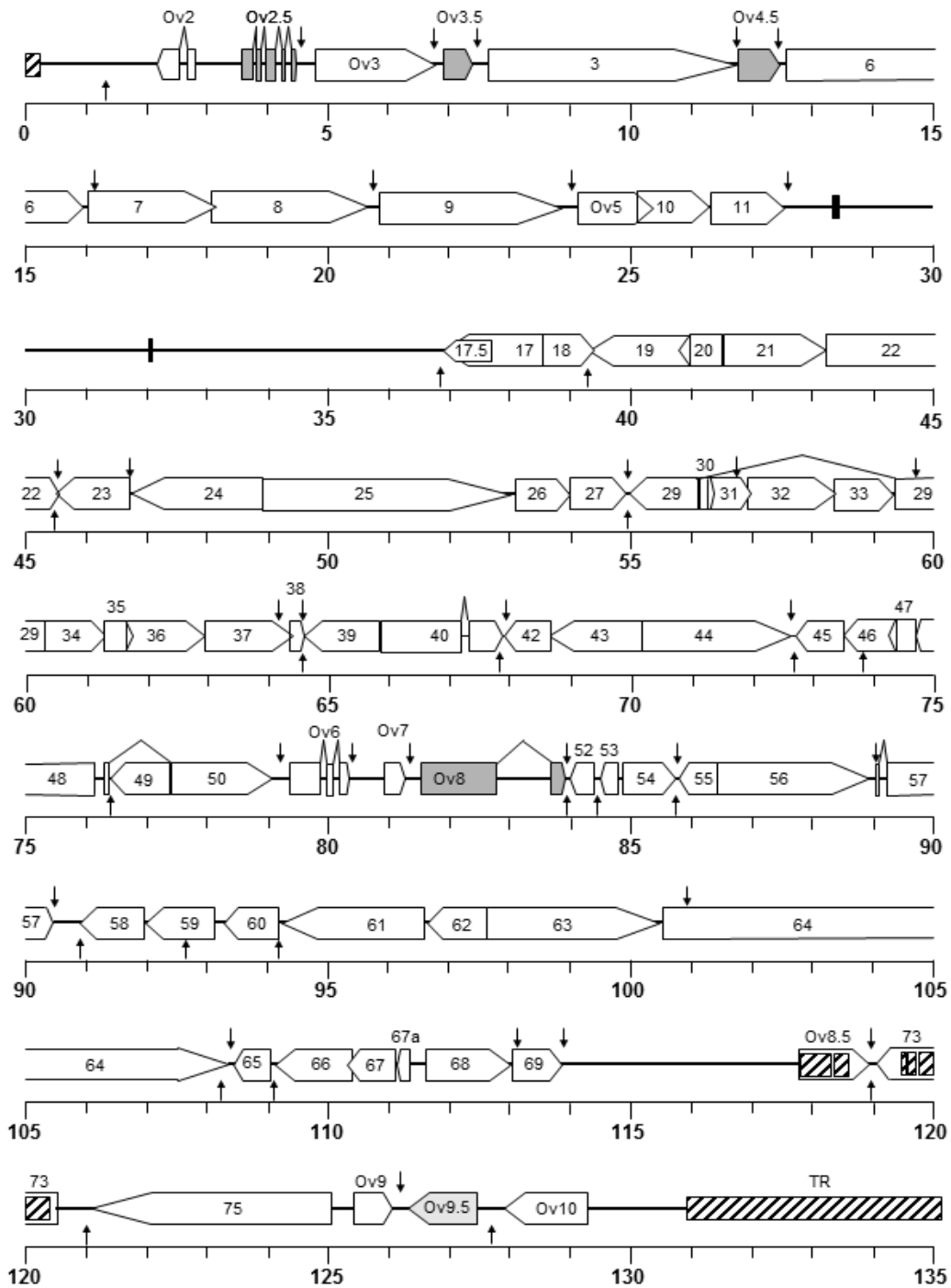


Figure 1-7. Organization of the OvHV-2 (isolate BJ1035) genome. Showing the direction of transcription, splice sites are shown as lines above connecting exons. Potential polyadenylation signals (AATAAA or ATTAATA) pointed by arrows. Major repetitive elements are shown as shaded rectangles, direct repeats as hashed blocks and inverted repeats as solid bars. Those highlighted in dark grey indicate unique OvHV-2 genes and the light grey indicates highly variable regions. Taken after (Hart *et al.*, 2007).

1.5 Clinical signs of MCF

MCF is usually a sporadic disease. The incubation period is not known, but with natural infections, it can be from a few weeks to as long as seven months. In experimental infections, the incubation period is shorter and was observed to be between 9-77 days (O'Toole *et al.*, 2007; Werner & Hugh, 2008). This variation could depend on the initial viral dose (Gailbreath *et al.*, 2010). MCF is frequently fatal, however, cases of animals that recovered from the disease have been recorded (O'Toole *et al.*, 1997; Penny, 1998). The clinical course is usually acute but can occasionally be chronic (Reid *et al.*, 1984). The morbidity and mortality of the disease varies greatly depending on the affected species, deer can die within two days and bison within three days, while cattle can have chronic course and even can recover from disease (O'Toole *et al.*, 1997; O'Toole *et al.*, 2002; Vanselow, 1980). The induction of clinical signs depend on the species and the initial virus dose that infect the animal (reviewed in (Li *et al.*, 2014)) as shown in Table 1-3.

In cattle, MCF has four overlapping clinical forms, a peracute, head and eye, alimentary, and a mild form; the head and eye form is the most common (Blood & Radostits, 1989). There are no significant clinical differences in MCF caused by OvHV-2 or AIHV-1 (Brown *et al.*, 2007). The disease starts with depression and high fever of 40 – 42 °C, tachycardia of 100-120 beats per minute, anorexia and agalactia (Blood & Radostits, 1989; Kahn & Line, 2010; Suhaila, 2011). The head and eye form is characterised by bilateral ocular and nasal discharges that are initially serous then become mucopurulent. Ocular signs range from corneal oedema to profuse lacrimation and conjunctivitis (Sharpe *et al.*, 1987; Zemljič *et al.*, 2012). Subsequently, necrosis, erosion, ulceration and crusting of the muzzle is seen (Selman *et al.*, 1974). Respiratory signs include dyspnoea, open-mouthed breathing, and coughing as disease progresses (Blood & Radostits, 1989). Upon clinical examination, enlargement of superficial lymph nodes and muscle tremor can be noticed (Ababneh *et al.*, 2012; Kirbas *et al.*, 2013). Alimentary signs vary from profuse diarrhoea to scanty, hard faeces or sometimes not passing faeces (O'Toole & Li, 2008; Selman *et al.*, 1974). As the disease progresses, neurological symptoms are observed, such as photophobia, behavioural changes, incoordination, inability to walk and eventually paralysis (Blood & Radostits, 1989). In some cases,

cutaneous lesions develop such as crusting and exudation on skin of neck, shoulder, axillae, back, udder, vulva and scrotum, and sometimes patchy excessive sweating (Selman *et al.*, 1974). In rare cases, MCF is only associated with cutaneous lesions or with fever and anorexia (David *et al.*, 2005; Holliman *et al.*, 1994; Penny, 1998). Haematological tests show severe leukopaenia characterised by a marked lymphopaenia but a relative neutrophilia (Brenner *et al.*, 2002; Reid *et al.*, 1984).

In bison and deer, clinical signs are slightly different from those in domestic cattle. They show severe depression, weight loss, haematuria, and bloody diarrhoea, but corneal opacity is less apparent (Li *et al.*, 2014; O'Toole *et al.*, 2002).

Table 1-3. Susceptibilities of different species to OvHV-2 infection and induction of MCF. Table adopted from (Li *et al.*, 2014) for OvHV-2 dose-response studies.

Species	OvHV-2 DNA copies intranasal inoculum							
	10 ²	10 ³	10 ⁴	10 ⁵	10 ⁶	10 ⁷	10 ⁸	10 ⁹
Sheep	Neg	Pos	Pos	Pos	Pos	Pos	Pos	MCF
Cattle				Neg	Pos	Pos	MCF	
Pig			Neg	Neg	MCF			
Rabbit			Neg	MCF				
Bison		Neg	MCF					

Abbreviations: Neg: no infection; Pos: subclinical infection; MCF: induction of MCF.

1.5.1 Gross pathological findings in MCF

MCF is multisystemic and affects many organs, gross lesions can vary greatly depending on the duration of disease and the affected species rather than the infecting virus (Li *et al.*, 2014). Cattle that die with the peracute form are dehydrated and emaciated, often with less obvious corneal opacity, and necropsy findings may include extensive inflammation of the mucosal linings (Maxie, 2007). However, in many acute cases there is multifocal petechial haemorrhage and heavy congestion of the alimentary tract, mucopurulent exudate covering the trachea; oedema, multifocal haemorrhage in the urinary bladder and bloody urine (Pardon

et al., 2009; Werner & Hugh, 2008). Patchy petechial haemorrhage, erosions and ulcers on the buccal mucosa and tongue as in some instances the necrotic lingual epithelium is detached and can be removed; in addition, abomasum and intestine may be covered with purulent exudate (Sharpe *et al.*, 1987; Suhaila, 2011). Mucopurulent exudate may cover the nasal turbinate, larynx and the mucosa of trachea (Costa *et al.*, 2009a). The brain is congested and exhibits multiple petichiae on the cerebral cortex (Vanselow, 1980). Mild to moderate enlargement of lymph nodes with congestion and haemorrhage can be seen. Certain species such as deer may have more severe enteritis (Reid *et al.*, 1984). Buffalo with MCF may exhibit haemorrhages epicardium and pericardium (Hoffmann *et al.*, 1984).

1.5.2 Histopathological findings in MCF

In MCF, there is lymphoid hyperplasia and basically two characteristic pathological changes: first, necrotising vasculitis with mononuclear cell infiltration and necrosis of the tunica media in medium calibre arteries and veins; and secondly, epithelial necrosis; these lesions involve most organs (Brown *et al.*, 2007; Liggitt & DeMartini, 1980a; b; Selman *et al.*, 1974; Vanselow, 1980). In vascular lesions, the infiltrating mononuclear cells are predominantly lymphoid cells (small and large T-cells), macrophages, plasma cells, neutrophils and a few B-cells (Ellis *et al.*, 1992; Selman *et al.*, 1974). A severe lymphocytic arteritis (and to a lesser extent phlebitis) that is characterised by swollen vacuolated endothelium and necrosis of the tunica media (represented by an eosinophilic coagulum of necrotic debris) is the main feature of MCF in cattle (Brown *et al.*, 2007). Epithelial lesions are represented by necrosis associated with lymphoid cell infiltration. The necrosis extends to the deeper mucosa and there is no or very few epithelial cell regeneration. These lesions occur in many epithelial tissues including those of the oral cavity, the eyes, the alimentary, respiratory and urinary tract, skin and in the choroid plexus (Liggitt & DeMartini, 1980b).

The eyes develop an erosive keratoconjunctivitis with corneal oedema, anterior uveitis particularly in ciliary body and processes and iris, and retinitis. Lymphocytic vasculitis is seen in vessels of uvea, ciliary, scleral and retinal vessels. Lymphocytic ciliary neuritis and optic meningitis are less common (Whiteley *et al.*,

1985). Skin lesions include hydropic degeneration and microvesicle formation in the epidermis that leads to erosion with exudate covering the epidermis. The underlying dermis exhibits oedema and lymphoid inflammation. In the alimentary tract, lesions vary in their severity. Besides a mononuclear infiltration, necrosis of the glandular epithelium and acantholysis that leads to collapsing of the epithelium is seen (Liggitt & DeMartini, 1980b). Hyperaemia, focal erosions and ulcerations that may extend to the submucosa are seen, often associated with fibrin and blood exudate on the surface (Brown *et al.*, 2007). In the respiratory tract, lesions vary from a mild congestion and serous exudation to severe degeneration in the upper respiratory mucosa (Brown *et al.*, 2007). In the lungs, there are peribronchial and perivascular lymphocytic cuffs, alveolar septal congestion with oedema, and interstitial lymphocytic pneumonia are seen (Vanselow, 1980). In the kidneys, interstitial, periglomerular and perivascular lymphoid infiltration, focal haemorrhages and infarcts, as a consequence of thrombi and degenerated arterioles, are seen. In the liver, there are vasculitis; periportal hepatocyte necrosis and accumulation of lymphocyte dominated mononuclear cells around portal vessels and bile ducts (Sharpe *et al.*, 1987). In lymph nodes, there is focal necrosis and haemorrhage, cortical lymphoid hyperplasia and central follicular depletion, Similarly in the spleen, changes can range from moderate lymphoid depletion to hyperplasia in the periarteriolar sheaths (Brown *et al.*, 2007).

A unique feature of MCF are the brain lesions, which are characteristically nonsuppurative meningitis, necrotising arteritis and lymphocytic adventitial infiltration (Brown *et al.*, 2007; Sharpe *et al.*, 1987). Despite those pathological changes, virus particles or inclusion bodies have never been found in MCF lesions (O'Toole *et al.*, 1995; Selman *et al.*, 1978).

1.5.3 Experimental MCF

Since there is no permissive cell culture system to support OvHV-2, researchers have used experimental animals to induce MCF under experimental conditions to study the pathogenesis of the disease. Naturally susceptible species such as cattle and bison have been used in experimental studies (Gailbreath *et al.*, 2010; Taus *et al.*, 2006). WA-MCF was induced experimentally by inoculating blood from

wildebeest or MCF affected cattle to naive cattle (Mettam, 1923). The first experimental SA-MCF was induced by intraperitoneal inoculation of a cell suspension from deer with MCF to a rabbit (Buxton & Reid, 1980). Rabbits show classical MCF lesions and therefore are a valuable model to study MCF pathogenesis as they were shown to exhibit similar pathogenic and infection features as bison (Buxton *et al.*, 1984; Cunha *et al.*, 2013). Deer were used in experimental contact transmission of OvHV-2 when housed close to sheep (Imai *et al.*, 2001). Pigs are naturally sporadically affected by SA-MCF and found to be a useful and comparatively low cost animal model for experimental MCF studies (Li *et al.*, 2012). Hamsters are also used as small animal models for WA-MCF and SA-MCF (Reid *et al.*, 1986). In a recent experiment, bison have been infected with AIHV-2 and two of six animals developed lesions consistent with MCF (Taus *et al.*, 2014).

1.5.4 Pathogenesis of MCF

The pathogenesis of MCF is not fully understood. However, the pathological changes including lymphoproliferation, vascular and epithelial lesions are well described (Maxie, 2007). The pathogenesis of OvHV-2-induced MCF in laboratory rabbits has been well studied (Buxton *et al.*, 1984). This study showed that even cyclosporine-A-mediated suppression of T cells did not inhibit development of the lesions. This suggests that not only the lymphoproliferation is responsible for the vascular and epithelial destruction, but that the virus itself plays a role. Furthermore, another study in rabbits extended understanding of the pathogenic mechanisms of the disease and differences between SA- and WA-MCF (Anderson *et al.*, 2007), as OvHV-2 was shown to cause more tissue necrosis and less lymphoid hyperplasia of predominantly CD8⁺ and few CD4⁺ and naive T cells, but no B-cells. Also in cattle MCF it was shown that predominantly cytotoxic CD8⁺ T cells are involved in lymphoid hyperplasia associated with vasculitis (Ellis *et al.*, 1992; Nakajima *et al.*, 1994; Nakajima *et al.*, 1992; Simon *et al.*, 2003). In rabbits with experimental MCF, CD4⁺ T cells were significantly depleted in lymph nodes, and numerous CD43⁺ (a T cell molecule important for adhesion to endothelial cells) and proliferating PCNA⁺ (Proliferating cell nuclear antigen) subepithelial lymphocytes were found in the esophagus, trachea and lacrimal gland (Schock & Reid, 1996). This

study suggested viral infection and *in situ* multiplication of lymphocytes or possibly antigen-presenting cells, and changes in lymphocyte function by altering a T cell activator, such as interleukin-2 (IL-2) (Schock & Reid, 1996). To further understand MCF, cell cultures of proliferating lymphocytes from lymph nodes have been established to study their features *in vitro*. These cells were shown to have a morphology of large granular lymphocytes (LGLs), have non-MHCII (major histocompatibility class 2) restricted cytotoxic activity and have features of activated natural killer (NK) cells (such as expressing CD4 or CD8, but always CD2, and not transcribing IL-1 β or IL-2) that respond to cytokines from other virus infected lymphocytes and start reacting against host's own cells (Burrells & Reid, 1991; Cook & Splitter, 1988; Swa *et al.*, 2001). Furthermore, a study have shown that the predominant CD8⁺ lymphocytes infiltrating in vascular lesions in bison with SA-MCF are cytotoxic CD8⁺/perforin⁺/WC1⁻ $\gamma\delta$ T cells of the innate immune system, not CD8⁺ $\alpha\beta$ T cells (Nelson *et al.*, 2010).

Despite the detailed pathological observations, very little is known about the virus in tissues. Although in rabbits with experimental MCF, OvHV-2 DNA was detected in lymph nodes by *in situ* hybridisation (Bridgen *et al.*, 1992). Later, viral DNA was found in CD8⁺ T lymphocytes associated with vasculitis in brain of cattle and bison with MCF by *in situ* PCR and immunohistochemistry (Simon *et al.*, 2003). Monocytes and macrophages were also detected in the lesions, but neither CD4⁺ T cells nor B cells. In another study, OvHV-2 structural proteins (ORF43 capsid and ORF63 tegument) were localised in the epithelial and M-cells (microfold cells) in the appendix of rabbits with experimentally induced MCF (Meier-Trummer *et al.*, 2009). This showed that M-cells and large intestinal epithelium are essential for viral replication and/or pathogenesis. This relates to a previous study because there was marked necrosis in lymphoid follicles of the appendix (Anderson *et al.*, 2007). These findings indicate that subepithelial lymphocytes are activated when exposed to cytokines from infected epithelial cells and start killing their host cells.

In an experiment to study AIHV-1-induced MCF in rabbits model, it was shown that the virus is associated with latent infection in proliferating CD8⁺ T, this was considered as a new model of pathogenesis (Dewals *et al.*, 2008). Here, rabbits were inoculated with AIHV-1 and treated with 5-Bromo-29-Deoxyuridine (BrdU),

then findings were summarized as: first, CD8⁺ T cell proliferation was detectable as early as two weeks after inoculation. Secondly, the viral loads in PBMC was undetectable during the incubation period, but increased sharply few days before death. At this stage, more than 10% of CD8⁺ cells had viral genome. Thirdly, RT-PCR analyses of mononuclear cells isolated from the spleen and the popliteal lymph node revealed no expression of ORF25 and ORF9, low or no expression of ORF50, and high or no expression of ORF73.

On the other hand, MHC-IIa allele polymorphism is probably associated with resistance or susceptibility to OvHV-2-induced MCF in bison (Traul *et al.*, 2007). Resistant bison show seroconversion with subclinical infection, but with very high viral doses, the resistant immune system status will be overwhelmed, like sheep when inoculated with a very high viral dose (Li *et al.*, 2005b). Concerning the virus's own role in pathogenicity, in an experimental OvHV-2 induced MCF in rabbits it was shown that severity of lesions is positively associated with the abundance of virus ORF25 which is a lytic transcript (Cunha *et al.*, 2013; Li *et al.*, 2011). While in WA-MCF in cattle, AIHV-1 ORF73 latent transcripts were detected in CD8⁺ T cells (Palmeira *et al.*, 2013). This suggests that different MCFV may have different pathogenic mechanisms.

The phenotype of lymphocytes in MCF and the little evidence about a direct viral damage to vascular and epithelial cells suggests that MCF is a consequence of immune dysregulation. The main question, i.e. which role the virus itself plays in the pathogenesis and tissue damage.

1.5.5 AIHV-1 and OvHV-2 infected cell lines

Since no permissive cell culture is available for OvHV-2 propagation, a useful way to study both the virus and behaviour of those cells is to culture OvHV-2 infected lymphoblastoid cell lines from MCF affected animals. The viral mechanisms that induce LGLs are unknown. Lymphocytes were derived from tissues (lymph node, thymus, spleen and cornea) of animals with SA-MCF, such as rabbits (Reid *et al.*, 1983) or cattle (Reid *et al.*, 1989). Some of these cell lines have been maintained indefinitely with feeder monolayers and/or IL-2. Moreover, some became independent of these factors after prolonged culture. As mentioned in the previous

section, these cells have the phenotype and cytotoxic activity of natural killer cells (Cook & Splitter, 1988; Reid *et al.*, 1989). OvHV-2 infected cell lines are responsive to Concanavalin A, express TNF- α , INF- γ , IL-4 and IL-10, but are negative for IL-1 β and IL-2 (Schock *et al.*, 1998; Swa *et al.*, 2001). AIHV-1 was readily detectable by DNA-ISH (Reid *et al.*, 1983) or by RNA-ISH (Cook & Splitter, 1988). OvHV-2 is maintained as circular DNA in bovine T cell lines, suggesting a predominant latent infection. In rabbit T cell lines, OvHV-2 have a linear genome with productive cycle gene expression of ORF75 (virion tegument protein) (Rosbottom *et al.*, 2002). In addition, viral capsids were seen for first time by TEM in infected rabbits T cells (Rosbottom *et al.*, 2002).

1.5.6 Ovine herpesvirus 2 microRNAs

MicroRNAs (miRNA) are a class of short (18-30 nt) non coding RNA molecules that regulate posttranslational gene expression (Olsen & Ambros, 1999). According to <http://mirbase.org/index.shtml> database, miRNAs exist in many organisms including viruses. In viruses, many miRNAs have been found and most of them are in double stranded DNA viruses, especially herpesviruses, that control viral and cellular gene expression (Plaisance-Bonstaff & Renne, 2011). EBV was the first virus in which viral encoded miRNA was found in the nucleus of various Hodgkin's and Burkitt's lymphoma cell lines (Pfeffer *et al.*, 2004). It was also shown that OvHV-2 encodes for at least eight miRNAs (Levy *et al.*, 2012). These miRNAs were identified from an immortalised lymphoblastoid cell line from a cow with MCF by parallel sequencing and northern hybridization. These miRNAs were encoded in two areas of the OvHV-2 genome that have no predicted protein coding and have no sequence similarity with other herpesvirus or cellular miRNAs. The role of OvHV-2 miRNAs in viral pathogenesis or latency was not clear. Further studies investigated the role of those miRNAs in virus biology and showed that ORF20 (cell cycle inhibition), ORF50 (reactivation) and ORF73 (latency maintenance) genes contains predicted targets for OvHV2-miR-2, miR-5 and miR-8 on those genes respectively (Riaz *et al.*, 2014). Relevant miRNAs have suppression effects on ORF50 and ORF20 but no effects on ORF73. Thus, the study hypothesized that miRNA-induced inhibition of viral genes functions to ensure that changes in OvHV-2 mRNA levels do not result in

reactivation when conditions are unfavourable for viral replication. In addition in a more recent study, by using RT-PCR, an additional 27 OvHV-2- miRNAs were identified (Nightingale *et al.*, 2014). In all OvHV-2 miRNAs, 30 was shown to be encoded in an approximately nine kb region that contains no predicted ORFs. There may be additional OvHV-2 miRNAs and until now the exact function of miRNAs is not fully understood in its pathogenesis.

1.5.7 Diagnostics of MCF

1.5.7.1 Clinical signs and histopathology

Formerly, the diagnosis of MCF was based on clinical signs, gross and microscopic pathological findings (Selman *et al.*, 1974). Also, the history of any recent mixing with the reservoir hosts supported the diagnosis, however, this is not always conclusive as in some disease cases the reservoir host contact is not apparent (Werner & Hugh, 2008). In animals with suspected MCF symptoms and lesions, a post mortem examination should be performed, tissue samples for histopathological examination to observe vasculitis and epithelial necrosis in organs and especially in the brain (Li *et al.*, 2014). However, because of the variable features of the different MCF forms and the similarity with some other viral diseases, a more confirmative laboratory diagnosis such as PCR is important (Holliman, 2005).

1.5.7.2 Serology

A number of immunological and serological tests have been developed to detect antibodies against viruses in MCF cases, such as immunoblotting (Herring *et al.*, 1989), enzyme-linked immunosorbent assay (ELISA) (Fraser *et al.*, 2006), competitive-inhibition (CI)-ELISA (Li *et al.*, 2001c; Li *et al.*, 1994; Li *et al.*, 1995; Powers *et al.*, 2005), indirect immunofluorescence and immunoperoxidase assay (Rossiter, 1981b), and complement fixation test (Sentsui *et al.*, 1996). Virus neutralisation was performed for AIHV-1 (Plowright, 1967). This assay works well with virus in natural hosts of the alcelaphine group in the wild or zoos, but works less well in clinical cases (Manual, 2013). Serological assays using antibodies can be non-specific because of antigenic cross-reactivity between herpesviruses, such as BoHV-1 and 4 (Dubuisson *et al.*, 1989). CI-ELISA is a sensitive test to detect

antibodies to a conserved complex glycoprotein 15A epitope of MCFVs (Li *et al.*, 1994). This technique was improved later as an indirect CI-ELISA and is a recommended simple serological method to detect antibodies against OvHV-2, AIHV-1, AIHV-2, CpHV-2 and WTD-MCF (Li *et al.*, 2001c).

1.5.7.3 Polymerase chain reaction

PCR has significantly improved the diagnostic options for MCF and allows specific identification of different MCFVs and even phylogenetic and epidemiologic studies (Russell *et al.*, 2014; Russell *et al.*, 2009). Several validated PCR protocols have been developed to amplify specific sequences of AIHV-1 and OvHV-2 or other MCFVs (Baxter *et al.*, 1993; Katz *et al.*, 1991; Li *et al.*, 1995; Murphy *et al.*, 1994; Wiyono *et al.*, 1994). A real-time PCRs have been developed to detect and quantify the MCFVs DNA loads (Hüssy *et al.*, 2001; Traul *et al.*, 2005). In studies using PCR and serological tests, it was shown that PCR was more sensitive than CI-ELISA (Li *et al.*, 1995). A multiplex PCR was developed to detect different MCFVs in clinical samples (Cunha *et al.*, 2009). In this protocol, a single set of primers plus different probes for MCFVs were used to detect OvHV-2, AIHV-1, CpHV-2, WTD-MCF and MCF-ibex. This assay is a good tool to test for most common MCFV in one reaction mix.

1.5.7.4 Differential diagnosis

There are other diseases that have similar clinical signs or gross lesions to MCF, especially those of head and eye or the alimentary form of MCF. Diseases that need to be considered as possible differential diagnoses when MCF is suspected include bovine viral diarrhoea-mucosal disease, bluetongue, epizootic haemorrhagic disease, infectious bovine rhinotracheitis, theileriosis, vesicular stomatitis and foot and mouth disease (Holliman, 2005; Kahn & Line, 2010). Oral lesions need to be distinguished from cases resulting from the uptake of corrosive substances, poisonous plants or mycotoxins (Werner & Hugh, 2008). The cutaneous lesions in MCF resemble those of skin hypersensitivity reactions, photosensitisation or generalised streptotrichosis in cattle (David *et al.*, 2005).

1.5.7.5 Treatment and control

Once an animal has MCF, there is no available treatment. However, in one farm where cattle showed distinct clinical signs of MCF, such as fever, ocular and oral lesions; and in a second farm, cattle only exhibited cutaneous lesions; affected animals were treated with systemic corticosteroids (dexamethasone), antibiotics (procaine penicillin) and eye drops (atropine, betamethasone and neomycin) (Milne & Reid, 1990; Penny, 1998). On the other hand, spontaneous recovery of cattle from MCF (confirmed by serology and PCR) has also been reported (O'Toole *et al.*, 1997; Penny, 1998).

Control of MCF may not be simple due to the large number of reservoir and susceptible hosts. Avoiding mixing or housing of reservoir animals with clinically susceptible animals helps to reduce the incidence of MCF (Werner & Hugh, 2008). However, this is not always achievable in places like zoos. In this case, the introducing of SPF animals may be a solution (Li *et al.*, 1999a). Also before mixing, MCFVs carrier species should be kept in quarantine and undergo molecular tests (Werner & Hugh, 2008). Vaccination is another control measure is discussed next.

1.6 Vaccine prospective

Currently there is no available licenced vaccine against MCF. Especially an effective vaccine is very necessary to limit the WA-MCF in cattle in Africa and OvHV-2 in the American bison industry and also in zoological parks with exotic animals (Li *et al.*, 2014). Both reservoir and susceptible hosts can produce antibodies against OvHV-2, as shown by indirect immunofluorescence and these antibodies can cross-react with L and E antigens of AIHV-1, meaning there are antigenic relationships between the two viruses (Rossiter, 1981a; 1983). In a study, wildebeest sera were used for immunoblotting against AIHV-1 structural antigens (Herring *et al.*, 1989). Rabbit and cattle were experimentally inoculated with AIHV-1, they produced antibodies, but the antibodies were not protective against MCF (Rossiter *et al.*, 1977). Antibodies in hyperimmune sera from diseased cattle and rabbits, shown to react with two early antigens in AIHV-1 WC11 infected cell culture (Rossiter *et al.*, 1978). Cattle were challenged by inoculation of different strains of AIHV-1, some produced several months immunity, but the experiment did not give rise to a practical vaccine

development, as immunity was not protective for other strains (Mirangi, 1991). In addition to this, cattle were used to study immunity in the oro-nasal-pharyngeal region against AIHV-1 MCF. For this purpose, attenuated C500 strain was injected intramuscularly, then the cattle were challenged with virulent C500. Nine of ten animals which had high levels of virus neutralising antibodies were protected (Haig *et al.*, 2008). To determine the duration of the protective immunity, an attenuated AIHV-1 vaccine was administered in a licensed adjuvant and was found to protect cattle from fatal intranasal challenge with virulent AIHV-1 for three to six months, but not for longer times (Russell *et al.*, 2012). The protected vaccinated cattle had higher initial anti-viral antibody titers in their plasma and nasal secretions before the inoculation, and these levels stayed stable afterwards. In comparison, in vaccinated cattle that succumbed to disease, antibodies levels has risen when they exhibit clinical signs. These results show that neutralising antibody in the mucosa is a crucial barrier to blocks infections of nasal entry (Russell *et al.*, 2012).

In the case of OvHV-2, due to lack of cell-free virus, immunological and vaccine experiments are limited to study *in vitro* virus antigens. Recently an *in vivo* system in sheep and rabbits was evaluated to see if antibodies can block virus entry following intranasal challenge (Li *et al.*, 2013b). Two inocula were prepared; one was treated (at 37 °C for one hour) with anti-OvHV-2 sheep sera, and the other was treated with negative sheep sera as a control. All Sheep became infected, but the positive serum reduced viral infectivity by 1000 folds based on detecting OvHV-2 DNA and seroconversion when compared to the control group. Rabbits that received immune sera were protected, but the control group developed MCF. This result shows the importance of antibody in blocking OvHV-2 entry (Li *et al.*, 2013b).

Another approach to produce a possible vaccine against MCF is by using recombinant viral vaccine technology. This is either by entire or partial virus genome cloning in a bacterial artificial chromosome (BAC) that could allow propagation of OvHV-2. This has been done for AIHV-1 as the viral genome has been cloned and the clones produced virions in permissive cells. When these virion inoculated to susceptible hosts, they induced MCF which was clinically and pathologically indistinguishable when compared to AIHV-1 induced MCF (Dewals *et al.*, 2006).

1.7 Aims of this study

SA-MCF is a worldwide disease in many ruminant species, including domestic cattle, bison, deer and other exotic animals. OvHV-2, the most frequent causative agent of MCF, cannot be propagated *in vitro*. OvHV-2 infection in sheep has not been studied in a great detail and there are fundamental questions to be answered. The virus is latent in blood, although there is controversy about which subset, T or B cells, and recent evidences suggest that alveolar epithelial cells can support viral reapplication and shedding. A critical question in the understanding of the pathogenesis, spread and control of OvHV-2 is where the virus normally resides, in which cell types, and to what extent, local or systemic. The aim of this project therefore was to investigate the precise cellular location of OvHV-2 in tissues derived from sheep at different ages and to clarify whether the virus was latent or productive. Also, very little is known about whether healthy cattle can carry OvHV-2, and if so, in which tissues. To answer these questions we used molecular biology and immunohistological techniques.

Answers to these questions will help in understanding the infection biology of OvHV-2 in its reservoir host (sheep), and the susceptible host (which is cows in this study) and this may help in the control of the disease.

CHAPTER TWO: MATERIALS AND METHODS

2.1 Animals and samples

2.1.1 Animals

Three groups of animals were used in this study: sheep (n=28), as the reservoir host of OvHV-2, cattle (cows) without clinical or pathological evidence of MCF (n=50), as a susceptible species, and cattle with MCF (n=12). Animals were of a wide range of age, as from foetuses to aged adults, with no discrimination to sex. Sheep and cattle without MCF were randomly collected; either they were normal cows from an abattoir in North England, or cows that were submitted for a diagnostic post mortem examination to the Institute of Veterinary Pathology, Vetsuisse Faculty, University of Zurich, Switzerland. The MCF materials were either provided by AHVLA in the UK as tissue blocks, or as formalin-fixed paraffin-embedded (FFPE) tissue sections from same the institute in Switzerland.

2.1.2 Sample collection and processing

In sheep and cattle without MCF, specimens were taken from a variety of organs, including lung, tongue, muzzle, spleen, mediastinal, mandibular and mesenteric lymph nodes, and blood. In bovine MCF, samples were collected from lungs, muzzle, tongue, lymph nodes, gastrointestinal tract, brain including rete mirabile, spleen and kidney. In animals at the abattoir, tissue samples were collected within about 1 h after slaughtering. In all tissue specimens, one piece of approximately 0.5 x 0.5 x 0.5 cm was kept in RNAlater[®] as a preservative (Sigma-Aldrich, Poole, UK) in a 1.5 ml plastic ependorf tubes, kept at 4 °C for 24 h (or frozen at -8 °C for 3 days in Zurich samples), then transferred to cryotubes and stored at -80 °C for future nucleic acid extraction. A larger piece of the same tissue was either fixed in 4% paraformaldehyde (PFA) pH 7.4 in 1 x PBS (used from a 5x stock of 42 g NaCl, 9.26 g Na₂HPO₄·2H₂O, 2.15 g KH₂PO₄ dissolved in 1 L dH₂O) for 24 h (abattoir samples) or fixed in 10% buffered formalin (post mortem samples) for 24 - 48 h. Tissue specimens were trimmed and routinely paraffin wax embedded by the technical staff of the Histology Laboratories, Veterinary Laboratory Services, School of Veterinary Science, University of Liverpool, or the Histology Laboratories, Institute of Veterinary Pathology, Vetsuisse Faculty, University of Zurich.

Samples in RNAlater from Zurich were sent to Liverpool frozen and then stored further at -80 °C. All cattle MCF cases were retrospective. In Zurich cases, paraffin blocks from formalin fixed tissue specimens were available from the archive (Table 3 in the Appendix). The UK cases were supplied by the AHVLA. From these, frozen tissue samples (at -20 °C) were provided and moved to -80 °C once they arrived at the department Infection Biology laboratories in University of Liverpool.

2.1.3 Histological examination

For routine histological examination, 3-5 µm tissue sections were prepared by the respective histology laboratories, and mounted on glass slides (Colormedia, Shrewsbury, UK) for routine haematoxylin and eosin staining and on PolyFrost™ (Poly Lysine adhesive coated White frosted Clipped Microslides; Solmedia Laboratory Supplies, UK) for immunohistology (IH) and RNA-ISH.

2.2 Polymerase chain reaction for detection and quantification of OvHV-2 DNA

To determine the amounts of OvHV-2 DNA in tissues of animals, a highly sensitive qPCR technique had to be established. List of primers that were used in the PCR are shown in Table 2-1. We tried to detect one virus genome copy per reaction. For this purpose, plasmid standards were used for quantification and two PCR setup were tried; a nested PCR trial, consisting of a first round conventional PCR and second round qPCR; and an optimised one-step qPCR. The second setup then was adopted in this study to test all samples.

2.2.1 Establishment of standards for viral DNA quantification

Partial sequence of the gene 12S subunit ribosomal RNA (12S hereafter) of sheep and cattle (Gatesy *et al.*, 1997) contained in a plasmid vector (p12SrRNA) was supplied by Professor Monika Engels (Table 2.1). To make large scale preparations, the 12S construct (in pCR2.0 TOPO, Invitrogen, Paisley, UK), was transformed into chemically competent *E.coli* and plasmids were obtained in higher quantities (see 2.3.1.5). The OvHV-2 gene contained in a plasmid (pOvHV2T63), was prepared by cloning the PCR product, generated from amplifications using first round primers (of

ORF63 gene), into vector (pCR2.0 TOPO, Invitrogen) (see 2.3.1.4). After elution in H₂O, plasmids concentrations were measured by UV spectrophotometry absorbency at 260 and 280 nm (QuBit[®] machine, Invitrogen) and the copy numbers then were calculated according to the following formula:

$$[\text{Number of copies}/\mu\text{l} = (\text{amount} \times [6.022 \times 10^{23}]) / (\text{length} \times 650)]$$

While (amount) is the measured concentration of plasmids in nanograms of DNA per 1 μl ; (6.022x10²³) is Avogadro's number; (length) is the base pairs calculation of the entire plasmid including the DNA insert of interest; (650) is average weight of one base pair (bp) in Daltons.

Tenfold serial dilution of plasmids were prepared in 100 ng/ μl non-mammalian carrier DNA (salmon sperm DNA, Sigma), dilutions were from 1 - 10⁶ copies for the OvHVT63 plasmid, and 10³ - 10⁸ copies for the 12S plasmid. Then the plasmids were tested with the qPCR to check reaction sensitivity.

Table 2-1. The qPCR specific primers and probes.

Gene	Primer	Oligoprimer Sequence 5' → 3'	Tm	Product Size(bp)
OvHV-2 ORF63 (Tegument)	OvHVT63 F first round	GGTTTGACTGCAGAGCCTC	59 °C	260
	OvHVT63R first round	GTGCGTGGAGACAACTCC	59 °C	
OvHV-2 ORF63 (Tegument)	OvHV63 Probe	FAM - GAGAACAAGCGCTCCCTACTGA - TAMRA	-	106
	OvHV63 F	CGTCAAGCATCTTCATCTCCAG	-	
	OvHV63 R	AGTGA ^C CTCAGACGATACAGCACGCGACA	-	
12S rRNA	12S F	GCGGTGCTTTATAY ^C CCTTCTAGAG	55 °C	76
	12S R	TTAGCAAG ^R ATTGGTGAGGTTTAT	52 °C	
	12S Probe	VIC - AGCCTGTTCTATAAY ^C GAT - MGBNFQ	-	

The qPCR specific primers and probes were suggested by Pro. Monika Engels, Institute of Virology, Zurich University and also kindly provided 12S rRNA construct.; **FAM**: 5' modification (6-Fluorescein amidite) maximum intensity 517 nm; **TAMRA**: 3' modification quencher; **VIC**: 5' modification: commercial fluorescent dye, maximum intensity 555 nm; **MGBNFQ**: 5' modification (minor groove binder nucleotide fluorescent) quencher); Y: (Pyrimidine) C or T; R: (purine) A or G.

2.2.2 Nested PCR

First round conventional PCR was performed starting with 200 ng extracted genomic DNA from samples and serial dilutions of both standards. The PCR reaction was performed in a 40 µl mix in a thin-walled 200 µl PCR tube. The reaction mix was prepared in a clean hood (Captair® Biohood, Erlab) treated by UV exposure for 20 min prior and after use. The reaction mix contained target DNA, 4 µl 10x PCR buffer (20 mM Tris-HCl, 50 mM KCl, pH8.4; Invitrogen), 2 µl of 50 mM MgCl₂, 2 µl of 2.5 µM each dATP, dTTP, dCTP and dGTP (Invitrogen), 1.5 µl of 10 pM each primers (Eurofins, UK), 2 units of DNA polymerase (platinum Taq DNA polymerase, Invitrogen) with appropriate primers (OvHVT63 primer for virus) and appropriate annealing temperatures (T_m °C) for 30 amplification cycles. The thermocycler used was a Thermo Hybaid MBS (Thermo Electron Corporation) machine.

Second round of the PCR was performed in a real-time PCR machine (Opticon Monitor 2, BioRad, UK) for quantification with TaqMan® system. A portion of 5 µl of first round PCR product was added to a 20 µl reaction mix. The mix contained, 12.5 µl TaqMan® universal PCR master mix (ABiosystem, life technologies, Paisley, UK), that composed of Gold® ultra-pure AmpliTaq DNA polymerase, Uracil-N glycosylase (UNG), dNTPs with dUTP, ROX™ passive Reference dye and buffer, 0.15 µl of each of 12S primers (100 pm stock) and 0.4 µl 12 S probe (of 10 pm stock); or 0.3 µl of OvHV63F and 0.8 µl OvHV63R primers [of 20 pm stock] and 0.2 µl OvHV63 probe [of 10 pm stock]). The total volume was finished to 25 µl with ddH₂O.

The qPCR cycling parameters were according to the 7900 HT emulation protocol and were composed of an initial 20 min incubation at 50 °C (to activate the UNG to cleave any carryover PCR products from previous qPCRs), an additional incubation for 10 min at 95 °C for DNA denaturing and UNG inactivation, then 40 amplification cycles of 15 sec at 95 °C and 60 sec at 60 °C. Results were analysed using with software system of the Opticon Monitor 3.1.32 (MJ Geneworks Incorporation, Bio-Rad laboratories) and the data analysed with Microsoft word excel.

2.2.3 Optimised one-step quantitative PCR

A more optimised one step (only second round of previous PCR) qPCR was established for OvHV-2 quantification as following: 100 ng of native DNA sample in 12.5 µl dH₂O was added to the reaction of 25 µl, containing, 12.5 µl TaqMan® universal PCR master mix and 2 µl of OvHV-2 primers (0.6 µl OvHV63F and 1 µl OvHVT63R [stocks of 20 pm] plus 0.4 µl probe [of 10 pm stock]). Reagents for the 12S were not changed (see 2.2.2). The qPCR cycling temperatures were same as previous second round, but the amplification rounds was increased to 45 cycles.

2.3 RNA *in situ* hybridisation

2.3.1 Preparation of riboprobes

2.3.1.1 Tissue grinding and DNA extraction

Mesenteric lymph node from an MCF-affected cow was used for generating riboprobes. The Qiagen® DNeasy blood and tissue DNA extraction kit (Qiagen, Manchester, UK) was used according to the manufacturer's manual. Initially, 25-35 mg of tissue was dissected to smaller fragments and placed in a 2 ml plastic tube with ceramic beads (Pqlab, VWR collection) containing 180 µl ATL buffer (Qiagen) and 20 µl proteinase K (Qiagen). Tissues processed in a bead beater machine for 10 seconds for optimal tissue grinding, the formed foam was reduced by adding 0.5 µl of an antifoaming DX reagent (Qiagen). Tubes were then incubated at 56 °C overnight in a waterbath for optimal tissue digestion. The following day, 200 µl AL buffer (Qiagen) was added, mixed by vortex, and further incubated at 56 °C for 10 min. Then, 200 µl of molecular grade absolute ethanol (Sigma) was added and mixed. The mixture was transferred into a spin column tube (Qiagen) and centrifuged at 6000 x g for 1 min and the flow through were discarded. Then 500 µl AW1 buffer (Qiagen) was added, spun at 6000 x g for 1 min, flow through discarded, another 500 µl AW2 buffer (Qiagen) was added, centrifuged at 20000 x g for 3 min and the flow through was discarded. The column then was placed in a new 1.5 ml Eppendorf tube and eluted with 200 µl dH₂O for 1 min incubation then 6000 x g spinning for 1 min. The spin column was discarded and the flow through is stored in -20 °C. Before storing, elutes DNA were measured by UV spectrophotometry

absorbency at 260 and 280 nm (QuBit® machine, Invitrogen) using DNA assay kit (Quant-iT™ dsDNA BR Assay Kit, Invitrogen).

2.3.1.2. Conventional PCR

With specific primers for the two genes in Table 2-2, a PCR reaction was performed using same parameters as in (2.2.2, first round PCR) with appropriate primers and annealing temperatures (Table 2-2). Thermal conditions were as following: an initial denaturation at 94 °C, then 40 of 94 °C for 30 sec, annealing step of 62 °C, 64 °C or 55 °C for 30 sec and an extension step at 72 °C for 45 sec, finished by a final extension at 72 °C for 5 min.

Table 2-2. Primers used in PCR to make specific riboprobes for OvHV-2 mRNAs.

Primer	Gene	Oligoprimmer Sequence 5' →3'	Annealing temperature	Amplicon size (bp)
N2.5L	Ov2.5 cDNA	ATGGCATTGGCCCACCAACTAC (22 nt)	62 °C	550
N2.5R		CTTGACCCCAAAGTAGCTTTCC (22 nt)		
OvORF65F	ORF65 DNA	TTGGTGGGTGGACTATCCACTGC (23 nt)	64 °C	521
OvORF65R		GCAGCTGCAAGTTCAGGTCCTC (22 nt)		

2.3.1.3 Agarose gel electrophoresis

Portions of 10 µl from the PCR products were mixed 3:1 with loading buffer (50 % v/v glycerol, 100 mM Tris-HCl pH 7.4, 10 mM EDTA, 0.02 % w/v orange G). Then were electrophoresed through 0.8 % w/v agarose gels (Invitrogen) containing fluorescent nucleic acid gel stain 1:1000 v/v Gel Red (VWR International, Lutterworth, UK) in 1x TAE buffer (40 mM Tris-base, 20 mM glacial acetic acid, 1 mM EDTA). Gels were placed horizontally in plastic tanks (BioRad), filled with 1x TAE buffer and ran at 110 Volts for appropriate time. Gels were then documented under ultraviolet light (Ultraviolet Transilluminator, BioRad), images taken and target DNA bands sizes compared with 1 kb or 100 bp DNA ladders (Invitrogen).

2.3.1.4 Plasmid cloning, transformation and bacterial culture

The PCR products were cloned into plasmids (pCR2.0 TOPO, Invitrogen) using the (TOPO TA cloning kit, Invitrogen) following manufacturer's manual. A cloning mix of 6 μ l was prepared containing 4 μ l of Taq amplified fresh PCR product mixed with 1 μ l supplied salt solution (1.2 M NaCl, 0.06 M MgCl₂) and 1 μ l plasmid (10ng/ μ l linearized plasmid, 50 glycerol, 50 mM Tris, 1 mM DTT, 0.1% 100X triton, 100 μ g/ml BSA, phenol red). The mixture was incubated for 5 min at RT and then kept on ice.

A volume of 2 μ l of the cloning reaction was mixed gently with chemically competent *Escherichia coli* (Mach1™ One Shot®, Invitrogen) in a vial, kept on ice for 30 min, heat shocked for 30 sec at 42 °C in a water bath then moved immediately on ice for 5 min. Then 250 μ l of supplied S.O.C medium (Invitrogen) was added to the vial of bacteria, capped tightly and incubated horizontally at 37 °C in an orbital shaker at 200 rpm for 1 h.

After incubation, 30 -50 μ l of the cells was spread on surface of Luria-Bretani (LB 1 % w/v tryptone, 0.5 % w/v yeast extract, 1 % w/v NaCl) with 50 μ l/ml ampicillin (Sigm) prewarmed and pre-spread agar plate with X-Gal (2 mg X-gal in 50 μ l LB, Promega, USA) for white/blue colony screening. Plates were then incubated overnight at 37 °C. On the following day individual white colonies were picked and cultured in 5 ml LB broth containing 50 μ l/ml ampicillin (minipreparation) then incubated 16 -18 h at 37 °C in universal tubes in orbital shaker at 200 rpm.

2.3.1.5 Plasmid purification (minipreparation and maxipreparation)

Plasmid purified from minipreparation (miniprep hereafter) of transformed *E.coli* using Qiagen plasmid purification kit (Miniprep Kit, Qiagen) following the manufacturer's manual. From the 5 ml cultured bacteria, 4 ml was pelleted by spinning at 6000 x g for 15 min at 4 °C. Supernatant was discarded and the bacterial pellet was homogenised in 300 μ l resuspension buffer P1 (50 mM Tris.Cl, 10 mM EDTA, 100 μ g/ml RNase A), then 300 μ l lysis buffer P2 (200mM NaOH, 0.1 w/v SDS) was added, mixed by vortex and incubated for 5 min at RT. Another 300 μ l of neutralization buffer P3 (3 M potassium acetate) was added and the mix was

incubated on ice for 5 min and centrifuged at 2000 x g for 10 min at 4 °C. The supernatant containing plasmids was pipetted into an equilibrated Qiagen tip 20 (Qiagen) by applying 1 ml equilibration buffer QBT (750 mM NaCl, 50 mM MOPS, 15% isopropanol v/v, 0.15% Triton X-100 v/v) and emptying by gravity flow. The tip then was washed twice with 2 ml QC buffer (1.0 M NaCl, 50 mM MOPS, 15% isopropanol v/v) by passing by gravity flow. The DNA was eluted with 0.8 ml QF buffer (1.25 M NaCl, 50 mM Tris-Cl, 15% isopropanol v/v), which was later precipitated by mixing with 0.6 ml isopropanol (Sigma) and spinning at 15000 x g for 30 min at 4 °C. The supernatant was carefully discarded and the pellet was further washed with 70% molecular grade ethanol (Sigma) and spun at 1000 x g for 10 min. The ethanol was decanted and the DNA pellet was air dried and resuspended in dH₂O. The DNA concentrations were measured by Qubit spectrophotometry machine using (Invitrogen) BR DNA assay kit solutions (Invitrogen).

For a larger scale bacterial culture to produce larger amounts of plasmid at maxipreparation (maxiprep hereafter), the Qiagen plasmid maxikit (Qiagen) was used according to manufacturer's manual. About 0.1 ml bacteria from previously produced minipreps with the right insert was cultured in 400 ml LB broth containing 50 µl/ml ampicillin in a 2 L conical flask. The flask was placed in shaker at 200 rpm and incubated overnight at 37 °C. On the following day, bacteria were centrifuged at 6000 x g for 15 min at 4 °C. The cell pellet was resuspended with 10 ml buffer P1 (50 mM Tris.Cl, 10 mM EDTA, 100 µg/ml RNase A), then the bacteria were lysed by adding 10 ml buffer P2 (200mM NaOH, 0.1 w/v SDS) and incubation for 5 min at RT. This followed by neutralizing with 10 ml buffer P3 (3 M potassium acetate) and incubation on ice for another 5 min. The mix was spun at 2000 x g for 10 min at 4 °C to reduce the air friction heat. The supernatant containing plasmids was decanted into Qiagen tip 500 (Qiagen) which was the pre-equilibrated by washing 10 ml equilibration buffer QBT (750 mM NaCl, 50 mM MOPS, 15% isopropanol v/v, 0.15% Triton X-100 v/v) that was allowed to drain by gravity flow. After adding plasmids, the tips were then washed with 2 x 30 ml QC buffer (1.0 M NaCl, 50 mM MOPS, 15% isopropanol v/v) and let to pass through by gravity flow. The DNA was then eluted with 15 ml QF buffer (1.25 M NaCl, 50 mM Tris-Cl, 15% isopropanol v/v). The eluted

DNA was precipitated with 10 ml isopropanol (Sigma) and pelleted by centrifugation at 5000 x g for 60 min at 4 °C. Pellets were further washed with 5 ml 70% molecular grade ethanol (Sigma), spun at 15000 x g for 10 min, air dried and resuspended in dH₂O. The DNA concentrations was measured same way as with minipreps.

2.3.1.6 DNA sequencing

The pCR2.0 TOPO plasmids containing the PCR product inserts were sequenced at Source Bioscience (Rochdale, UK) using universal primers (M13 R and M13F) to sequence both sense and antisense DNA strands. Sequence data were compared to other available sequences on Genbank (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) using BLAST (Basic Local Alignment Search Tool) programme.

2.3.1.7 Restriction digestion for preparation of plasmid template

To prepare DNA templates, plasmids had to be linearized using appropriate restriction enzymes to produce right DNA cut as shown in Table 2-3. An amount of 10 µg of DNA was mixed with 10 µl of (20 unit/µl) restriction enzyme with 10 µl of its optimal buffer, 1 of 10x filtered bovine serum albumin (BSA), the mixture volume then finished to 100 µl with nuclease free H₂O and incubated for 2 hours at 37 °C. A volume of 5 µl of the digested DNA and a 100 ng of uncut plasmid beside of 1Kb DNA ladder were electrophoresed on an agarose gel (2.3.1.3) to compare sizes and speed of migration (supercoiled uncut plasmids electrophoresed slower through the agarose gel). Digested DNA was then purified using Phenol:Chloroform:Isoamyl alcohol (25:24:1), Chloroform:Isoamyl alcohol (24:1) and ethanol (Sigma), resuspended in 50 µl RNase-free water and stored at -20 °C.

Table 2-3. List of genes, plasmids and restriction enzymes used in pre-preparation for probe synthesis.

OvHV-2 Gene	Plasmid	Preparing sense probe (enzyme; buffer)	Preparing anti-sense probe (enzyme; buffer)
Ov2.5 *	pCR2.0 TOPO	XhoI; buffer 4 (New England Biolabs)	BamHI, buffer 3 (New England Biolabs)
ORF65	pCR2.0 TOPO	XhoI; buffer 4 (New England Biolabs)	BamHI, buffer 3 (New England Biolabs)

*Ov2.5 cDNA construct was provided by Professor James Stewart.

2.3.1.8 *in vitro* transcription of DIG-labelled RNA probes

Appropriately linearized DNA template was used for *in vitro* riboprobe synthesis using DIG RNA labelling kit (Roche, USA) to produce Dig-labelled RNA probes as per manufacturer's instruction. A volume of 1 µg of purified template DNA was made up to 13 µl with RNase-free dH₂O in an RNase-free reaction tube and placed on ice. The following reagents were then added: 2 µl 10x NTP labelling mixture, 2 µl 10x transcription buffer, 1 µl protector RNase inhibitor and 2 µl on any of appropriate RNA polymerase. To generate anti-sense probe, T7 polymerase was used and for the sense probe T3 polymerase was used. The mixes were incubated at 37 °C for 2 h to produce labelled RNA. The template DNA was then degraded by incubation with 2 µl DNase I at 37 °C for 15 min and the reaction was stopped by adding 2 µl 0.2M EDTA. Carrier yeast tRNA (1 µl of 1 mg/ml) was added to the DNase digested probes, precipitated by ethanol and then resuspended in 50 µl RNase-free water.

2.3.1.9 Dot Blot analysis of generated probes

To check the quality of generated probes, they were tested by dot blot hybridisation. Ten-fold serial dilutions of DIG-labelled RNA probe was prepared in 10 ng/µl baker's yeast tRNA (Roche) and 5 µl portions were spotted on a Hybond N⁺ membrane (Amersham Biosciences, UK, Buckinghamshire) of delutions starting from 10⁻⁵ to 10⁻¹. After drying, the probes were fixed to the membrane by a UV cross-linker (Stratagene). The membrane was then washed in a tray with 100 ml washing buffer (0.1 M maleic acid, 0.15 M NaCl pH 7.5, 0.3 % v/v Tween 20) on a shaking platform for 2 min at RT. Then incubated with blocking solution (10 x blocking solution [Sigma] in 100 ml Maleic acid buffer [0.1 M maleic acid, 0.15 M NaCl, pH 7.5]) for 30 min n shaker. Later, the membrane was incubated with 20 ml labelled anti-DIG antibody solution (anti-Digoxigenin-AP FAb fragments [Roche] diluted 1:5000 in blocking solution) for 30 min. The membrane was then washed twice with washing buffer for 15 min and equilibrated in detection buffer (0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5) for 3 min. One tablet of the color substrate (Sigma Fast BCIP/NBT [5-bromo-4-chloro-3-indolyl-phosphate/4-nitro blue tetrazolium chloride]) was dissolved in 10 ml RNase-free water, added to the tray and the membrane placed in the dark, periodically observed for dot color development.

Approximately after 1 h the reaction was stopped by washing the membrane in TE buffer for 5 min.

2.3.1.10 Slides preparation, proteolysis and acetylation

Tissue sections were first deparaffinised by dipping twice in xylene (VWR chemicals) for 5 min in a glass tanks, rehydrated through twice in 100% ethanol, once in each 96%, and 70% ethanol for 5 min and in DEPC-treated water (diethyl pyrocarbonate, Sigma) in a glass tank for 5 min. They were then placed in Hellendahl glass jars (Solmedia Lab Supplies) and washed with 1x PBS for 5 min at RT.

To perform proteolysis, sections were incubated in 0.2 N HCl for 30 min at RT, then twice in 2x sodium saline citrate (SSC, MP Biomedicals, UK) with 5mM EDTA for 30 min at 50°C, followed by digestion in 60 ml proteinase K solution (150 µg Proteinase K recombinant PCR grade [Roche] with 1 ml 1 M Tris pH 8.0, 1 ml 0.1 M CaCl₂ in 60 ml of DECP-treated H₂O) for 15 min at 37°C. A further fixation step followed, through washing in 60 ml 0.2% glycine PBS for 5 min at RT and subsequent 4 min washing in 4 % PFA at RT, a 2 min wash in 1x PBS and a 15 min wash in 60 ml of 5 mM MgCl₂ in 1X PBS.

Acetylation was performed by incubation with 0.25 % v/v acetanhydride in 0.1 M triethanolamine pH 7.5 (VWR chemicals) for 10 min at RT, followed by three washes in 1x PBS twice for 1 min and once for 15 min.

2.3.2. Hybridisation of riboprobes

2.3.2.1 Prehybridisation, hybridisation and post-hybridisation

Sections were prepared for hybridisation by incubating the slides in the jar for 1 h at 52°C in 50 ml prehybridisation buffer (0.1 mg/ml salmon sperm DNA [Sigma] and 0.25 mg/ml yeast tRNA [Roche] in a prehybridisation stock mix of 30% v/v 20X SSC, 45% v/v 100% deionised formamide [Ambion], 10% v/v 50x Denhardt's solution [Invitrogen] with 14% DEPC-treated dH₂O).

For hybridisation, slides were removed from the jars, placed on metal trays and covered with 40-50 µl hybridisation buffer composed of 250 µg/ml tRNA, 250 µg/ml ssDNA [salmon sperm DNA], 6.2 mg/ml dextran sulfate solution, 2-4 µl riboprobes) added to a 700µl stock of hybridisation mix (60% v/v 100% deionised

formamide, 32% of [30 mM EDTA pH 8.0 and 30 mM piperazin-N,N'bis 2-ethanesulfate-acid-PIPES, pH 7.0, Sigma], 0.9 M NaCl, 6x Denhardt's solution, 0.01 % v/v Triton X-100, and 20,000 U heparin, Sigma). The hybridisation mix contained the DIG-labelled riboprobes at a dilution of 1:100 of each Ov2.5 or ORF65 genes. Slides were covered with the hydrophobic face of gel-bond film (Dako, Glostrup, Denmark), boundaries glued with Fix-O-Gum gel (Marabu, Tamm, Germany) and incubated at 52°C in a moist box overnight.

On the following day, the gel-bond films were removed and the slides returned to the Hellendahl jars for post-hybridisation washes with SSC. Washes were twice at 42 °C for 15 min in 6x SSC with 45% v/v formamide (Analar Normapur, VWR chemical), followed by two washes with 2x SSC for 5 min at RT and two final washes with 0.2x SSC at 50 °C for 5 min.

2.3.2.2 Detection of hybridized probes

After washing with buffer 1 (0.1 M Tris, 0.1 M NaCl, pH 7.5) for 1 min and 30 min with blocking solution (53 ml buffer 1 with 1.2 ml sterile neutral sheep serum [NSS, Sigma] and 1.8 ml 10X Triton). Slides were removed from the jars, lined at both ends of the sections with speial water-repellent pen (Dako), and placed into a moist metal chamber. Sections were covered with 0.4 ml of antibody solution (30 µl anti-digoxigenin antibody AP conjugate Fab fragments [Sigma], 62 µl NSS, 188 µl 10x Triton and 6 ml buffer 1) and incubated for 2 h at 25 °C. Slides were then replaced into the glass jars and washed twice for 15 min with buffer 1, and once for 2 min with freshly prepared buffer 3 (0.1 M Tris, 0.1 M NaCl and 0.05 M MgCl₂.6H₂O, pH 9.5). Finally, slides were incubated in the dark for 3-8 h (depending on the speed of the staining reaction) in 60 ml of staining solution (three tablet of BCIP/NBT and 30 mg levamisole [Sigma] in 60 ml buffer 3). Slides were periodically examined under the light microscope to check the development of the signals (dark blue colour in cell cytoplasm) and to stop at the appropriate time point for each individual slide. This was by checking positive control sections (lymph node of mouse for MHV-68 viral tRNA probe of previously validated ISH, and lung sections from MCF affected cattle 12L-0200, see Table 3 in Appendix for the later). The reaction was stopped by moving the slides into a glass jar with buffer 4 (10 mM Tris with 1mM EDTA) in

which they were incubated for 10 min. Slides were then kept in distilled water until counterstaining or subsequent immunohistological staining (see 2.3.5). To perform RNA-ISH negative controls, either sense probes were used in sheep, cattle and MCF affected animals tissue section alongside antisense probes, or the antisense probes was tested on non-related species tissue sections such as canine (dog).

Counterstaining was performed by dipping the slides in haematoxylin (Papanicolaou's solution 1b Haematoxylin solution S, Merck Millipore, USA) for 10 sec, followed by washing in running tap water for 5 min. Slides were then covered with glass coverslips using glycer-gel mounting medium (Dako). They were subsequently stored in the dark to avoid fading of the signals.

2.4 Immunohistology

The immunohistology (IH) was employed for the demonstration of OvHV-2 antigen *in situ*, and for the demonstration of T cells and B cells in selected lymph nodes, spleens and lungs of sheep and cattle, and in selected MCF lesions (see Tables 1,2 and 3 in the Appendix). Information on the antibodies and references for their use in bovine tissues are provided in Table 2-4. The IH was performed by the technical staff of the Histology Laboratories, Veterinary Laboratory Services, School of Veterinary Science, University of Liverpool (CD3, Pax-5), or the Histology Laboratories, Institute of Veterinary Pathology, Vetsuisse Faculty, University of Zurich (OvHV-2 Ov8).

Table 2-4. List of antigens and antibodies used for immunohistology.

Antigen	Antibodies	Specificity	Sources	References
Ov8	Rabbit anti-OvHV-2 Ov8	OvHV-2 glycoprotein	Homemade, Professor James Stewart	Unpublished data
CD3	Rabbit anti-human CD3	Pan T-cell marker	DAKO, Glostrup, Denmark (A045229)	(Rosbottom <i>et al.</i> , 2008)
Pax5	Mouse anti-human Pax-5 clone 24/Pax-5	Pan B-cell marker	BD Transduction Laboratories, Kentucky, USA (610862)	(Agostinelli <i>et al.</i> , 2010)
CD20	Mouse anti-human clone L26	Pan B-cell marker	DAKO, Glostrup, Denmark	(Kämmerer <i>et al.</i> , 2001)

2.4.1 Immunohistological staining for CD3 (T-cells)

The ABC technique was performed as previously described (Kipar *et al.*, 1998). First, slides were dewaxed in xylene (VWR chemicals) twice for 5 min, rehydrated for 2 min each in 100% and 96% ethanol. Then immersed in methanol with 0.5% v/v (Perhydrol 30% H₂O₂ P-a, Fisher scientific) for 30 min at RT to inactivate endogenous peroxidase. Washed in Tris-buffered saline tween pH 7.6 (TBST; 100 ml of 10x Tris stock, 7.2 g NaCl, 500 µl Tween 20; finished with 900 ml dH₂O) for 5 min. For antigen retrieval, slides were incubated in 10 µM citrate buffer (0.9 % v/v 0.1 M citric acid, 1 % v/v 0.1M sodium acetate pH 6.0) for 25-30 min at 97 °C in screw cap coplin jars in a water bath. After cooling, slides were placed with cover plates in Sequenza racks (Thermo Shandon) and washed in TBST for 5 min, followed by blocking of non-specific antiserum binding by incubation in goat serum ([Sigma] diluted 1:10 in TBST) for 10 min at RT. Slides were then incubated with polyclonal rabbit anti-CD3 (1:10 in 20% swine serum in TBST) overnight (15-18 h) at 4°C, then washed three times for 5 min in TBST. This was followed by a 30 min incubation at RT with biotinylated goat anti-rabbit IgG (Vector Laboratories Ltd, Peterborough, UK; 1:100 in TBST) and a 5 min wash in TBST. Followed by a 30 min incubation at RT with avidin biotin complex (Vectastain ABC-Kit, Vector Laboratories Ltd; 0.9 µl A + 0.9 µl B to 100 µl TBST). Slides were then washed with TBST, removed from the cover plates and incubated for 10 min at RT in 0.1 M diaminobenzidine tetrahydrochloride (DAB) (0.1 M imidazole, 0.42 M HCl 2:3 v/v pH 7 [Fluka Chemie AG] with 0.01% H₂O₂ [Perhydrol 30% H₂O₂ P-a, Fisher Scientific]). This was followed by three washes in TBST for 5 min each and a final wash with distilled water for 5 min. The slides were counter stained with Papanicolaou's haematoxylin (Merck) for 1 min, blued in running tap water for 5 min, dehydrated in graded ethanol 96% and 100% for 1 min and 5 min respectively, cleared in xylene three times for 2 min, and mounted with cover slips with Distyrene Plasticizer Xylene (DPX; BDH brand, VWR chemicals, Lutterworth, UK).

2.4.2 Immunohistological staining for Pax5 (B-cells)

The peroxidase anti-peroxidase (PAP) technique was used as previously described (Kipar *et al.*, 1998). Deparaffinising and blocking of endogenous peroxidase was

performed as described for CD3. The slides were then washed for 5 min in TBST. Antigen retrieval was achieved by protease treatment. The sections were washed in 1x PBS (pH 7.2) for 5 min at 37 °C, then for 5 min in 0.05% protease (bacterial protease type XXIV, Sigma, UK) in pre-warmed PBS at 37 °C, followed by three washes for 5 min each in ice-cold TBST. The slides were then placed with coverplates in Sequenza racks (Thermo Shandon) and washed for 5 min with TBST. Followed by blocking of non-specific binding of antiserum by incubation in 10% rat serum (ABD Serotec, Kidlington, UK) in TBST for 10 min at RT. The slides were then incubated overnight (15-18 h) at 4 °C with monoclonal mouse anti-human Pax5 (1:40 in TBST). After washing in TBST for 5 min, slides were incubated for 30 min at RT in rat anti-mouse IgG (1:100 in TBST, H&L, Jackson ImmunoResearch, Suffolk, UK), washed for 5 min wash in TBST and incubated for 30 min at RT with PAP mouse (1:500 in TBST; Jackson ImmunoResearch). Subsequently, the slides were treated as described for the immunohistological staining for CD3 (see 2.3.1).

2.4.3 Immunohistological staining for CD20 (B-cells)

A horseradish peroxidase staining (Envision, DAKO) was used. The staining process took place in a DAKO Autostainer. After routine deparaffinisation, slides were incubated for 1 h at RT with the primary antibody diluted 1:1,000 in DAKO ChemiMate™ Antibody Diluent (Dako). After washing with TBST, endogenous peroxidase was blocked by incubation for 10 min at RT with Dako REAL™ peroxidase Blocking Solution (Dako). Slides were then subjected to the secondary antibody solution (EnVision Rabbit System HRP; Dako), followed by routine visualization with DAB and counterstaining with haemalaun (1:20) for approximately 2 sec. This staining was only applied on tissues of animals with MCF.

2.2.4 Immunohistological staining for OvHV-2 Ov8 antigen

The labeled streptavidin biotin (LSAB) method was followed, a biotinylated secondary antibody and a horseradish-streptavidin solution, was used. After routine deparaffinisation, antigen retrieval was performed by incubation of the slides in EDTA buffer pH 9.0 for 20 in at 98 °C. Endogenous peroxidase was blocked by incubation for 10 min at RT with Dako REAL™ peroxidase Blocking Solution (Dako).

The staining process took place in a Dako Autostainer. The slides were incubated for 1 h at RT with the primary antibody diluted 1:1,000 in DAKO ChemiMate™ Antibody Diluent (Dako), and were then subjected to the secondary antibody solution (biotinylated goat anti-mouse and anti-rabbit immunoglobulins; Dako REAL™ Link). Followed by streptavidin conjugated to horseradish peroxidase (REAL™ Streptavidin Peroxidase) which were both provided in the ready-to-use DAKO Real™, Peroxidase/ARC rabbit/mouse kit for use with Dako automated immunostaining instruments (Dako). Slides were washed with TBST between each incubation step. This was followed by routine visualization with DAB and counter staining with haemalaun (1:20) for approximately 2 sec.

2.4.5 Combined RNA-ISH and immunohistology

On selected cases and tissues (see Tables 1, 2 in the Appendix), RNA-ISH for the demonstration of viral transcripts and immunohistology for T and B cells (CD3, PAX-5) were combined to demonstrate in which cells OvHV-2 replicates. For this purpose, section underwent the full RNA-ISH protocol apart from the counterstaining, at which point (see in 2.3.2.2) the sections were subjected to the immunohistology staining (see in 2.4.1 and 2.4.2).

CHAPTER THREE: RESULTS

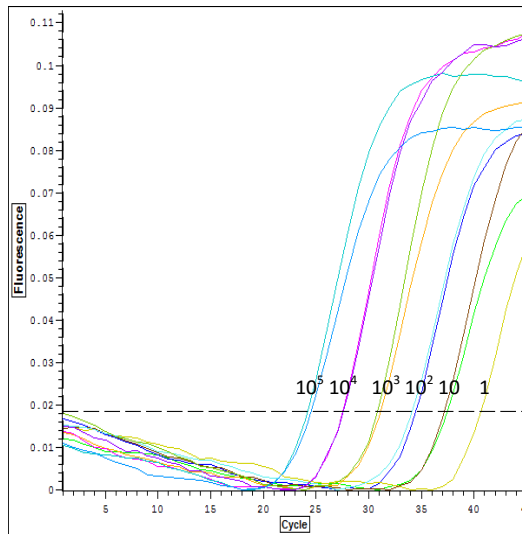
3.1 Detection of OvHV-2 in sheep

3.1.1 Establishment of the one-step qPCR assay

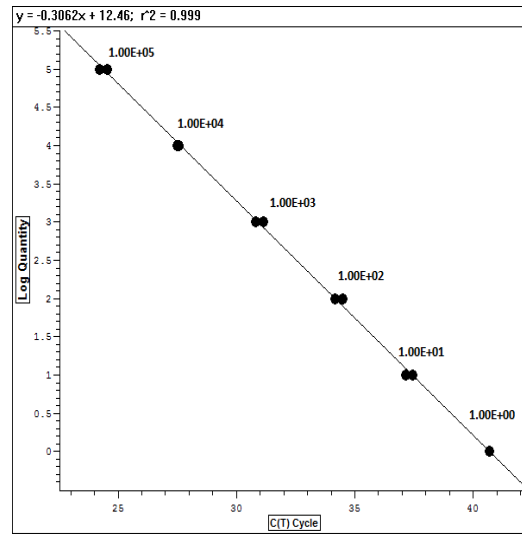
The one-step qPCR assay was optimised to a sensitivity level that can detect one viral copy per reaction. Absolute quantification was achieved by using a reference plasmid containing OvHV-2 target DNA (pOvHV2T63). Amplification of the OvHV-2 standard plasmid produced a linear deviation curve when tested on tenfold serial dilution from 1 - 10⁵ copies of pOvHVT63 per qPCR reaction (Figure 3-1 A,B). To normalise the reactions and to take account of individual variations in DNA content and amplification efficiency, a control reaction was performed using the cellular gene that encodes 12S ribosomal RNA using the reference plasmid containing the target DNA (p12SrRNA). The same procedure as for pOvHV2T63 was followed for the internal control plasmid, but the tenfold serial dilution were between 10³ and 10⁹ copies per qPCR reaction (Figure3-1 C,D).

After validation of the qPCR, the assay was applied for the testing of samples from the three animal groups; sheep, cows without MCF and cattle with MCF. Detailed information for each group is given in the following sections of this chapter.

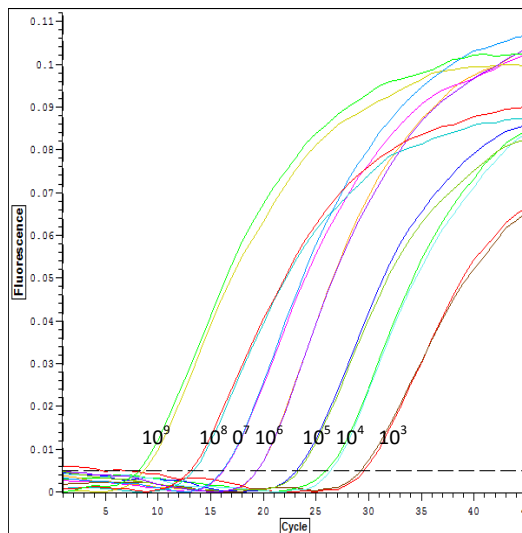
A. pOvHV2T63 fluorescent curves.



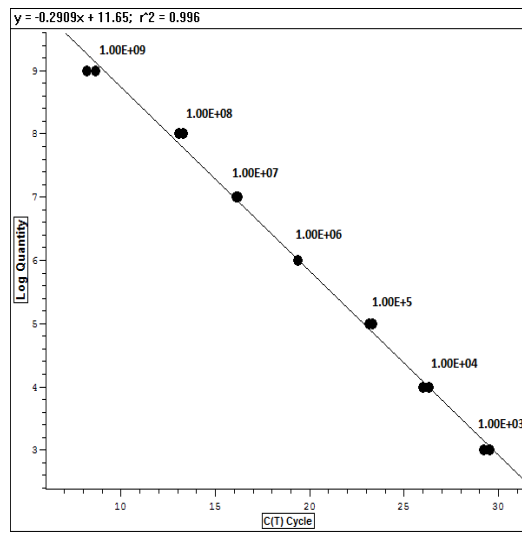
B. pOvHV2T63 standards line.



C. p12SrRNA fluorescent curves.



D. p12SrRNA standards line.

**Figure 3-1.** An example of amplification of standards at 10-fold serial dilutions.

A. Amplification fluorescent curves of the standard of plasmid pOvHV2T63. **B.** pOvHV-2 standard line equation, r2 value on the top. **C.** Amplification fluorescent curves of the standard of plasmid p12SrRNA. **D.** p12SrRNA standard line equation, r2 value on the top.

Y axis represents fluorescence measurements. X axis represents the 45 cycle numbers.

3.1.2 Quantification of OvHV-2 DNA loads in sheep

A total of 28 sheep were tested for the presence and amount of OvHV-2 DNA in a range of samples, including a variety of organs (in the majority at least lung, mediastinal lymph nodes and spleen), peripheral blood leukocytes (PBL) and/or nasopharyngeal swabs (Table 3-1; for detailed information of each individual animal, see Appendix, Table 1a and 1b). Of the 17 sheep of which organs were tested, 15 (88 %) tested positive for OvHV-2 DNA. All five 8-month-old sheep (100 %), of which only the PBL were tested, were found to be viraemic, i.e. PCR-positive, with viral loads of 2-9 copies per 100 ng genomic DNA in the PBL. Of the six sheep, for which only nasopharyngeal swabs were examined, three (50 %) were positive, with 6, 14 and 297 virus copies, respectively. In tissues, viral DNA loads were highly variable, not only between the different tissues within an animal, but also between individual animals and between animals at similar ages, and in general viral loads were relatively low (generally <300). Young lambs (neonates to less than five weeks old) were negative. By the age of seven months, animals were positive, but with very low virus titres (usually below 10 copies in organs), overall ranging between 1 - 215 copies in positive organs. Among those tested at eight months of age and over, only one sheep had high copy numbers (case no. 13L-2592C, eight-month-old), with 4200, 3200, 3300 virus copies in lung, mediastinal lymph node and spleen, respectively. In animals aged over ten months, the viral loads were still variable among organs, but had risen to above 100 copies in the most frequently tested samples, such as lung, lymph node, spleen, muzzle, tongue and nasopharynx. Notably, in the two older sheep (3.5 and 14 years) the viral loads were very low again.

Generally, among most tested organs, the most frequently positive ones were spleen (91 %), muzzle (85 %), followed by both lung and mediastinal lymph node (81 %) (Table 3-2). Overall, in all samples, the peak viral load (4200 copies) was found in the lungs and the relatively the lowest load was in the nasopharynx.

Table 3-1. Results of the OvHV-2 PCR and viral DNA loads in organs from sheep.

Case number/ age	Result	Organs											
		L	Ln	S	M	To	Na	Tu	U	Th	PI	Other	
S13-1453 14 y, Mother	+		6		-		-					2	
S13-1453 neonate	-	-		-						-	-		
S13-1353 5 we	-	-	-	-		-		-		-			
S14-0017 3 mo	+	-	-	-	1			-	-				
13L-2593 7 mo	+	4	2				215						
13L-2595 7 mo	+	2	5				2						
13L-2594 7 mo	+	1	45				22						
13L-2592B 8 mo	+	18	24	2									
13L-2592C 8 mo	+	4200	3200	3300									
13L-2592A 8 mo	+	1	-										
13L-4220 A-C 10 mo	+	143	229	276	648	97							
13L-4220D,E 10 mo	+	23	61	667		36							
13L-4218A-C 11 mo	+	15	27	9	56		4						Tes 23
13L-4218 D,E 11 mo	+	57	344	108	290	222							
13L-4219A-C 11 mo	+	131	556	24	5	345							
13L-4219D-F 11 mo	+	136	122	5	251	2							Ep 12
S13-1516 3.5 year	+	61	2	1	-	1		-	-				

Abbreviations: mo: months; we: weeks; y: years; L: lung; Ln: mediastinal lymph node; S: spleen; M: muzzle; Na: nasopharynx; To: tongue; Tu: Turbinate; U: uterus; Th: thymus; PI: placenta; Tes: testis; Ep: epididymis; "-": negative; empty boxes: not examined.

Table 3-2. Number of OvHV-2-PCR-positive samples and DNA loads in organs, PBL ^[2] and nasopharyngeal swabs in sheep.

Sample	Samples ^a		OvHV-2 DNA copies		
	Total	Pos	Min ^b	Max ^c	Med ^e
Lung	16	13 (81%)	1	4200	45
Mediastinal LN ^[1]	16	13 (81%)	2	3200	91
Spleen	12	11 (91%)	1	3300	24
muzzle	8	5 (63%)	1	667	160
Tongue	7	6 (85%)	1	648	154
Nasopharynx	5	4 (80%)	2	215	13
Turbinate	3	0			
Uterus	2	0			
Thymus	2	0			
Placenta	1	1	2		
Testis	1	1	23		
Epididymis	1	1	12		
PBL ^[2]	5	5 (100%)	5	9	6
Swabs ^[3]	6	3 (50%)	6	297	14

a. Samples tested, **total** is the number of all samples taken for that particular type of specimen. **Pos:** is number and percentage of samples tested positive. Table arranged according to the most frequently tested tissues. **b.** Minimum number of virus copies detected in the particular specimens. **c.** Maximum number of virus copies detected in the particular specimens. **d.** Median number of the virus copies. [1] LN refers to lymph node. [2] PBL: peripheral blood leukocytes. [3] nasopharyngeal swabs. [2 and 3] are the sole samples taken from those sheep.

3.1.3 Identification of cells harbouring OvHV-2 mRNA and antigen in sheep

The generated riboprobes (Ov2.5 and ORF65) were controlled for their functionality and reliability prior to use with test animal samples. For this purpose, riboprobes underwent dot blot analysis and yielded signal intensities according to their concentration. For the test controls, the antisense probes of Ov2.5 and ORF65 were tested on tissues of cattle with MCF as a positive control and on tissues from dog as a negative control. In the cow MCF case, ISH signals were detected in infected cells (Figure 3-24 C-E), while in dog tissues there were not any ISH signal (Figure 3-12 E,F). The sense probes of both genes were also tested on tissue sections of the same the cow with MCF, but no ISH signal was detected (Figure 3-30 A,B).

A selection of tissues were tested by RNA-ISH and immunohistology, to demonstrate viral mRNA and antigen, respectively, in an attempt to identify the cells that harbour the virus. RNA-ISH probes were specific for the Ov2.5 and ORF65

genes. The former should pick up both latently and productively infected cells and the latter productively infected cells. The anti-viral antibody was specific for the OvHV-2 Ov8 glycoprotein and should label productively-infected cells. In addition, double staining (RNA-ISH for viral transcripts in combination with immunohistology to highlight B cells [Pax5-positive] and T cells [CD3-positive]) was performed on lung, mediastinal lymph node and spleen of selected cases to identify the lymphocyte subtype positive for the viral transcript. The results for each case are provided in detail in the Appendix, Table 1a. The presence of viral transcripts was represented by dark blue cytoplasmic signals (Figure 3-2). This was seen with a similar intensity and cell range for both antisense probes (Ov2.5 and ORF65) (Figure 3-2 A,B).

Of the sheep tested by qPCR, samples from a total of 19 sheep were tested by RNA-ISH (organs and PBL), ranging in age from neonate lamb to 14 years. The neonate lamb tested negative by both qPCR and RNA-ISH, but only had a faint reaction for Ov8 antigen in spleen possibly in stromal fibroblasts, otherwise negative in other organs. The five-week-old lamb was PCR-negative and only positive in the RNA-ISH with Ov2.5 probe in the tongue (weak signals in sporadic cells in epithelium) (Figure 3-5 A). All other sheep were positive by qPCR, with variable virus copy numbers, and by RNA-ISH, and, when tested, in more than one organ. An attempt was made to find a relation between qPCR, RNA-ISH and immunohistology results. However, in general, despite the high variability of virus loads, the RNA-ISH signals were rather consistent in their intensity, but sometimes varied in the amount of positive cells. In contrast, the number of viral antigen-positive cells was positively correlated with the virus load. In terms of correlation between the RNA-ISH and viral IH, the viral IH was always positive where the RNA-ISH testes was positive in those animals tested, except for a 14-year-old sheep (S13-1453), where two of its organs (muzzle and nasopharynx) were only positive for viral antigen. In addition, the cell types positive by RNA-ISH and viral IH were similar and sharing certain cell types, however the viral IH, was most frequently detected in specific cell types. The results, cell types and correlation between the qPCR and RNA-ISH and viral IH are shown in Table 3-3.

In sheep tested, all organs were positive by all tests, except for few cases (foetus, five-week, three-month and the 14-year-old) were partially positive. Taken together, the qPCR and RNA-ISH results confirm that sheep are infected at an early age (at or before five weeks of age), with increasing viral loads up to approximately 1 year; the virus is readily detectable by qPCR over seven months of age.

Table 3-3. Correlation between OvHV-2 DNA loads and the presence of viral mRNA and antigen in sheep tested positive with either method.

Case Age	Tissue	Results		
		qPCR	RNA-ISH	OvHV-2 Ov8 Ag
S13-1453.4 neonate	Spleen	-	-	few stromal fibroblast
	Thymus	-	-	-
	Lung	-	-	-
	Placenta	3	-	-
S13-1353.6,7 5 we	Lung	-	-	ND
	Thymus	-	-	ND
	Spleen	-	-	ND
	Med. LN	-	-	ND
	Tongue	-	Some papillae EpC	ND
	Nares	-	-	ND
	Turbinates	-	-	ND
	Conchae	-	-	ND
S14-0017. 1, 2 3 mo	Lung	-	Perivascular LC, REC and VEC	ND
	Spleen	-	Occasional LC, VEC, vessels fibroblasts	ND
	Med. LN	-	Few LC, VEC, arterial SMC	ND
	Uterus	-	-	ND
	Muzzle	1	Focal area in EpC	ND
	Nares	-	-	ND
13L-2593A, B 7 mo	Lung	4	LC, VEC (arteries), occasional REC	ND
	Med. LN	2	numerous LC	ND
	Nasoph.	215	Some EpC in salivary gland, surrounding LC	ND
13L-2595A, B 7 mo	Lung	5	Some p.v LC, REC, glandular EpC, VEC and SMC of arteries	ND
	Med. LN	2	Numerous LC	ND
	Nasoph.	2	Submucosal LC, arteries VEC, intravascular leukocyte	ND
13L-2594A, B 7 mo	Lung	45	Many p.v LC, REC, glandular EpC, VEC and arterial SMC	Mph/FDC in BAL, type II AlvC
	Med. LN	1	Numerous LC	Moderate Mph/FDC
	Nasoph.	22	Strong EpC, LC, VEC	apoptotic bodies, occasional FDC
13L-2592B 8 mo	Lung	18	Several LC in BAL, REC, VEC	VEC, type I,II AlvC, PIM
	Med. LN	24	Numerous LC, TBM, VEC	Mph/FDC, VEC
	Spleen	2	Numerous LC, TBM,	Mph/FDC, VEC
13L-2592C 8 mo	Lung	4200	some LC in BAL, Rec, AlvC	ND
	Med. LN	3200	See 13L-2592B, but weaker	ND

13L-2592A 8 mo	Lung	1	Numerous LC in BALT, REC, VEC, AlvC	ND
	Med. LN	1	Numerous LC, TBM, VEC	ND
13L-4220A, B, C 10 mo	Med. LN	229	Some LC in follicles	ND
	Spleen	276	Very weak signal in some LC	ND
	Muzzle	648	LC and EpC	ND
	Tongue	97	Few patches of EpC	ND
	Lung	143	-	ND
13L-4220D, E 10 mo	Lung	23	Some LC in BALT, occasional glandular EpC	VEC, type I,II AlvC, aslo Mph, PIM, leukocytes in BALT
	Med. LN	61	Some LC	Mph, FDC in follicles, VEC
	Tongue	667	Weak patchy in EpC	VEC, infiltrating leukocytes, fibrocytes
	Spleen	36	Some LC in follicles	Mph, FDC, few LC and VEC
13L-4218A, B, C 11 mo	Lung	15	Occasional LC, VEC	ND
	Nasoph.	4	EpC and underlying LC	ND
	Med. LN	27	Some LC in follicles	ND
	Spleen	9	Some LC in follicles	ND
	Muzzle (epidermis)	56	Hair follicle EpC, some infiltrating LC, VEC	Dermis fibroblasts, Mph
	Epididymis	32	Ductal EpC, VEC, arterial SMC, fibroblasts	Stromal fibroblasts
13L-4218 D, E 11 mo	Lung	57	LC in BALT, AlvC II	Mph, VEC, Mph/DC in BALT, AlvC II
	Med. LN	344	Few Mph, LC	Many Mph and/or FDC
	Tongue	222	Individual EpC	Fibroblasts, VEC
	Muzzle	290	hair follicle EpC, occasional infiltrating LC	Strong in many in basal epidermal cells, some dermal fibroblasts
13L-4219A, B, C 11 mo	Med. LN	556	Few Mph, LC	ND
	Spleen	24	Weak some LC in follicles	ND
	Tongue	345	-	ND
	Muzzle	5	Weak in hair follicle EpC	ND
	Lung	131	LC in follicle-like BAL, gland EpC, REC	ND
13L-4219D, E, F 11 mo	Lung	136	LC in follicle-like BAL, gland EpC, REC	ND
	Med. LN	122	Some LC in follicles	ND
	Muzzle	251	Hair follicle EpC, EpC	ND
	Tongue	2	Weak in quamous EpC	ND
S13-1453.2,3 14 y	Med. LN	6	Few cells in sinuses, follicles	Mph and FDC
	Muzzle	-	-	Mph and FDC
	Nasoph.	-	-	Fibroblasts, few VEC
	Placentom e	2	Maternal placental epithelium	Weak in stroma fibroblasts

Abbreviations: ND: not done; qPCR: virus copy numbers; RNA-ISH: either Ov2.5 or ORF65 or both; IH: immunohistology for viral antigen; med.LN: mediastinal lymph node; mo: month-old; we: week-old; y: year-old; nasoph.: nasopharynx; "-": negative; EpC: epithelial cells; FDC: follicular dendritic cells; REC: respiratory epithelial cells; VEC: vascular endothelia cells; AlvC: alveolar cells; LC; lymphocyte; p.v: perivascular; Mph: macrophages TBM: tingible body macrophages.

3.1.3.1 Lung

In the lung, viral transcripts were detected in a range of cells such as type II pneumocytes, respiratory and glandular epithelial cells of bronchi and bronchioles, arterial, venous and capillary endothelial cells, a proportion of cells in the follicle-like lymphocyte aggregates of the bronchus associated lymphoid tissues (BALT), and occasional macrophages (Figures 3-2 A,B; 3-3 A; 3-4 A). Combined RNA-ISH and immunohistology identified the Pax5⁺ cells (B cells) as the lymphocytes carrying the virus (Figure 3-3 A-D). The distribution of viral Ov8 antigen was different from that of viral transcripts; only occasional vascular endothelial cells were found to be positive, and in the BALT, only macrophages and follicular dendritic cells (FDCs) were found to express viral antigen. In addition, occasional possible type II pneumocytes and sporadic peribronchial fibroblasts and chondrocytes were positive (Figure 3-4 B-F). Of the 17 lungs tested by RNA-ISH, two were negative. These were the qPCR-negative lungs from the neonate and the five week old lamb. Of the 15 RNA-ISH positive lungs, one was qPCR-negative. The latter was from a three month-old sheep (S14-0017.1,2, see Appendix, Table 1) in which spleen, mediastinal lymph node and muzzle positive by ISH, but the only PCR-positive tissue was muzzle. In the lungs which were positive for RNA-ISH, the viral loads ranged between 1 and 4200 virus copies (Table 3-1).

One point to be mentioned is the pale yellowish to orange colour of the B cells in the double RNA-ISH and immunohistology staining for Pax5 in B cells (they normally should appear brown as with the immunohistology staining), which made the B cells less visible. This was because of the RNA-ISH was performed prior to the immunohistology; this affected the Pax-5 antigen detection in the B cells in lung BALT, lymph node and spleen. But this did not happen with ISH/immunohistology for T cells.

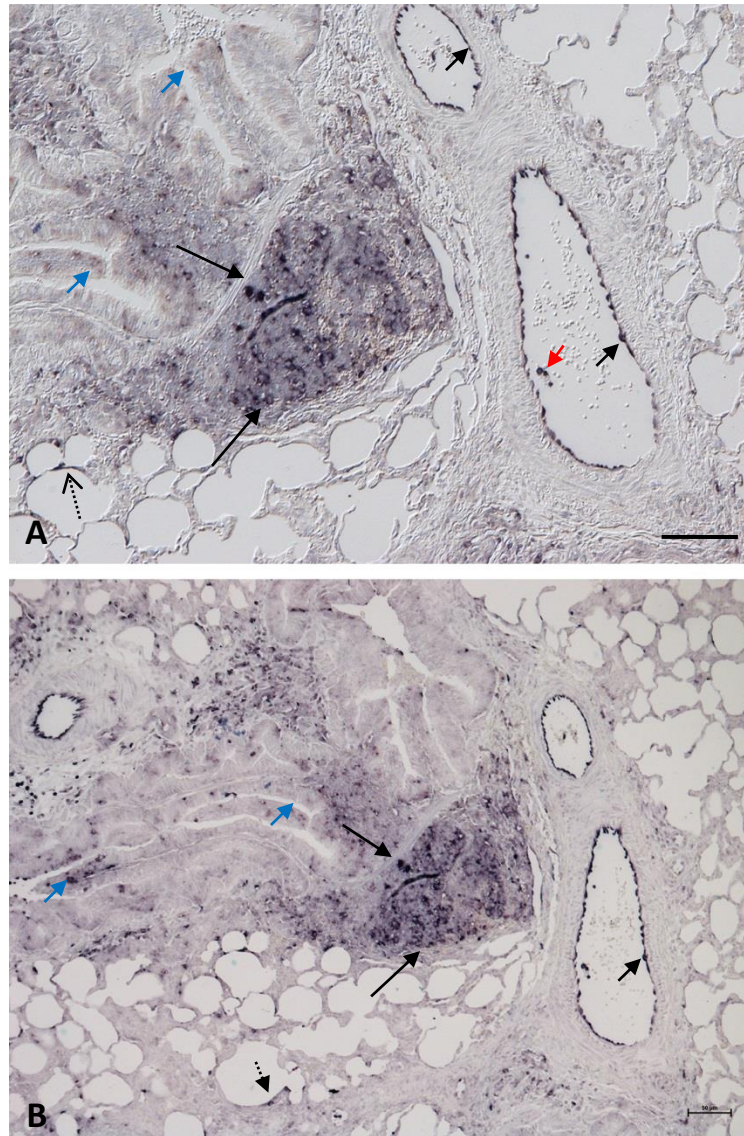


Figure 3-2. RNA-ISH Lung in an eight month-old sheep (13L-2592B) with a viral load of 18 virus copies.

RNA-ISH for OV2.5 (A) and ORF65 (B). In both slides there are strong ISH signals (dark blue) are seen in arterial endothelial cells (short black arrows), in lymphocytes and probably macrophages (in the peribronchial lymphatic tissue [long black arrows]), in individual type II pneumocytes (dashed black arrow) and in circulating leukocytes in the vessel lumen (red arrow). Weaker signals are seen in a few bronchiolar respiratory epithelial cells (blue arrows). Bars= 100 (A), 50µm (B). BCIP/NBT, haematoxylin counterstain.

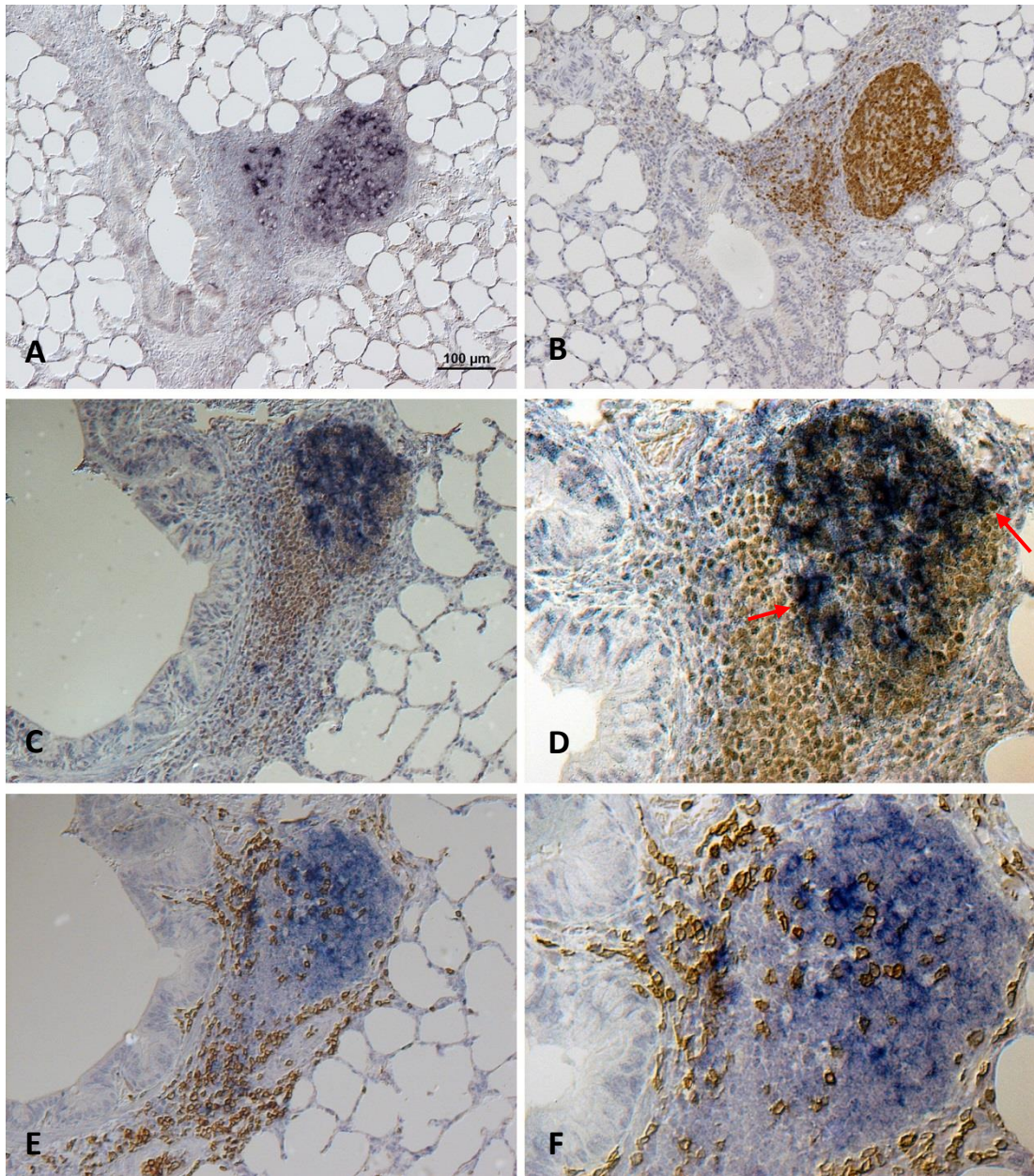


Figure 3-3. RNA-ISH and immunohistochemistry for T and B cells. Serial sections of lung of an eight month-old sheep (13L-2592B); see also Figure 3-2.

(A) RNA-ISH for Ov2.5, showing positive cells (dark blue) in the follicle-like structures (B cell zones) of the BALT. **(B)** Immunohistochemistry for Pax5, confirming that the follicle-like structures are predominantly composed of B cells (brown colour) (200x). **(C)** Double RNA-ISH and Pax5 staining, confirming the co-localisation of cells with viral transcripts and B cells (brown) (200x). **(D)** Higher magnification of C, confirming that a proportion of PAX5⁺ B cells exhibit a positive RNA-ISH signal (red arrows), (400x). **(E)** Double RNA-ISH and CD3 staining, showing that CD3⁺ cells (T cells, brown colour) are scarce in the follicle centres, but otherwise located around the follicles (200x). **(F)** Higher magnification of E, confirming that that the CD3⁺ T cells in the follicle centre do not exhibit any ISH signal (400x). BCIP/NBT (RNA-ISH), DAB (IH), haematoxylin counterstain.

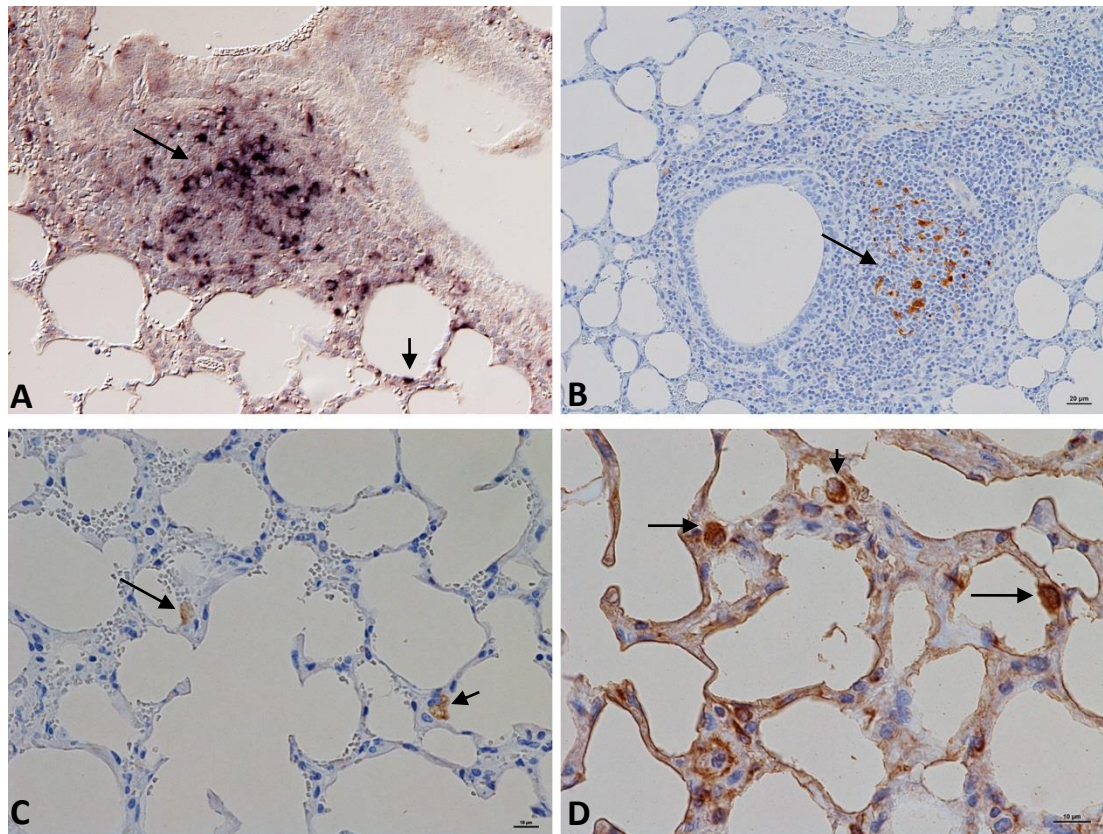


Figure 3-4. RNA-ISH and immunohistochemistry for viral Ov8 antigen in lung of sheep. A-C is a seven month-old sheep (13L-2594 A) with a virus load of 45 virus copies. D is a ten-month-old sheep (13L-4220 D) with a virus load of 23 virus copies.

(A) RNA-ISH for Ov2.5 (dark purple), strong signals in BALT leukocytes (long arrow) and in the alveoli cells (short arrow), (200x). **(B)** IH for viral antigen (brown), the antigen is expressed in a proportion of leukocytes in the BALT, bar= 20 μ m. **(C)** Higher magnification, the viral antigen is weakly expressed in type II alveolar cells (long arrow) and alveolar macrophages (short arrow), bar= 10 μ m. **(D)** Higher magnification of alveoli, the viral antigen is seen in possible sporadic individual type II pneumocytes or pulmonary macrophages (arrows), and in the PIM (short arrow) inside the capillary with positive endothelial cells, bar = 10 μ m. BCIP/NBT (RNA-ISH), DAB (IH), haematoxylin counterstain.

3.1.3.2 Tongue and muzzle

Of six tongues, one was PCR-negative, but ISH-positive (five week-old lamb, case no. S13-1353), all other tongues were qPCR/ISH-positive except one case was PCR-negative/ISH-negative (11 month-old sheep, case no. 13L-4219). The viral DNA loads were generally relatively high (only two cases were 1-2 virus copies, in the others were 22-345 virus copies). In two tested PCR/ISH-positive animals, the virus antigen was positive as well. In the young aged lamb (five week-old), a weak RNA-ISH signal was seen in the epithelium of in tongue papillae (Figure 3-5A), while it was negative by qPCR. In contrast, viral Ov8 antigen expression was only seen as a weak reaction in a few sub-epithelial vascular endothelial cells. In general, in the tongue, the RNA-ISH signals were patchy. Viral transcripts were usually detected in epithelial cells in the most superficial layers in the stratum spinosum, in the papillae and occasionally in a few leukocytes in the submucosa (Figure 3-5 B). viral Ov8 antigen was relatively strongly expressed in vascular endothelial cells and in infiltrating leukocytes and fibrocytes/fibroblasts beneath the epithelium (Figure 3-5 C,D).

Of the seven muzzles tested by both PCR and RNA-ISH, only one was negative by both (14 year-old sheep) and all the others were positive by both tests. Except for three animals where there were low viral loads in muzzle, it was relatively high in other sample (56 - 648 viral DNA copies). In animals where tongue was positive by qPCR, the muzzle was positive also, but not always with the RNA-ISH. In these PCR-positive muzzles, the presence of viral transcripts was demonstrated by RNA-ISH. In general, the viral transcripts were detected in patchy areas in the squamous epithelial cells, in hair follicles and occasionally in infiltrating leukocytes (Figure 3-5 E). viral Ov8 antigen expression was more widespread and was observed in basal epithelial cells of the epidermis, a few fibroblasts and macrophages in the dermis and vascular endothelial cells (Figure 3-5 F).

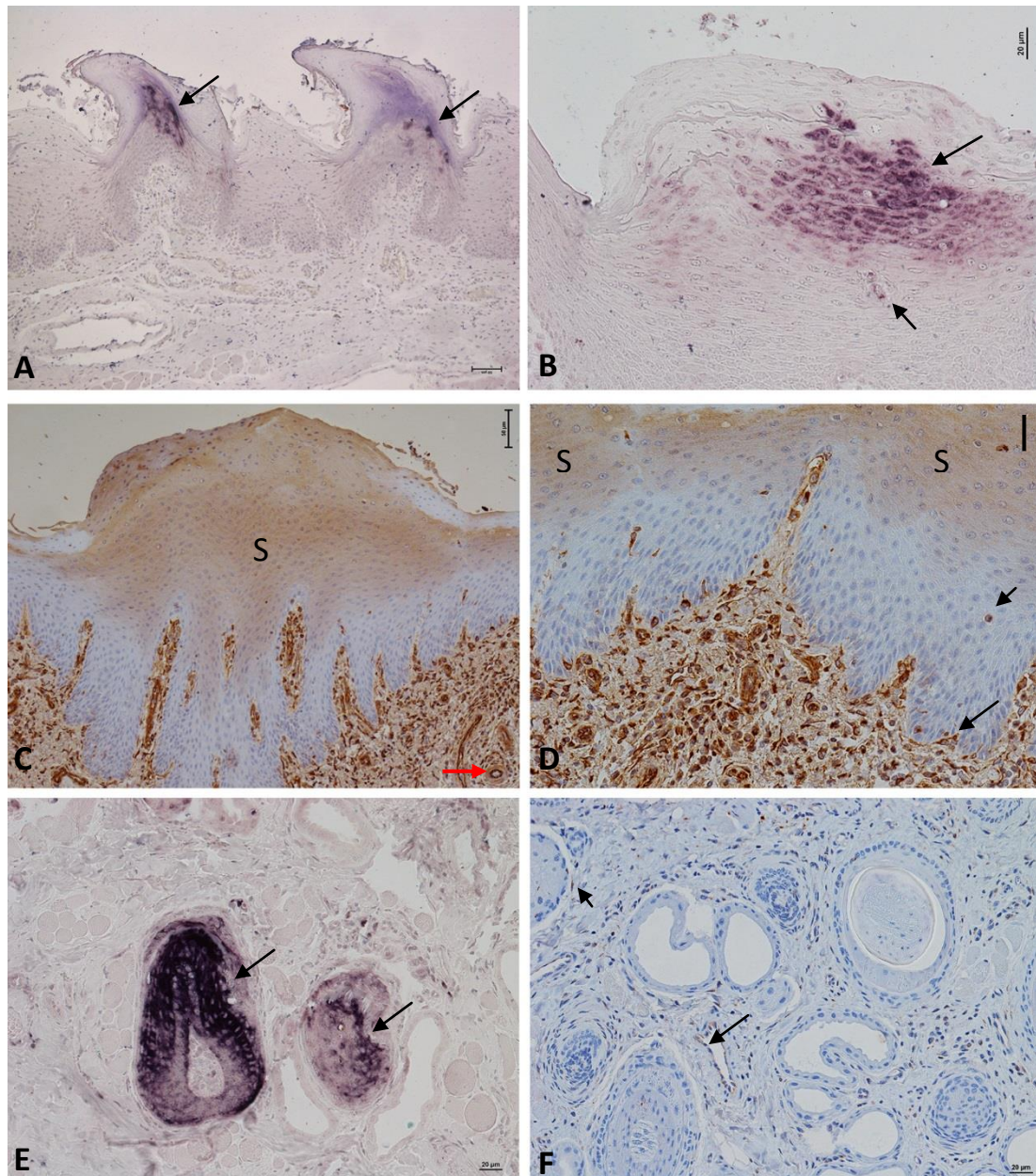


Figure 3- 5. RNA-ISH and immunohistochemistry for viral Ov8 antigen in tongue and muzzle of sheep. **(A)** RNA-ISH for Ov2.5 in tongue of a five-week-old sheep (S13-1353.7) with negative PCR result. There are ISH signals in few epithelial cells in the filiform papillae (arrow), bar=50μm. **(B)** RNA-ISH for Ov2.5 in tongue of a 10 month-old sheep (13L-4220 E) with a viral load of 667 virus copies. ISH signals in aggregates of epithelial cells in stratum spinosum (long arrow) and very weak signals in a few cells beneath (short arrow), bar=20μm. **(C & D)** IH for viral antigen in tongue in B, overall strong reaction in VEC (red arrow), in infiltrating leukocytes (short black arrow) and in fibrocytes/fibroblasts beneath the epithelium (long black arrow), also there are weak reaction in the superficial epithelial cells (S), bar=50μm in A, 20μm in B. **(E)** RNA-ISH for Ov2.5 in muzzle of a 11 month-old sheep (13L-4218 E) with a viral load of 290 virus copies. Muzzle epidermis, showing strong ISH signals in hair follicle epithelial cells, bar=20μm. **(F)** IH for viral antigen of the muzzle dermis in E., the antigen is weakly expressed relatively numerous in basal fibroblasts (short arrow) and in VEC (long arrow), bar=20μm. BCIP/NBT (RBA-ISH), DAB (IH), haematoxylin counterstain.

3.1.3.3 Mediastinal lymph nodes

Of the 15 lymph nodes tested by both qPCR and RNA-ISH, two were negative by both tests. These were the neonate and the five week-old lambs, one sample was only ISH-positive on the three month-lamb, and all the remainder were positive by both tests. The viral transcripts were mainly seen in B cell zones, i.e. in relatively numerous lymphocytes in the follicles of the cortex, sometimes in tingible body macrophages (TBM) and occasionally in interfollicular lymphocytes (Figures 3-6 A-F; 3-7 A,B; 3-8 A,B). Staining of consecutive sections with the B and T cell markers and double RNA-ISH and IH staining confirmed that the cells carrying viral transcripts are B cells (Figures 3-7 A-D). The viral Ov8 antigen was detected in possible macrophages or FDCs in follicles, and in a few macrophages in medulla and sinus (Figure 3-8 C,D). Totally, of the 17 lymph nodes (the above 15 plus another two tested only by RNA-ISH) tested by RNA-ISH, one was negative (case no. S14-0017, a three month lamb). This animal was also negative in the qPCR in all organs except in the muzzle.

The intensity of the RNA-ISH signals was not always correlated with the viral load, as the signals in a lymph node sample with 24 virus copies (Figure 3-6 A,B) were very similar to those of other lymph node with only one virus copy (Figure 3-7 A), whereas the extent of viral Ov8 antigen expression in the lymph nodes appeared to be rather consistent with viral loads detected by qPCR (Figure 3-8 C,D).

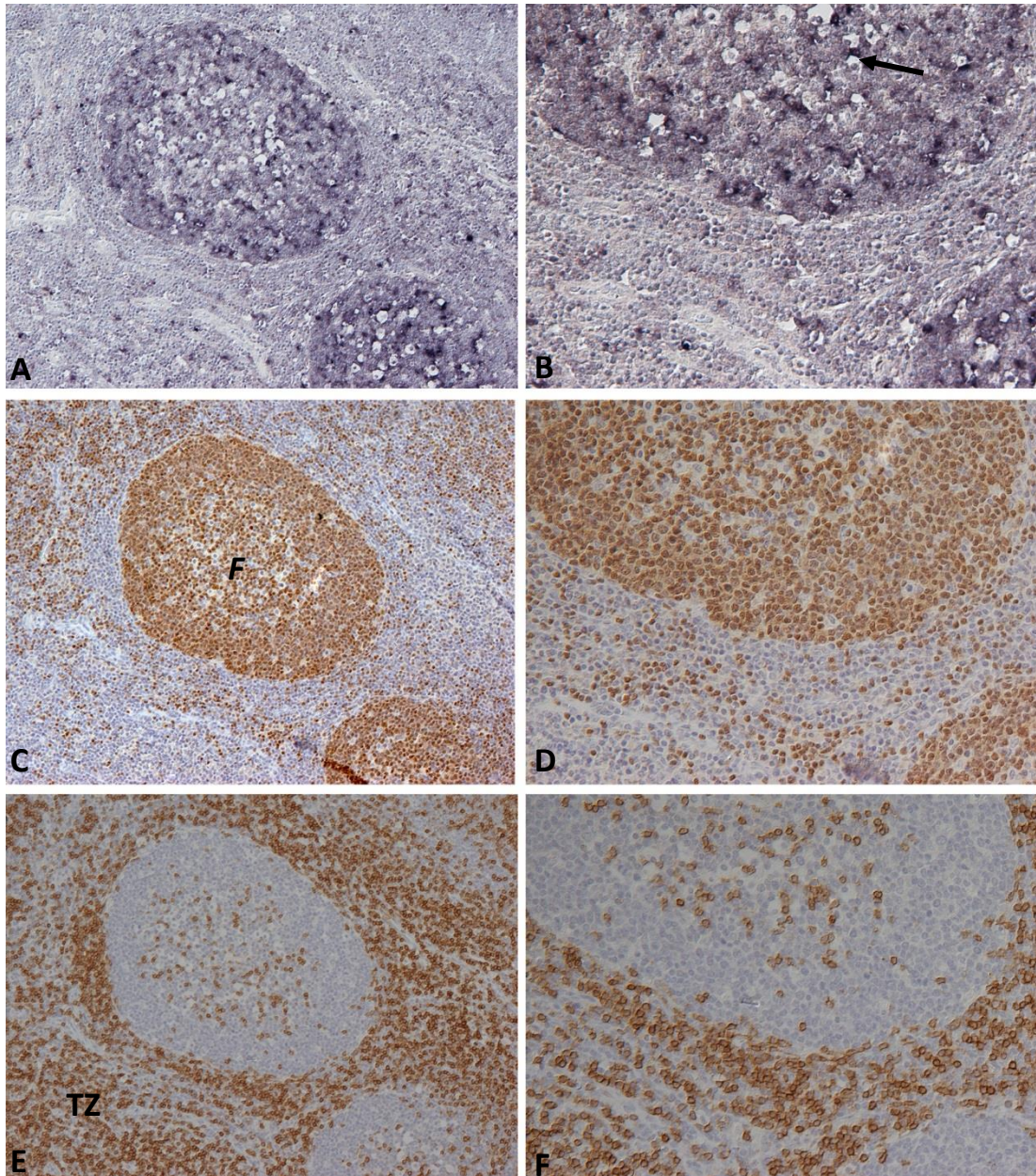


Figure 3-6. RNA-ISH and immunohistochemistry for B or T cells. Serial sections of the mediastinal lymph node of an eight month-old sheep (13L-2592 B) with a viral load of 24 virus copies.

(A) RNA-ISH for Ov2.5, cortical lymphoid follicle, showing an ISH signal in lymphocytes in the follicles and occasional lymphocytes in interfollicular areas (200x). **(B)** Higher magnification of A, showing positive lymphocytes and TBM (arrow) within the follicle (400x). **(C)** IH for Pax5, confirming that B cells (brown) comprise the follicles (Italic F) (100x). **(D)** Higher magnification of C (400x). **(E)** IH for CD3, showing that T cells (brown) are predominantly located outside the follicles in the interfollicular T cell zones (TZ) (200x). **(F)** Higher magnification of E showing few T cells are located in the B follicles (400x). BCIP/NBT (RNA-ISH), DAB (IH), haematoxylin counterstain.

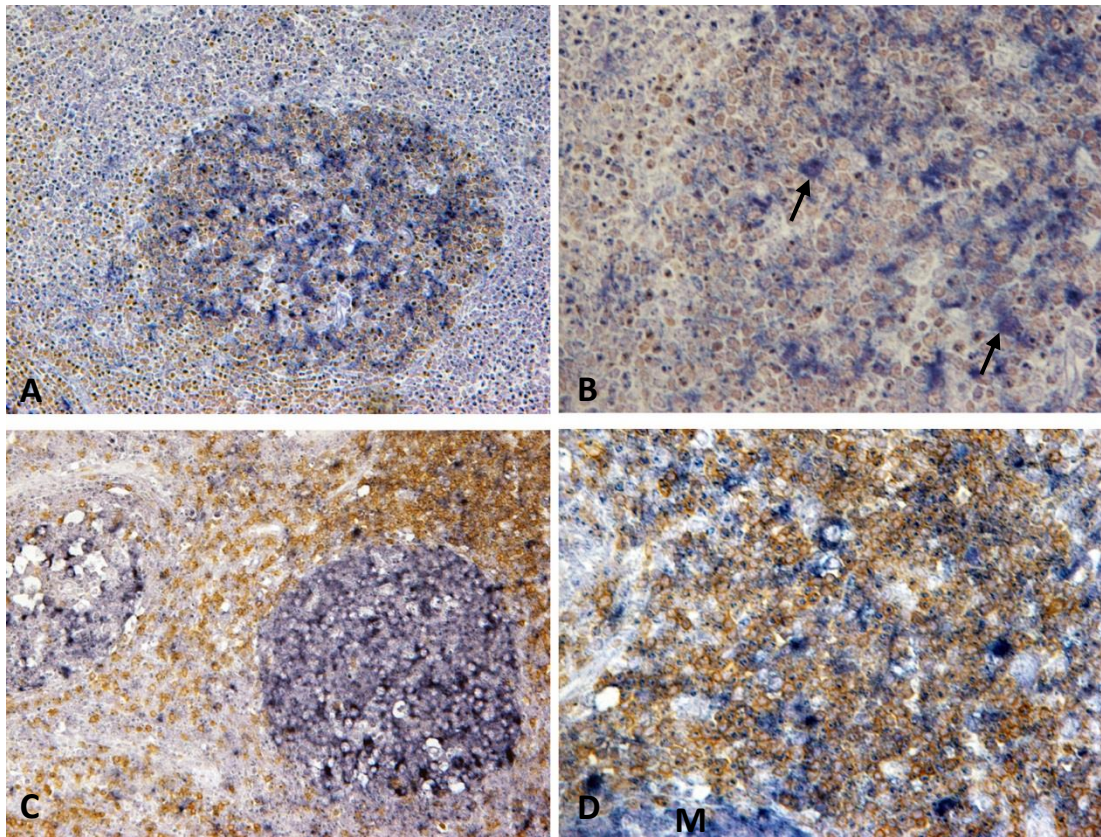


Figure 3-7. RNA-ISH and immunohistochemistry for T and B cells. Mediastinal lymph node of a seven-month old sheep (13L-2592 A) with a viral load of one virus copy.

(A) Double RNA-ISH and Pax5 staining, confirming the co-localisation of cells with viral transcripts and B cells (yellowish brown) (200x). **(B)** Higher magnification of A, confirming that a proportion of PAX5⁺ B cells exhibit a positive RNA-ISH signal (arrows), (400x). **(C)** Double RNA-ISH and CD3 staining, showing that CD3⁺ cells (T cells, brown colour) outside the follicle centres, but otherwise located around the follicles (200x). **(D)** Higher magnification of C, margin of the follicle (M), confirming that that the CD3⁺ T cells outside the follicle centre do not exhibit any ISH signal (400x). BCIP/NBT (RNA-ISH), DAB (IH), haematoxylin counterstain.

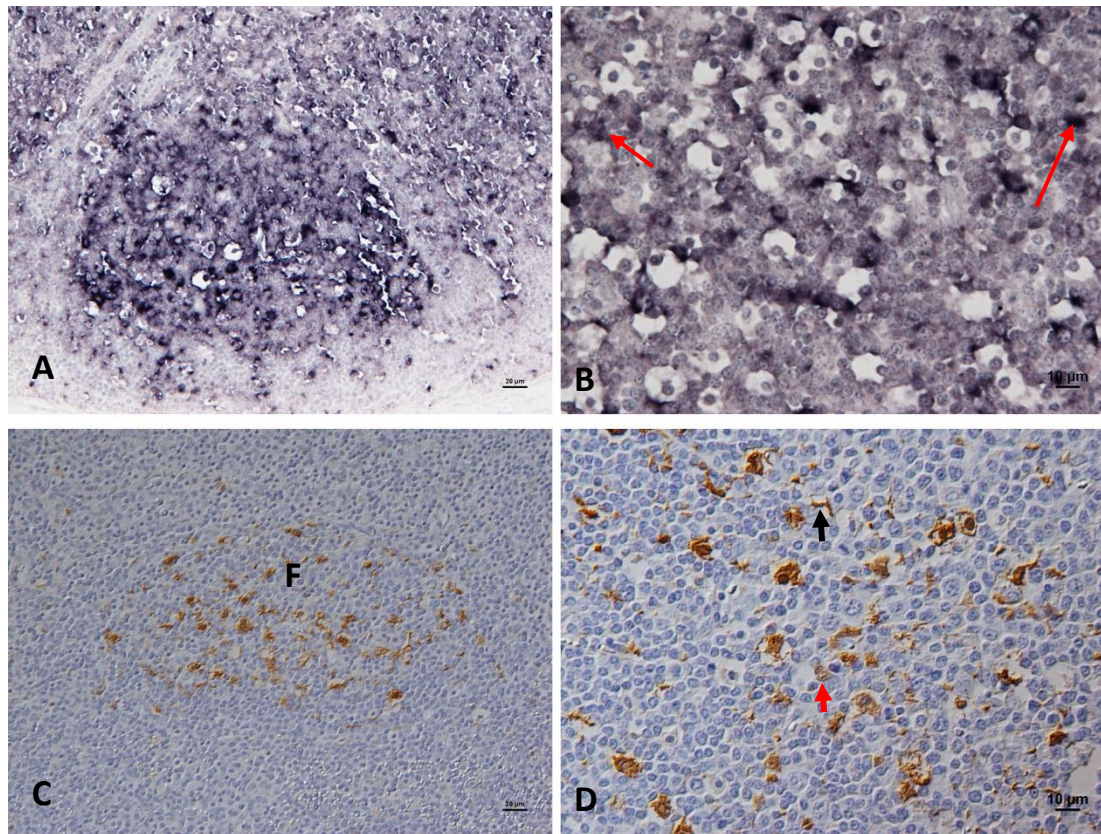


Figure 3-8. RNA-ISH and immunohistochemistry for viral Ov8 antigen. Mediastinal lymph node of a seven month-old sheep (13L-2594 A) with a viral load of one virus copy; see also Figure 3-7.

(A) RNA-ISH for Ov2.5, cortical follicle, a large proportion of leukocytes in the follicle exhibit ISH signal, bar=20µm. **(B)** Higher magnification of A, confirming that both lymphocytes (long arrow) and TBM (short arrow) are positive, bar=10µm. **(C)** IH for viral antigen (brown), virus antigen are expressed within the follicle (F), bar=20µm. **(D)** Higher magnification of C, showing viral antigen expression in either FDCs (arrow) or macrophages (red short arrow). BCIP/NBT (RNA-ISH), DAB (IH), haematoxylin counterstain.

3.1.3.4 Spleen

The spleen was the most frequently PCR-positive organ for vial DNA. Of the eight spleens was positive by both qPCR and RNA-ISH, six were qPCR-positive with a viral DNA load of 24 virus copies as the median number, while seven were positive by RNA-ISH. Similar to the lymph nodes, in the spleen, the viral transcripts (Ov2.5 and ORF65) were mainly seen in follicles, but not in the T cell zones of the white pulp. In secondary follicles, positive cells were mainly found in the mantle zone, but were only sparse in germinal centres (Figure 3-9 A,B). The morphology of positive cells confirmed these as predominantly lymphocytes and occasional TBMs. In addition, a few leukocytes in the red pulp and the vascular endothelial cells were also positive. In some cases, fibroblasts around vessels also showed a positive signal. When consecutive sections were tested for either RNA-ISH and IH for either CD3 or Pax5 markers (Figure 3-9 A-D), and with double RNA-ISH and IH staining it was confirmed that the cells carrying viral transcripts are B cells. viral Ov8 antigen was found to be expressed in a proportion of macrophages and FDCs in the white pulp, in vascular endothelial cells and in lymphocytes (Figure 3-9 E,F).

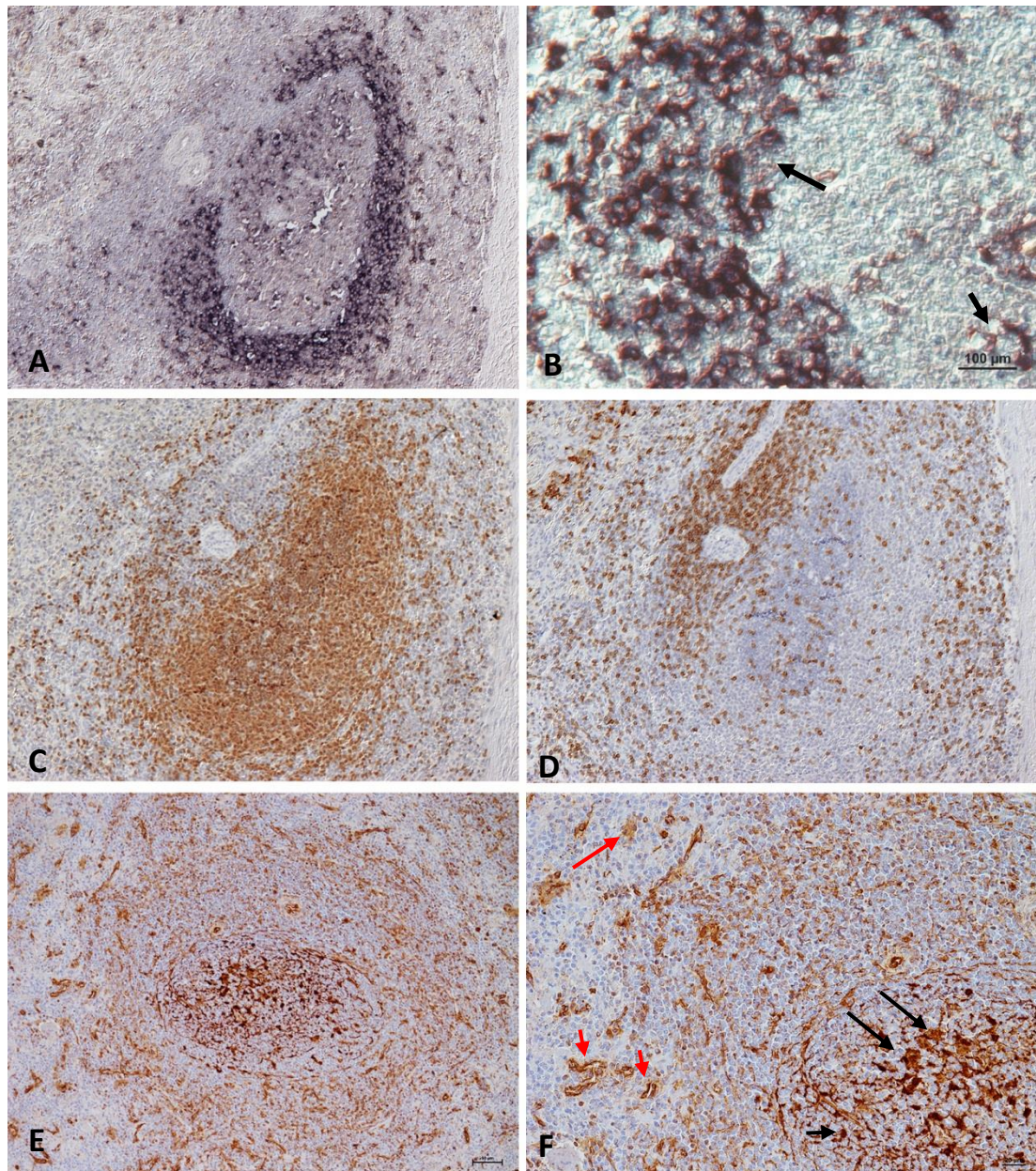


Figure 3-9. RNA-ISH and immunohistochemistry for B or T cells and for viral Ov8 antigen in sheep spleen. Serial sections of spleen of an eight months-old sheep (13L-2592 B) with a viral load of two virus copies (A-D). Spleen of a ten-month-old sheep (13L-4220 E) with a viral load of 32 virus copies (E&F). **(A)** RNA-ISH for ORF65, white pulp, signals are seen in a proportion of cells predominantly at mantle zone of the follicle and in fewer cells in the germinal centre, and also in individual cells in the red pulp (100x). **(B)** Higher magnification of A, mantle zone, showing signals in individual lymphocytes (arrow) and possible TBM (short arrow). **(C)** IH for Pax5, confirming that B cells (brown) comprise the follicle (100x). **(D)** IH for CD3, showing that T cells (brown) are predominantly located outside the follicle and are scarce (200x). **(E)** IH for viral antigen, there overall strong reaction in the white and red pulp, bar=50µm **(F)** Higher magnification of E, showing viral expression in macrophages (and FDC) in follicles (arrows) and in macrophages in red pulp (red arrow), in a few lymphocytes (short black arrow) and in VEC (short red arrows), bar=20µm. BCIP/NBT (RNA-ISH), DAB (IH), haematoxylin counterstain.

3.1.3.5 Peripheral blood leukocytes

Of the five PBL samples taken from sheep, two were tested by both qPCR and RNA-ISH. Viral loads were at five and eight virus copies per two eight-month-old sheep (13L-4883A and 13L-4883B respectively). In both samples, viral transcripts were exhibited in about 40-50 % of cells and with morphology consistent with lymphocytes or monocytes (Figure 3-10 A). viral Ov8 antigen was not detected in PBLs, and also based on the immunohistological staining of consecutive sections for CD3 or Pax5, it was not possible to comment on the lymphocyte subtype that showed positive signals, since both B and T cells were present in similar ratios (about 30 % B cells and 50 % T cells) (Figure 3-10 B,C).

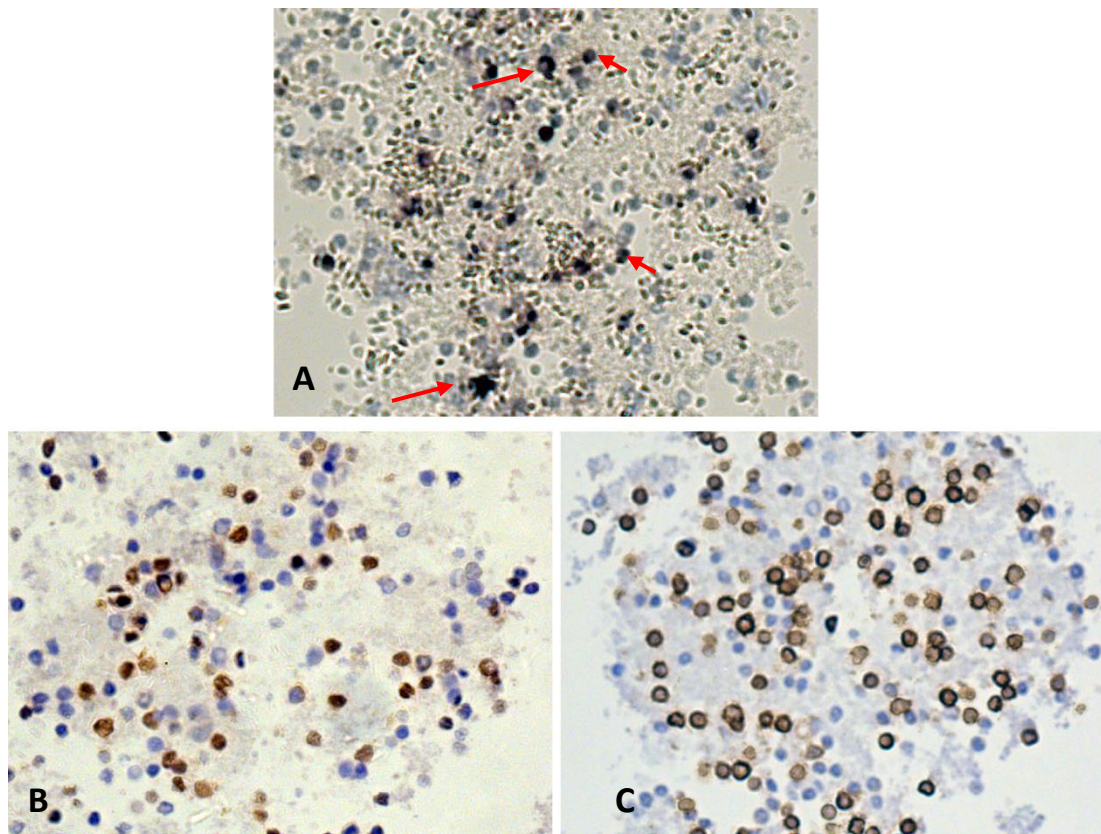


Figure 3-10. RNA-ISH and immunohistology for B or T cells. Pelleted peripheral blood leukocytes of a nine month-old sheep (13L-4883 B) with viral load of five virus copies. **(A)** RNA-ISH for Ov2.5, approximately 5-6 % of the leukocytes show ISH signals that morphologically appear as lymphocytes (short arrows) and monocytes (long arrows) (200x). **(B)** IH for Pax5, the B cells comprise approximately 10-20 % of WBCs (200x). **(C)** IH for CD3, the T cells comprise 30-40 % of WBCs (200x). BCIP/NBT (RNA-ISH), DAB (IH), haematoxylin counterstain.

3.1.3.6 Uterus and placenta

The uterus was tested in two sheep, a three-month and a 3.5-year-old sheep (case no. S13-1516), Neither viral DNA nor viral transcripts or viral Ov8 antigen was detected in these samples. However second animal was tested by qPCR only, and had a high viral DNA titres in lung with viral load of 61 copies (see Appendix, Table 1).

From a gravid ewe (S13-1453.3, aged 3.5 year, see Appendix, Table 1), maternal placentome and foetal placenta (S13-1453.4, crown-rump-length 17.5 cm) were tested. The qPCR identified two virus copies in the ewe's placentome, and the viral transcripts were found as weak signals in part of the maternal placental epithelium (Figure 3-11 A), while the viral Ov8 antigen was detected in a proportion of stromal fibroblasts, but not in epithelial cells (Figure 3-11 B). In the foetus, the placenta was PCR-positive (three virus copies), but was negative for the RNA-ISH or viral antigen.

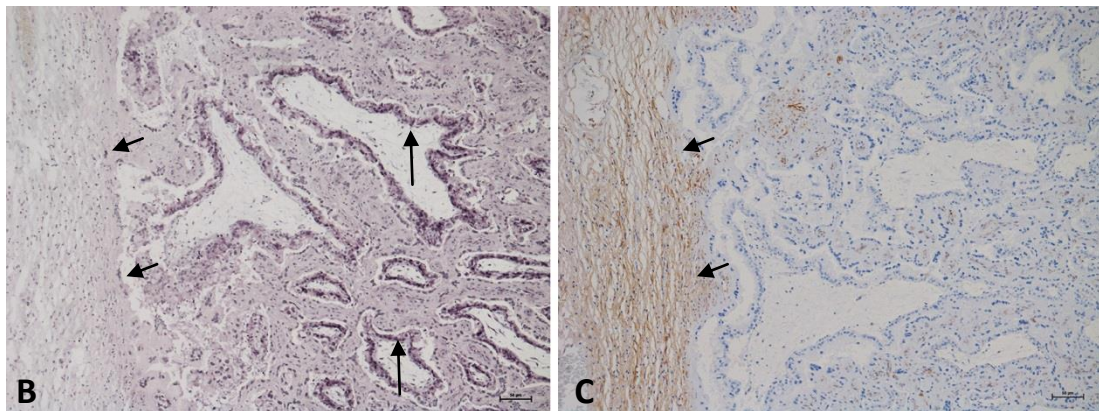


Figure 3-11. RNA-ISH and immunohistochemistry for viral Ov8 antigen in the sheep placentome. A 14 year-old ewe (S13-1453.3) with a viral load of three virus copies.

(A) RNA-ISH for Ov2.5, there are relatively strong ISH signals in glandular epithelial cells (long arrows) and stromal fibroblasts (short arrow), bar= 50µm. (B) IH for viral antigen, the viral antigen is weakly expressed in stromal fibroblasts (arrows), bar= 20µm. BCIP/NBT (RNA-ISH), DAB (IH), haematoxylin counterstain.

3.1.3.7 Controls

Negative control sections served to check the specificity of the probes and reliability of the results obtained with the antisense probes. Testing of sense probes in lung, lymph node and spleen sections, did not yield any signal (Figure 3-12 A-D), however, sometimes ORF65 sense probe had a diffuse background staining on some sections. In addition, antisense probes were applied to sections of a dog lung and lymph node, and did not yield any signal (Figure 3-12 E,F).

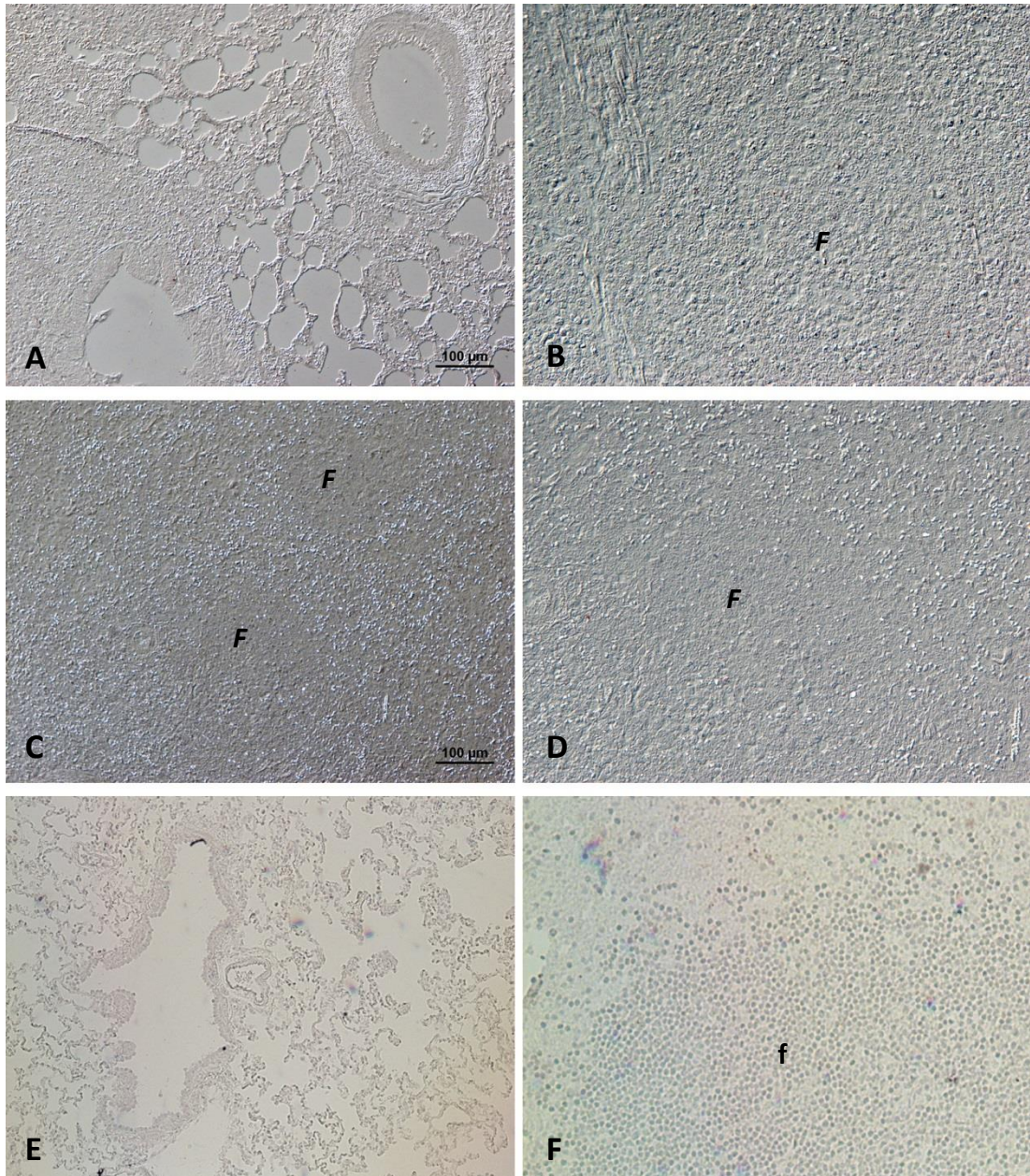


Figure 3-12. RNA-ISH negative controls in sheep and dog.

(A) RNA-ISH with Ov2.5 sense, lung of an 11-month-old sheep (13L-2592A), with a viral load of one virus copies. There are no any definitive ISH signals. **(B)** RNA-ISH with Ov2.5 sense, mediastinal lymph node of the same sheep as A, with a viral load of one virus copies, cortical follicle (*italic F*), there are no ISH signals, (200x). **(C)** RNA-ISH with Ov2.5 sense, spleen (13 L-2592B) with a viral load of two virus copies, there are no any signal in the red or the white pulps (*italic F*), (100x). **(D)** Higher magnification of C, showing the follicle (*italic F*) with no ISH signals (200x). **(E, F)** RNA-ISH with Ov2.5, lung in a dog (13L-5017B) lung (E) and lymph node (F). Neither of the tissues exhibit any ISH signals). BCIP/NBT, haematoxylin counterstain.

3.2 Detection of OvHV-2 in cows without MCF

3.2.1 Detection and quantification of OvHV-2 DNA loads by qPCR

The second group of animals tested for the presence and amount of OvHV-2 were cattle without MCF. A total of 49 cows were examined by qPCR, ranging in age from a six month-old foetus to mature cattle of 12 year-old. In 40 animals (including seven foetuses), a variety of organs were tested (at least lung, mediastinal lymph node and spleen). In the remaining nine cattle, only PBLs were tested. In the 40 cattle of which organs were tested, 26 (67 %) animals were positive for viral DNA (Table 3-4). Of the nine cattle where the PBL were tested, six (66 %) were positive and thereby found to be viraemic.

The amount of OvHV-2 DNA varied significantly among individual animals and even in different tissues within the same animal (between 1 and 6500 copies per 100 ng genomic DNA). Among tissues, the most frequently positive ones were tongue (45 %), spleen (38 %), lung (36 %), and then mediastinal lymph node (30 %), all organs and tissues tested are listed in Table 3-5. The amount of the virus was also highly variable in tissues in a given animal, and in the majority of animals, viral loads were low, between 1 and 417 virus copies, but in two cases, a stillborn foetus (described below) and a one-year old animal (13L-2591), the viral loads in organs were relatively high, with 6500, 5800, 3900, 3600 virus copies in the submandibular lymph node, lung, tongue and spleen respectively. In nine animals, aged one week to eight month, only four were positive, and in sporadic organs and with loads below five virus copies, but in a seven-month-old calf (case S13-1419), all tested organs except the thymus were positive, with viral loads ranging from 12 to as high as 417 (spleen) copies. Generally, in positive cows, usually at least 2-3 organs or more were positive for the virus presence. In the viraemic cattle (PCR-positive PBLs), viral loads were between 1 and 96 copies in the PBL. However only nine samples were tested, blood was among the most frequently PCR-positive tissue compared to other tissues. The amount of positive samples and OvHV-2 DNA loads in different cattle tissues are listed in Table 3-5.

Two of the four gravid cows were PCR-positive, but their foetuses were negative. The third cow was negative, but its foetus was positive. The fourth cow and its foetus were negative. In total, seven foetuses were tested, three (42 %) of

which were positive for viral DNA. In two, the viral loads were very low as ranged between 1-4 virus copies, and they were only positive in mediastinal lymph nodes and spleen. While in the third positive, a stillborn foetus (case no. S13-1438), all tested organs, i.e. lung, mediastinal lymph node, spleen and thymus were positive and the lymph node exhibited the highest viral load (6300 copies); in the other three organs loads were 5-25 copies. Interestingly, the thymus of this animal was the only thymus of the 15 tested that was positive.

Table 3-4. Results of the OvHV-2 qPCR and viral DNA loads in organs from cows without MCF. Cases ordered in ascending age order.

Case number/age	Results	Organs									
		L	Ln	S	To	Th	Na	Tu	U	M	Other
S13-1350/ Fo, 6 mo	-	-	-	-		-					
S13-1360/Fo, 7 mo	+	-	1	-		-					
S13-1355/ Mt, 5 y	+	-	1	-			2		56		_ ^[6]
S13-1355/ Fo, 8 mo	+	-	3	4		-					
S13-1483/ Mt, 5 y	+	10		5	16		49		0		
S13-1483/ Fo.8 mo	-	-	-	-		-					
S13-1452/ Mt, 12 y	-	-	-	-			-		-		
S13-1452/Fo, 8 mo	-	-	-	-		-					_ ^[6]
S13-1360/Mt, 8 y	+	-	-	-	1		3	-	1		1 ^[6]
S13-1448/ Fo, 8 mo	-	-	-	-		-					
S13-1438/Stilb., 9m	+	6	6300	25		5					
S13-1418/ 1 we	-	-	-	-	-	-	-	-			
S13-1329/ 10 d	+	-	-	-	-	-	-	4			
S13-1377/ 5 we	+	-	-	-	-	-	-	1			
S13-1419/ 7 we	+	156	129	417	12	-	12	50			
S13-1373/ 7 we	+	1	-	1	1						
S13-1434/ 8 we	-	-	-	-	-	-	-	-	-		
S13-1460/ 13 we	-	-	-	-	-	-	-	-			
S13-1459/ 18 we	+		3	-		-					
S13-1352/ 8 mo	-	-	-	-	-		-	-			
13L-2591/ 1 y	+	5800		3600	3900						6500 ^[1]
13L-2599/ 19 mo	+	5	-								2 ^[2]
13L-2596/ 20 mo	+	14	8	9							
13L-2597/ 20 mo	+	7		3							2 ^[2]
13L-2598/ 20 mo	+	-		5							0 ^[2]
13L-4213A,B/ 2 y	+	-	6	4	-					16	
13L-4213D/ 2 y	+	1	-	-	4						
13L-4214A,B/ 2 y	-	-	-	-	-						
13L-4214 C,D/ 2 y	+	-	1	1	-					2	
13L-4215A,B/ 2 y	+	8	9		2						3 ^[3] , 1 ^[4]
13L-4215C,D/ 2 y	+	1	-	1	-					-	
13L-4216/ 2 y	-	-	-	-	-					-	- ^[4]

13L-4216/ 2 y	+			5						2 ^[3]
13L-4217/ 2 y	-	-	-	-	-					
13L-4221/ 2 y	+									1 ^[5]
13L-2600A,B/ 2.5 y	+	13	-	-	8					
13L-2600C,D/ 2.5 y	+	3	-	-	8					
S13-1515/ 3 y	+	-	-	-	6	-	-	-	-	
S13-1425/ 4 y	-	-	-	-	-	-	-	-	-	

Abbreviations: mo: month-old; we: week-old; y: year-old; Fo: foetus; Mt: mother; [-]: negative; L; lung; LN: mediastinal lymph node; S: spleen; M: muzzle; Na: nasopharynx; Stillb: stillborn; T: tongue; Tu: turbinate; U: uterus; Th: thymus; [1]: submandibular lymph node; [2]: bronchial lymph node; [3]: trachea; [4]: aorta; [5]: mammary lymph node; [6]: placenta; "-": negative; empty boxes: not examined.

Table 3-5. Amount of positive samples and OvHV-2 DNA loads in tissues and PBL samples of cow without MCF.

Sample	Tested ^a		OvHV-2 DNA copies		
	Total	Pos (%)	Min ^b	Max ^c	Med ^d
Lung	36	13 (36 %)	1	3600	5
Mediastinal LN ^[1]	33	10 (30 %)	1	5800	7.5
Spleen	33	13 (39 %)	1	129	14
Tongue	23	10 (43 %)	1	3900	6
Thymus	15	1 (6 %)	2	5	4
Nasopharynx	13	4 (30 %)	1	49	25.5
Turbinate	9	3 (33 %)	1	50	12.5
Uterus	6	3 (50 %)	1	56	28.5
Muzzle	3	2 (66 %)	2	16	9
Placenta	3	1 (33 %)	1	1	2
Trachea	2	2	2	3	2.5
Bronchial LN	3	1	2		
Aorta	2	1	1		
Submandibular LN	1	1	6500		
Mammary LN	1	1	1		
Cerebellum	1	1	2		
PBL ^[2]	9	6 (66 %)	1	86	5

a. Samples tested, **total** is the number of all samples taken for that particular type of specimen. **Pos:** is number and percentage of samples tested positive. Table arranged according to the most frequently tested tissues. **b.** Minimum number of virus copies detected in the particular specimens. **c.** Maximum number of virus copies detected in the particular specimens. **d.** Median number of the virus copies. [1] LN refers to lymph node. [2] PBL: peripheral blood leukocytes.

3.2.2 Identification of cells harbouring OvHV-2 mRNA and antigen in cows without MCF

Cattle without MCF were also tested by RNA-ISH and immunohistology in tissues, to demonstrate viral Ov2.5 and ORF65 transcripts and Ov8 antigen, respectively, in an attempt to identify the cells that harbour the virus. In addition, a double staining (RNA-ISH for viral transcripts in combination with immunohistology to highlight B cells [Pax5-positive] and T cells [CD3-positive]) was performed on lung, mediastinal lymph node and spleen of selected cases to identify the lymphocyte subtype positive for viral Ov2.5 and ORF65 transcripts. The results for each case are provided in detail in Table 2a in the Appendix. The presence of viral transcripts was represented by dark blue cytoplasmic signals (Figure 3-13 A,B). Generally, the results obtained with both probes (Ov2.5 and ORF65) were similar with regards to the cellular range and signal intensity (Figure 3-16 A-D). Of the 50 cattle examined by either qPCR, RNA-ISH (and a selection by IH), 44 animals were tested by RNA-ISH, 41 were tested positive with both qPCR/RNA-ISH. One foetal calf was only tested by RNA-ISH.

In seven foetuses tested by both PCR/RNA-ISH, two were negative by both techniques, two were PCR-negative but ISH-positive (one of which additionally tested for viral Ov8 antigen and was positive), and the remaining three were positive by both tests (one of which additionally tested for viral Ov8 antigen and was positive) in at least one tissue (Table 3-5). Except for the stillborn foetus that had relatively high virus loads and exhibited viral transcripts in all organs and viral Ov8 antigen in the mediastinal lymph node, the positive foetuses exhibited very low copy numbers and were sporadically positive in the RNA-ISH. An additional foetus aged nine months (H04-981 G) was only tested by RNA-ISH and immunohistology for viral antigen, with negative results for both tests. Of the remaining 34 cattle (excluding those of which only PBL were tested), 31 cattle (91 %) were positive by RNA-ISH, 24 by qPCR and only three animals were negative in both tests. Overall, more tissues were RNA ISH-positive than PCR-positive. The results indicate that infection occurs during foetal development or at an early age (one week) as detected by RNA-ISH, but the virus becomes more readily detectibly by qPCR over one year age. Results for each animal is provided in Table 2a, b in the Appendix.

An attempt was made to find a relationship between qPCR, RNA-ISH and immunohistology results. However, in general, despite the high variability of virus loads, the RNA-ISH signals were rather consistent in their intensity, but sometimes varied in the amount of positive cells. In contrast, the number of viral antigen-positive cells was positively correlated with the virus load. Immunohistology for viral Ov8 antigen was performed in eight cattle (five adult and two foetuses), the relation between results of RNA-ISH and viral IH was quite variable, as in a mother and foetus (case no. S13-1483), these two tests were not always positive together (Table 3-3), even with a stillborn and 10-day-old calf the PCR/ISH/IH were not consistently related. While in the three animals (from seven-week, one and two-year-old; case no. S13-1329, S13-1419, 13L-2591 respectively) all the three tests results were consistently related, when most viral DNA was detected in most of these animals' organs. In addition, the cell types positive by RNA-ISH and viral IH were similar and sharing certain cell types, however the viral IH, was most frequently detected in specific cell types. Animal test results, positive cell types and correlation between the qPCR and RNA-ISH and viral IH are shown in Table 3-6.

Although the qPCR results were very variable through all ages, the RNA-ISH (and viral IH) results were more consistent after seven month age and especially after one-year-old, most of tested organs were positive by either RNA-ISH/viral IH. The correlation between the qPCR and RNA-ISH is shown in Table 3-6. In terms of specific correlation between ISH-positive organs within individual animals, there was not any specific relationship, however, generally in animals the lymph node and spleen, the RNA-ISH and viral IH results were positively related.

Taken together, the qPCR and RNA-ISH results confirm that a proportion of cattle without-MCF are infected whilst are intrauterine, and the viral DNA loads increased from approximately one year (except for few cases; a stillborn and seven month-old lamb, case no. S13-1438 and S13-141 respectively). Also the viral DNA loads become more readily detectable by qPCR at about 18 months of age and more cells have will be positive for viral transcripts. Then as the animal gets older (after 5 to 12 years), generally the viral DNA loads tend to decline and the number of ISH-positive organs will be less frequent or the animal will be negative (as in the 12 year old cow).

Table 3-6. Correlation between OvHV-2 DNA loads and the presence of viral mRNA and antigen in cattle without MCF tested positive with either method.

Case and age	Organs	Results		
		qPCR	RNA-ISH	Viral IH
S13-1350 Aborted foetus 6 mo	Lung	-	-	ND
	Thymus	-	-	ND
	Spleen	-	-	ND
	Med.LN	-	-	ND
S13-1448 Stillborn foetus 8 mo	Lung	-	Some capillaries VEC, REC	ND
	Thymus	-	-	ND
	Spleen	-	VEC of capillaries	ND
	Med LN	-	-	ND
S13-1355 Mother, 5 y	Placenta	-	Weak in stromal cells	ND
	Uterus	56	Glandular EpC, VEC, SMC	ND
S13-1355 Foetus 8 mo	Lung	-	Individual type II AlvC; Alv Mph	ND
	Thymus	-	few cells (LCs?) in medulla	ND
	Spleen	4	Random cells in red pulp	ND
	Med LN	3	Few Mph, LC in cortex, around follicles	ND
S13-1483 Mother 5 y	Lung	10	-	Individual interstitial fibroblasts and Mph
	Spleen	5	-	FDC, apoptotic/ autolytic cells
	Conchae	-	-	-
	Tongue	16	-	-
	Nasoph.	49	-	-
	Uterus	-	Majority of EpC	Stromal fibroblasts
S13-1483 Foetus 8 mo	Lung	-	-	-
	Thymus	-	-	-
	Spleen	-	-	-
	Med LN	-	Individual cells (LC?, Mph?)	Some FDC, and Mph outside follicles
S13-1452 Mother 12 y	Lung	-	-	ND
	Spleen	-	-	ND
	Med LN	-	-	ND
	Uterus	-	-	ND
S13-1452 Foetus 8 mo	Lung	-	-	ND
	Thymus	-	-	ND
	Spleen	-	-	ND
	Med LN	-	-	ND
	Placenta	-	-	ND
S13-1360 8 y	Lung	-	Individual cells in BALT (LC)	ND
	Spleen	-	Numerous (LC) in mantle zones	ND
	Med LN	-	Strong signals in LC at follicular periphery	ND
	Buccal mucosa	-	Proportion of infiltrating LC	ND
	Tongue	1	small patchy area EpC	ND
	Nasoph.	3	-	ND
	Uterus	1	Proportion of glandular EpC, patches of surface EpC	ND
	Placenta	1	Trophoblasts, stromal cells	ND

S13-1438 Stillborn calf 9 mo	Lung	6	Focal bronchiolar EpC, VEC	-
	Thymus	5	Few LC, Mph	-
	Spleen	25	Sporadic cells in white pulp, few LC in follicles	Individual Mph in red pulp
	Med LN	6300	Strong sporadic LC in cortex/medulla, few LC in follicles, individual Mph in medulla	-
S13-1418 1 we	Lung	-	-	ND
	Thymus	-	-	ND
	Spleen	-	-	ND
	Med LN	-	-	ND
	Tongue	-	Basal EpC, papillary EpC	ND
	Nasoph.	-	Some glandular EpC	ND
	Nasal epithelium	-	Basal EpC, ORF65 in inflamed glandular ducts too	ND
S13-1329 10 d	Spleen	-	Occasional cells	-
	Thymus	-	-	-
	Med LN	-	LC in follicles, some LC in medulla	Individual FDC/Mph in follicles
	Nasoph.	-	-	Weak stromal fibroblasts
	Tongue	-	Strong in EpC in taste buds	Weak stromal fibroblasts
	Lung	-	-	-
	Nares	4	Few glandular/surface EpC	Weak stromal fibroblasts
S13-1377 Calf 5 we	Lung	-	-	ND
	Thymus	-	-	ND
	Spleen	-	-	ND
	Med LN	-	-	ND
	Tongue	-	-	ND
	Turbinate	1	Many glandular EpC, VEC, subepithelial fibroblasts	ND
	Nasoph.	-	Several cells in a follicle-like LC aggregates	ND
S13-1419 7 we	Lung	156	REC (bronchioles)	Few PIM
	Thymus	-	-	-
	Spleen	417	Weak occasional cells	Weak FDC/Mph in follicles, scattered Mph in red pulp
	Med LN	129	Few cells in follicles	Weak FDC/Mph in follicles
	Tongue	12	Variable basal EpC	Weak in stromal fibroblasts, VEC
	Nasoph.	12	Most of mucosal EpC	See tongue
	Turbinate	50	Basal epidermal, hair follicle EpC	See tongue
S13-1373 7 we	Lung	1	Few cells in BALT, few REC	ND
	Spleen	1	Few cells in follicles (Mph?)	ND
	Thymus	-	-	ND
	Med LN	-	Few cells in and outside of follicles (Mph?)	ND
	Tongue	1	Patches of EpC in taste buds	ND
S13-1434 calf 8 we	Lung	-	Scattered type II AlvC	ND
	Thymus	-	-	ND
	Spleen	-	Cells in follicles, red pulp	ND

	Med LN	-	Some cells in cortex, medull	ND
	Tongue	-	Patches (3-4 eEpC) in taste buds	ND
	Uterus	-	Glandular and surface EpC	ND
	Nasoph.	-	Patches of positive EpC	ND
S13-1460 3 mo	Thymus	-	-	ND
	Spleen	-	-	ND
	Med LN	-	-	ND
	Lung	-	-	ND
	Tongue	-	Patches of EpC in taste buds	ND
	Nasoph.	-	-	ND
	Nares	-	-	ND
S13-1459 Calf 4 mo	Thymus	-	-	ND
	Spleen	-	Sporadic cells in follicles and medulla	ND
	Med LN	3	Cells in white pulp	ND
S13-1352 Calf 8 mo	Tongue	-	Patches of basal EpC	ND
	Nasoph.	-	Few infiltrating, few glandular EpC	ND
	Lung	-	Proportion of REC	ND
	Med LN	-	Many cells in cortex, paracortex, VEC	ND
	Spleen	-	Cells in follicles and red pulp	ND
	Turbinates	-	Surface and glandular EpC, chondrocytes, VEC	ND
13L-2591 12 mo	Spleen	3600	Mantle zone LC, outside the follicle, some in red pulp	FDC, Mph in red pulp, VEC
	Submandibula LN	6500	LC in outer mantle zone	FDC, Mph in sinus and medulla, VEC
	Lung	5800	Few arteries VEC	ND
	Tongue	3900	Most basal EpC, gland EpC, VEC	ND
13L-4893 18 mo	DRG	-	-	ND
	Cerebellum	2	Neurons and Purkinje cells	ND
	Hippocampus	-	Neurons	ND
	Pituitary gland	-	-	ND
13L-2599 19 mo	Lung	5	Individual leukocytes; VEC; individual type II AlvC, AlvC Mph; Few LC, Mph in BALT	ND
	Med LN	-	Occasional mantle zone LC, sinus Mph	ND
	Bronchial LN	2	LC in outer mantle zone, few Mph	ND
13L-2596 20 mo	Spleen	9	Cannot be assessed	FDC
	Lung	8	Occasional type II AlvC	ND
	Med LN	14	Mantle zone LC, sinus Mph	ND
13L-2597 20 mo	Lung	7	REC, type II AlvC, VEC	ND
	Spleen	3	-	ND
	Bronchial LN	2	-	ND
13L-2598 20 mo	Lung	0	REC, VEC, some LC in BALT, type II AlvC?, arterial SMC	ND
	Spleen	5	LC in mantle zone, in red pulp	ND
	Bronchial LN	-	Some LC in mantle zone	ND
13L-4213 A,B,C 2 y	Tongue	-	Patchy basal EpC, salivary gland	ND
	Muzzle	16	Few Mph(?) in dermis, most fibrocytes, salivary gland EpC, VEC in some vessels	ND
	Med LN	6	Few disseminate LC	ND

	Spleen	4	Few LC in mantle zone , few Mph in the centre	ND
13L-4213 D,F 2 y	Lung	1	-	ND
	Tongue	4	-	ND
	Med LN	-	-	ND
	Spleen	-	Few LC in mantle zone , few Mph in the centre	ND
13L-4214 A,B 2 y	Lung	-	Few Mph and LC in BALT	ND
	Tongue	-	EpC; some LC and Mph	ND
	Med LN	-	Few LC in periphery , few Mph in the centre	ND
	Spleen	-	-	ND
13L-4215 A,B 2 y	Lung	8	Occasional arterial VEC, AlvC	Weak in some arterial VEC, occasional cells in interstitium, AlvC
	Med LN	9	Few LC in periphery , few Mph in the centre	Weak in few FDC
	Tongue	2	Patches of basal EpC	ND
	Trachea	3	REC, VEC, infiltrating LC, gland EpC	ND
	Aorta	1	VEC of vasa vasorum and media fibrocytes	ND
13L-4215 C,D 2 y	Lung	1	Several LC in BALT	ND
	Med LN	-	Few LC in periphery , Mph in centre of follicle	ND
	Tongue	-	Weak mid/basal EpC	ND
13L-4216 A,B,C 2 y	Lung	-	Some LC in BALT, around bronchiolar EpC	ND
	Med LN	-	Few LC in periphery , Mph in centre of follicle	ND
	Spleen	-	-	ND
	Aorta	-	-	ND
	Muzzle	-	-	ND
	Tongue	-	-	ND
13L-4216 D,E 2 y	Spleen	5	Few cells in mantle zones	ND
	Trachea	2	Numerous subepithelial LC	ND
13L-4217 2 y	Lung	-	Some LC, few arteries VEC	ND
	Tongue	-	Few LC in follicles	ND
	Med LN	-	Few LC (and Mph?) in follicles	ND
	Spleen	-	Several LC in mantle zone	ND
13L-4221 , 2 y	Mammary LN	1	Few LC in periphery , Mph in centre of follicle	ND
13L-2600 A,B 2.5 y	Lung	13	Arterial VEC and SMC, type II AlvC, LC in BALT	ND
	Med LN	-	Few Mph in follicle centres and LC outside	ND
	Tongue	-	Superficial EpC in mucosa	ND
	Spleen	-	Few Mph in follicles	ND
13L-2600 C,D 2.5 y	Lung	3	Arterial SMC, bronchiolar EpC, some LC	ND
	Tongue	8	Rare LC in subepithelial infiltrates	ND
	Spleen	-	Few Mph in follicles	ND
S13-1425	Lung	-	-	ND

4 y	Med LN	-	-	ND
	Spleen	-	-	ND
	Tongue	-	Basal EpC, subepithelial fibrocytes	ND
	Nasopharynx	-	Few infiltrating LC	ND
	Turbinate	-	Several LC, VEC, in glandular EpC	ND

Abbreviations: ND: not done; qPCR: virus copy numbers; RNA-ISH: either Ov2.5 or ORF65 or both; IH: immunohistology, monoclonal antibodies for OvHV-2 Ov8 protein; mo: month-old; we: week-old; y: year-old; AlvC: alveolar epithelial cells; Mph: macrophages; BALT: bronchus associated lymphoid tissue; SMC: smooth muscle cells; LC: lymphocyte; VEC: vascular endothelial cells; FDC: follicular dendritic cells; EpC: Epithelial cells; “-”: negative; “+”: positive.

3.2.2.1 Lung

From the 28 lung samples examined by RNA-ISH, in 21 the viral transcripts were detected, while only 12 of those were PCR-positive. In the RNA-ISH positive lungs, the viral loads were greatly variable and generally low, with a median viral load of 7.5 virus copies, except for one case (case no. 13L-2591, one year-old), where 5800 virus copies per 100 ng genomic DNA were found. In addition, in that given animal all other organ (tongue, submandibular lymph node, spleen) have similar virus loads and were all positive for RNA-ISH and for viral Ov8 antigen (in spleen and lymph node). The signal intensity was variable, regardless of the amount of virus copies. Generally, viral transcripts were detected in a variable proportion of type II pneumocytes, bronchiolar respiratory epithelial cells, vascular endothelial cells, a proportion of lymphocytes in the BALT and occasional individual lymphocytes or macrophages in the interstitium (Figure 3-13 A-C, 3-14 A,B). Viral Ov8 antigen was detected in endothelial cells in some small arteries, in bronchiolar epithelial cells and in type II pneumocytes (Figure 3-13 D-F). With the combined RNA-ISH and immunohistology, However, B cells were not detected in the BALT (probably due to digestion of Pax5 antigen with the RNA-ISH processing), but a few T cells were detected around the bronchioles and in the alveoli that were negative for viral transcripts (Figure 3-14 A-D), (see Appendix, Table 2).

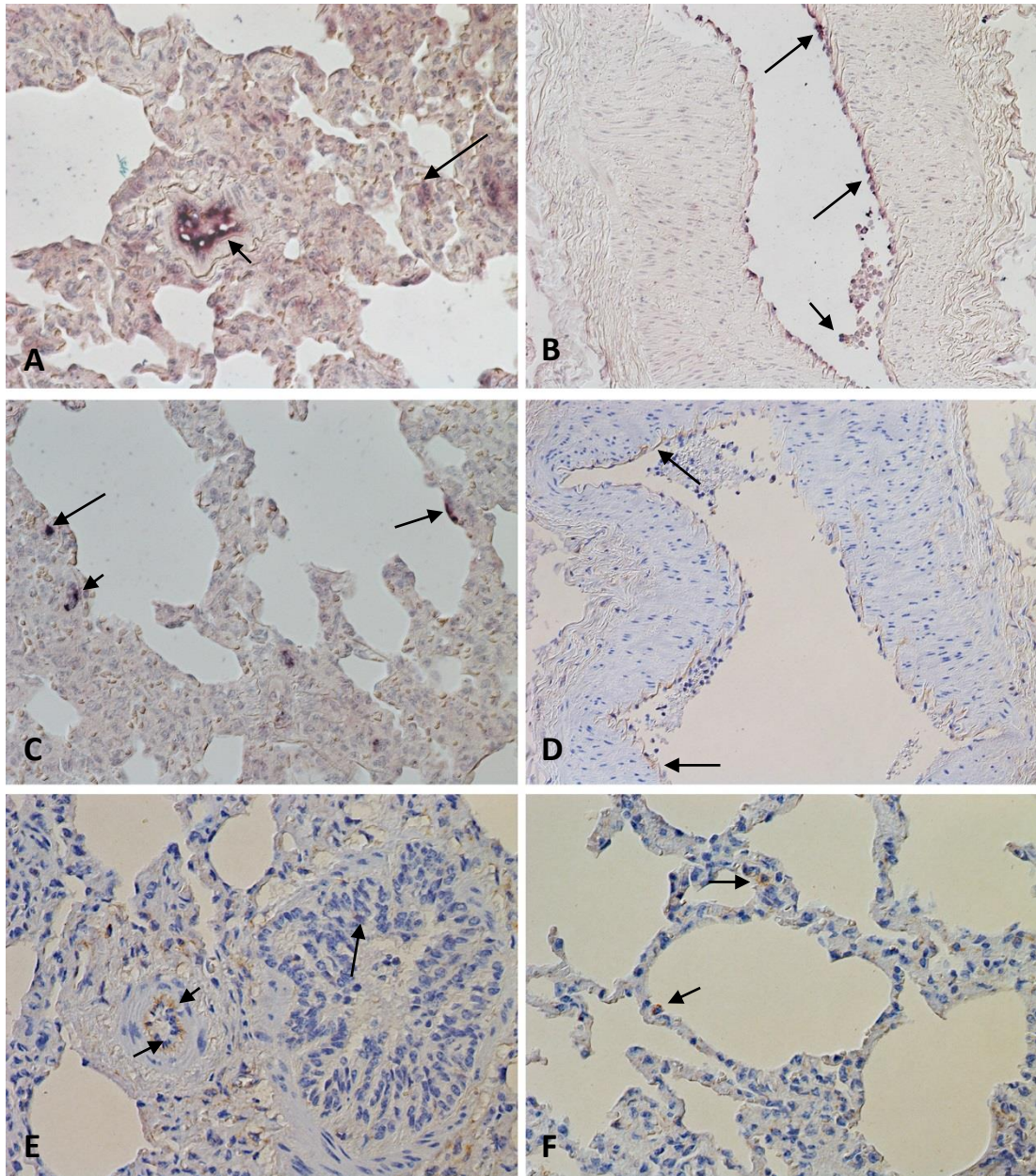


Figure 3-13. RNA-ISH and immunohistochemistry for viral Ov8 antigen. Lung of a two year-old cow without MCF (13L-4215 A) with a viral DNA load of eight virus copies.

(A) RNA-ISH for Ov2.5 (dark purple), strong signals in arteriolar endothelial cells (short arrow) and weaker signals in other cells between the alveoli cells (long arrow), (200x). **(B)** RNA-ISH for Ov2.5, signals in most of endothelial cells in the arteriole (long arrows) and in the circulating leukocytes in the vessel lumen (short arrow), (200x). **(C)** RNA-ISH for Ov2.5, alveoli, there are signals in individual pneumocytes (arrows) and a possible alveolar macrophage (short arrow), (400). **(D)** IH for viral antigen (brown), weak reaction in arteriolar endothelial cells (arrows), (200x). **(E)** The viral antigen is expressed in VEC (short arrows) and very weakly in bronchiolar epithelial cells (long arrow), (200x). **(F)** Higher magnification of alveoli, virus antigen is seen in sporadic individual type II pneumocytes (arrows), (400). BCIP/NBT (RNA-ISH), DAB (IH), haematoxylin counterstain.

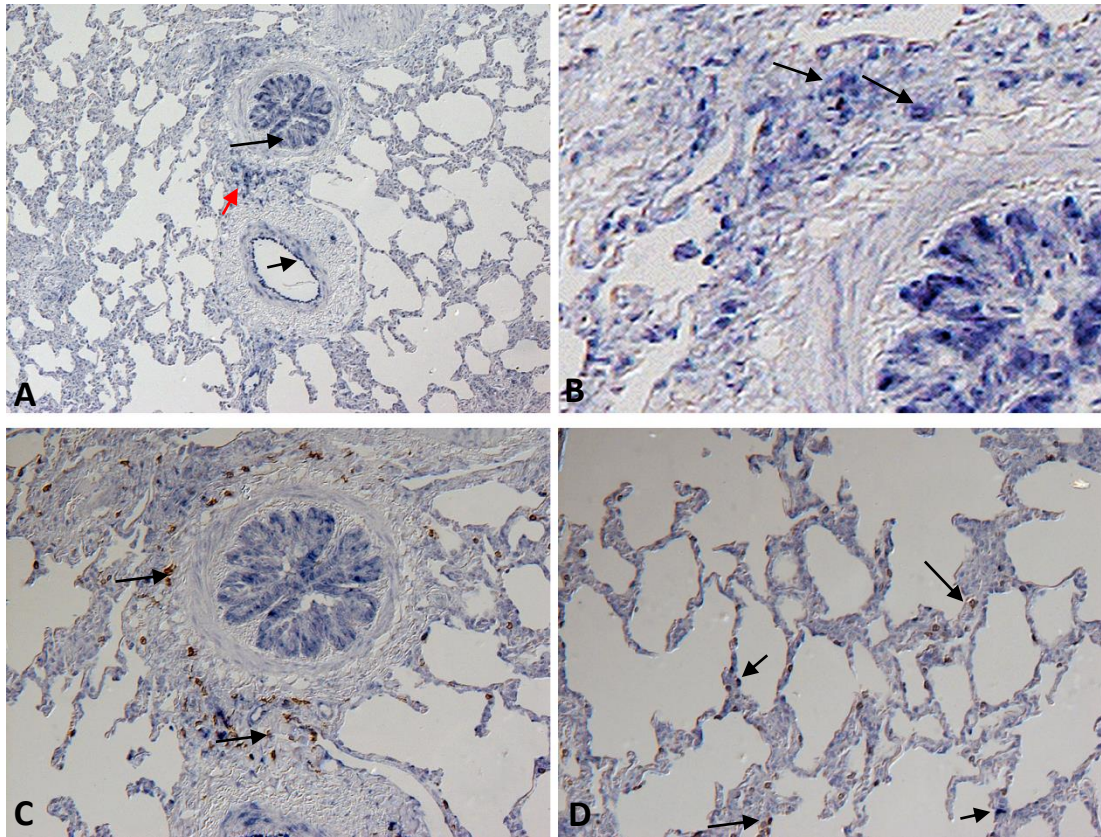


Figure 3-14. RNA-ISH and immunohistology for T and B cells. Lung of a one year-old cow without MCF (13L-2591 B) with a viral load of 5800 virus copies.

(A) Double RNA-ISH and IH for Pax5 staining, there are no detectable B cells in the section, but the viral transcripts are seen in a large proportion of bronchiolar epithelial cells (arrow), BALT cells (red arrow) and arterial endothelial cells (short arrow), (100x). **(B)** Higher magnification of A, a couple of B cell with a faint colour are seen in the BALT. **(C)** Double RNA-ISH and IH for CD3 staining, showing that CD3⁺ cells (T cells, brown colour) surrounding the bronchiole (arrows), but they do not show ISH signal (200x). **(D)** Double RNA-ISH and IH for CD3, showing few T cells (brown, arrows) are disseminated in the alveoli, but do not show ISH signals, while the type II pneumocytes show ISH signals (short arrows), (400x). BCIP/NBT (RNA-ISH), DAB (IH), haematoxylin counterstain.

3.2.2.2 Tongue and muzzle

A total of 23 tongue samples from cattle were examined by both qPCR and RNA-ISH. Of these, eight were qPCR-positive and 17 were positive by RNA-ISH. From those 11 were ISH-positive/PCR-negative and in two cases, (five-year, case no. S13-1483.6, two-year, case no. 13L-4213) tongues were PCR-positive/ISH-negative. In addition, when the tongue was positive for RNA-ISH it was also positive for viral Ov8 antigen. In the qPCR-positive tongues, the viral DNA load had a median of eight copies (generally 1-16 copies), except for one case it was 3900 copies (one year-old case no. 13L-2591). Ov2.5 transcripts were generally seen in basal epithelial cells (Figure 3-15 A,B), while ORF65 transcripts were located in more superficial epithelial cells. RNA-ISH signals were patchy in the mucosal epithelium of those samples with low viral loads, while there were strong signals in those with a relatively high virus DNA loads (3900 copies) (Figure 3-15 A,B). The viral Ov8 antigen was expressed in the stromal fibroblasts and vascular endothelial cells (Figure 3-15 C).

The muzzle was examined in two cases. First, a two-year-old cattle (case no. 13L-4213) by both qPCR and RNA-ISH, and with positive results in both. The second with qPCR only. The viral DNA copies were 16 and 2, respectively. Viral transcripts were detected in epidermal epithelial cells (Figure 3-15 D), also in salivary glandular epithelial cells and vascular endothelial cells of some vessels, some fibrocytes, occasional hair follicles, and possibly in macrophages in the dermis.

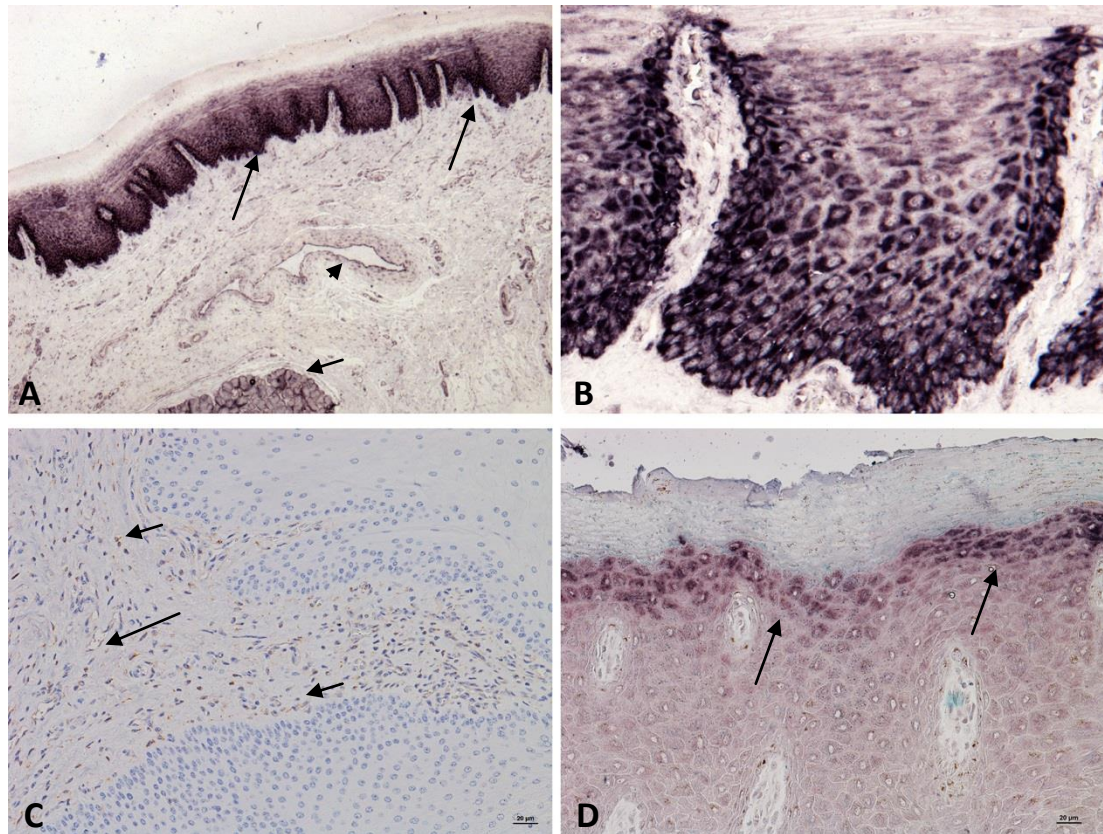


Figure 3-15. RNA-ISH and immunohistology in tongue and muzzle in cows without MCF at different ages and different viral loads.

(A) RNA-ISH for Ov2.5, tongue mucosa, of a one-year-old cow without MCF (13L-2591 B) with a virus load of 3900 virus copies. Very strong ISH signals in the basal epithelial cells in the stratum basale (long arrows) and in fewer signals in stratum granulosum, glandular epithelial cells (short arrow) and in VEC (arrowhead), (100x). **(B)** Higher magnification of A, showing the positive epithelial cells (400x). **(C)** IH for viral antigen, tongue of a seven-week-old cattle (S13-1419.6) with a viral load of 12 virus copies. Weak reaction in stromal fibroblasts (short arrows) and in VEC (long arrow), bar= 20µm. **(D)** ISH for Ov2.5, muzzle of a two-year-old cattle (13L-4213B) with a viral load of 16 virus copies. ISH signals are seen in epithelial cells in the epidermis (arrows), bar= 20µm. BCIP/NBT (RNA-ISH), DAB (IH), haematoxylin counterstain.

3.2.2.3 Lymph nodes

A total of 32 different lymph nodes (27 mediastinal, three bronchial and one of each submandibular and mammary lymph nodes) from cattle were tested by qPCR and/or RNA-ISH. Of the 32 lymph nodes tested, 11 were positive by qPCR, while 21 were positive by RNA-ISH. The mediastinal lymph node, which was tested most frequently (n=27), exhibited a median of 7.5 virus copies per 100 ng genomic DNA. However, some lymph nodes, such as the submandibular lymph node in a cow (case no. 13L-2591), had a much higher viral load of 6500 viral DNA copies. The RNA-ISH signal intensity and the number of positive cells were generally variable and not always correlated with the viral load. Also, in 10 qPCR negative lymph nodes, viral transcripts were detected. In cattle older than five years and foetus, the majority of lymph nodes were negative, but with increasing age, i.e. after seven weeks age, the lymph nodes were positive by RNA-ISH (and viral IH) with undetected viral DNA (the same for other organs). After one year age, animals lymph nodes were positive as well as other organs, however sometime lymph nodes were negative, but there were positive organs in a given animal. Viral transcripts were detected in relatively numerous lymphocytes within follicles, a few TBM and occasional interfollicular lymphocytes as well as vascular endothelial cells (Figures 3-16 A-D, 3-18 A). Double staining of sections for the B and T cell markers and by RNA-ISH confirmed that the cells carrying viral transcripts are B cells (Figure 3-17). The viral Ov8 antigen expression was seen in FDCs and macrophages (based on the morphology) (Figure 3-18 B-D). In lymph nodes with higher viral loads, viral Ov8 antigen expression was seen in a wider range and more number of cells, including a proportion of lymphocytes, macrophages, vascular endothelial cells and stromal fibroblasts (Figure 3-18 E,F).

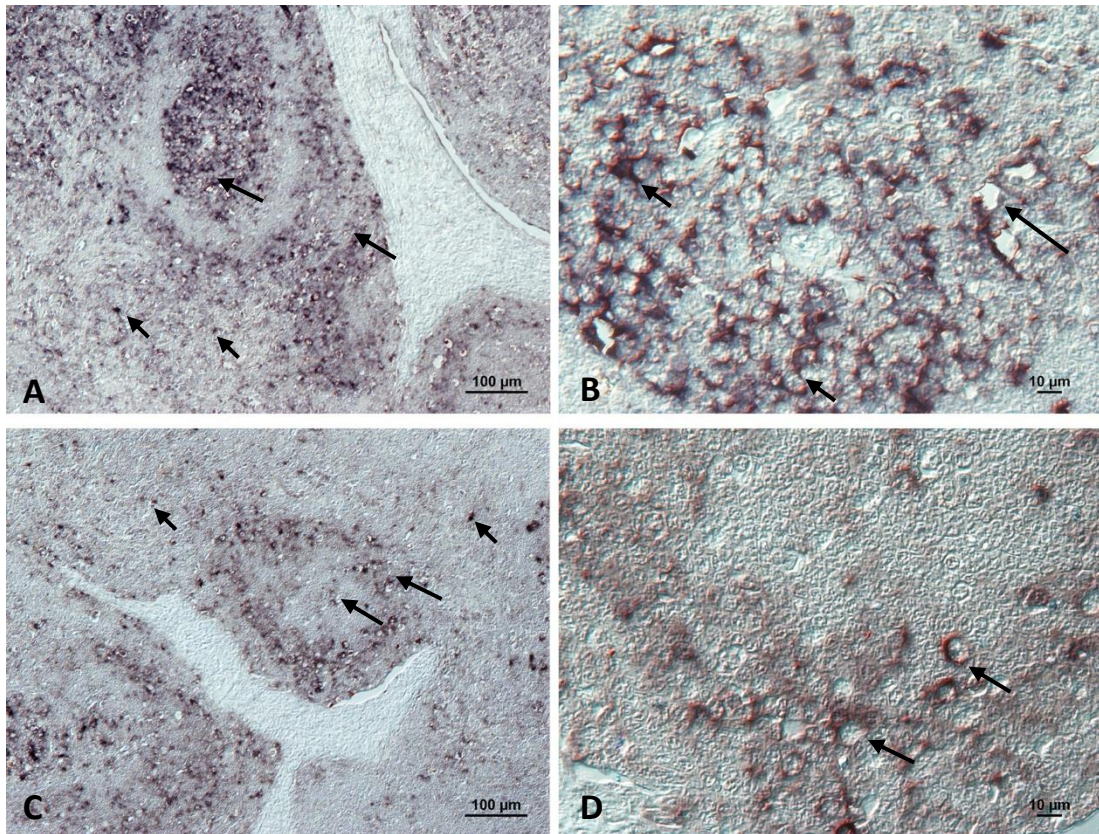


Figure 3-16. RNA-ISH for submandibular lymph node. A one year-old cow without MCF (13L-2591A) with a viral load of 6500 virus copies.

(A) RNA-ISH for ORF65, cortical follicles, strong signals in cells inside and at the periphery of the follicle (long arrows) and in individual cells in the interfollicular zones (short arrows). **(B)** Higher magnification of the follicle centre of A, ISH signals are in lymphocytes (short arrows) and in the TBM (long arrow). **(C)** RNA-ISH for Ov2.5, ISH signal are similar as ORF65, but with less number of cells. **(D)** Higher magnification of C, ISH signals are in lymphocytes (short arrows). BCIP/NBT, haematoxylin counterstain.

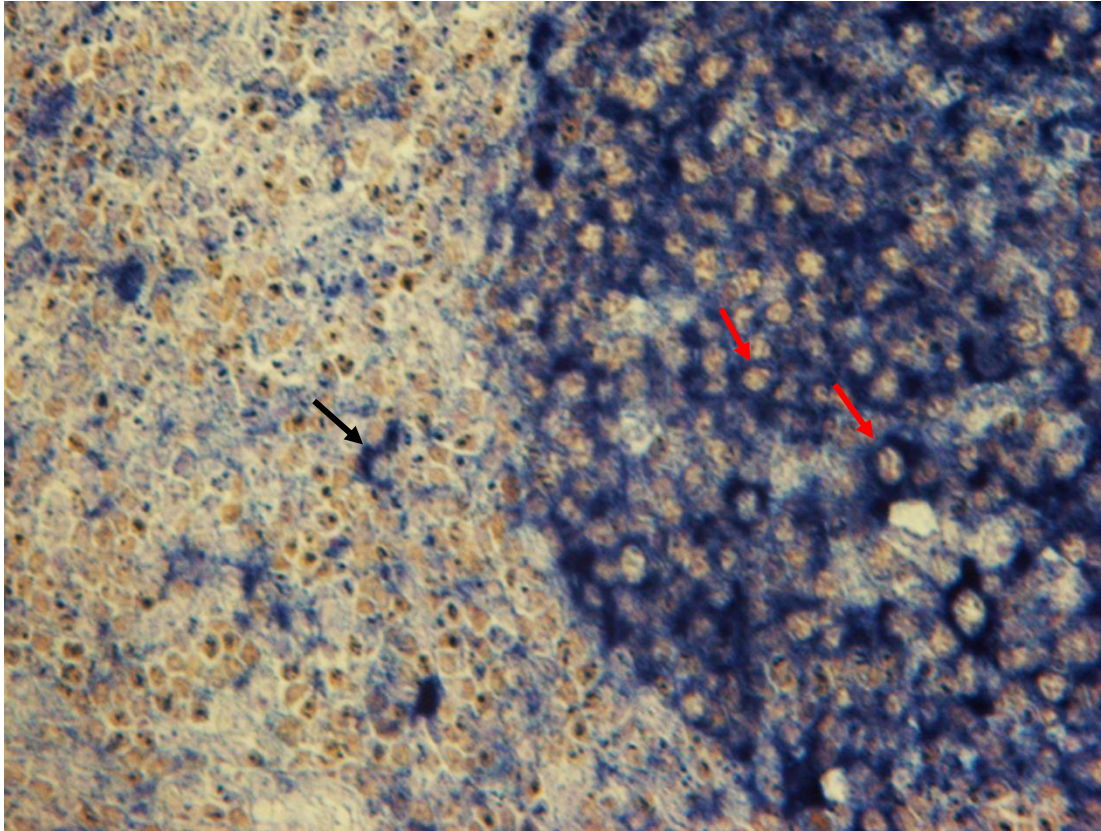


Figure 3-17. Double RNA-ISH for Ov2.5 and immunohistochemistry for Pax5 (B cells). A submandibular lymph node of a one year-old cow without MCF (13L-2591 B, see also Figure 3-16) with a viral load of 6500 virus copies.

The double staining is confirming the co-localisation of viral transcripts in the B cells (Pax5⁺, yellowish brown, red arrows). There are strong ISH signals in the B cells and sporadic non B cells, and possibly macrophages outside the follicle (black arrow), (400x). BCIP/NBT (RNA-ISH), DAB (IH), haematoxylin counterstain.

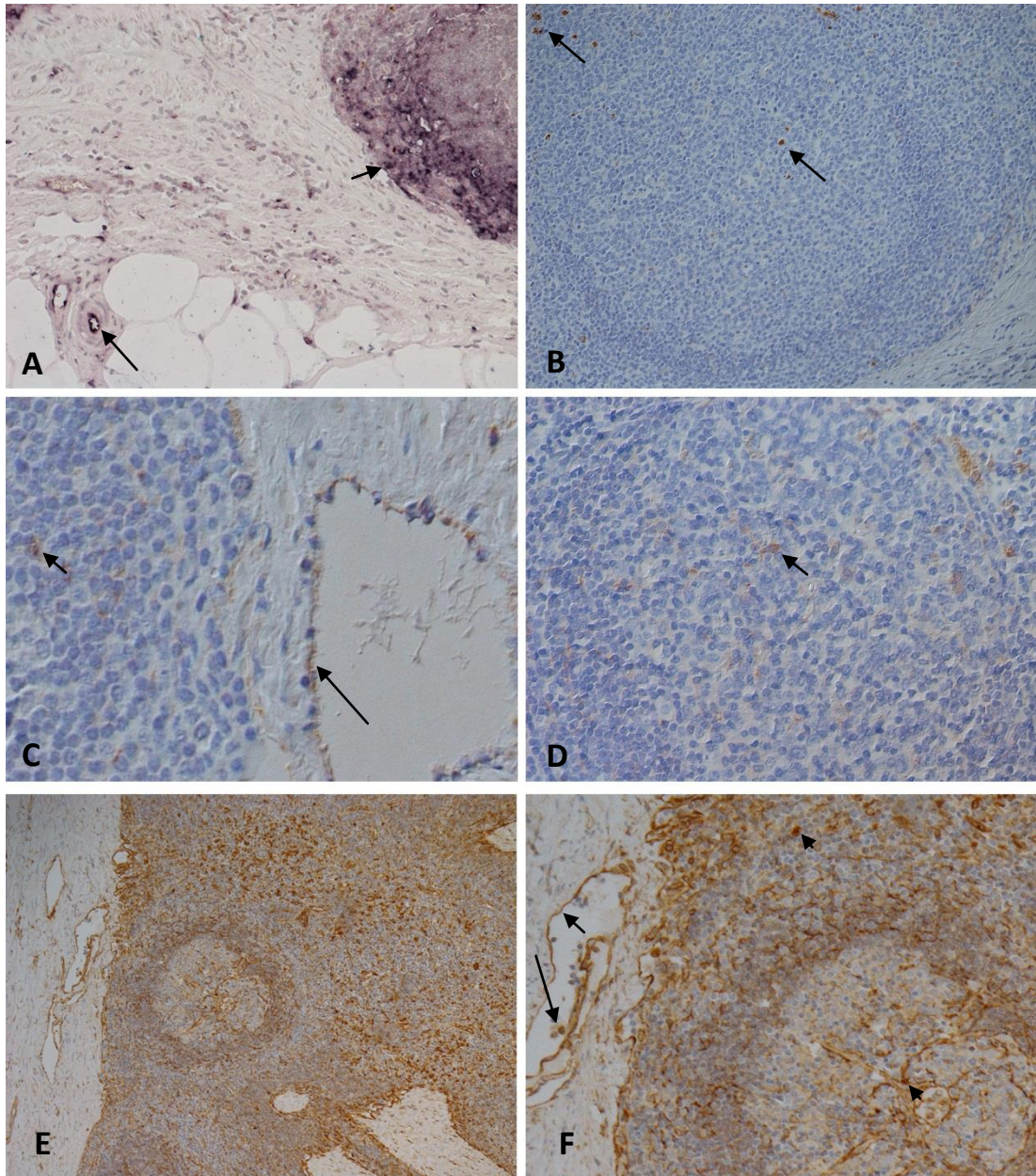


Figure 3-18. RNA-ISH and immunohistochemistry for viral Ov8 antigen in cows. Lymph node of two cows without MCF, the first is a two year-old cow ([13L-4215A], A-D) with a viral load of eight virus copies. Second is a one year-old cow (13L-2591A E & F [see Figure 3-11 for ISH]) with a viral load of 6500 virus copies.

(A) RNA-ISH for Ov2.5, mediastinal lymph node, ISH signals are seen in cells mainly at the periphery of the lymphoid follicles (short arrows) and in vascular endothelial cells (VEC), (arrows). **(B, C and D)** IH for viral antigen (brown), same cattle in A, the viral antigen is expressed weakly in VEC (long arrow) and FDCs in the follicles (short arrows), (100x in B, 400x in C&D). **(E)** IH for viral antigen (brown), submandibular lymph node, there are extensive expression of viral antigen in through the section (100x). **(F)** Higher magnification of E, the antigen expression is seen in the cells in the follicle and in the interfollicular zones (arrowheads), VEC (short arrow) and in individual circulating leukocytes in the vessel lumen (long arrow), (200x). BCIP/NBT (RNA-ISH), DAB (IH), haematoxylin counterstain.

3.2.2.4 Spleen

In total, 28 spleen samples were examined. Of these, 23 were RNA-ISH-positive, whereas only 11 were qPCR-positive, with variable virus loads, with a median number of 15 virus copies. Similar to the lymph nodes, the RNA-ISH signal intensity was not always, but sometimes correlated with the qPCR results (Figure 3-19 A,B), whereas the viral Ov8 antigen expression was positively correlated with the viral load (Figure 3-19 C,D). Generally, when viral transcripts were detected in spleen, it was usually also detected in mediastinal lymph nodes.

Viral Ov2.5 and ORF65 transcripts were mainly found in a proportion of lymphocytes in the follicular mantle zones and in the red pulp, in vascular endothelial cells and in fibroblasts around vessels and occasionally in TBM (Figure 3-19 A,B). viral Ov8 antigen was mainly expressed in FDCs and macrophages and in vascular endothelial cells (Figure 3-19 C,D). Double RNA-ISH and IH staining confirmed that the cells carrying viral transcripts in the mantle zones are B cells, whereas T cells were negative in the RNA-ISH (Figure 3-20 A-D).

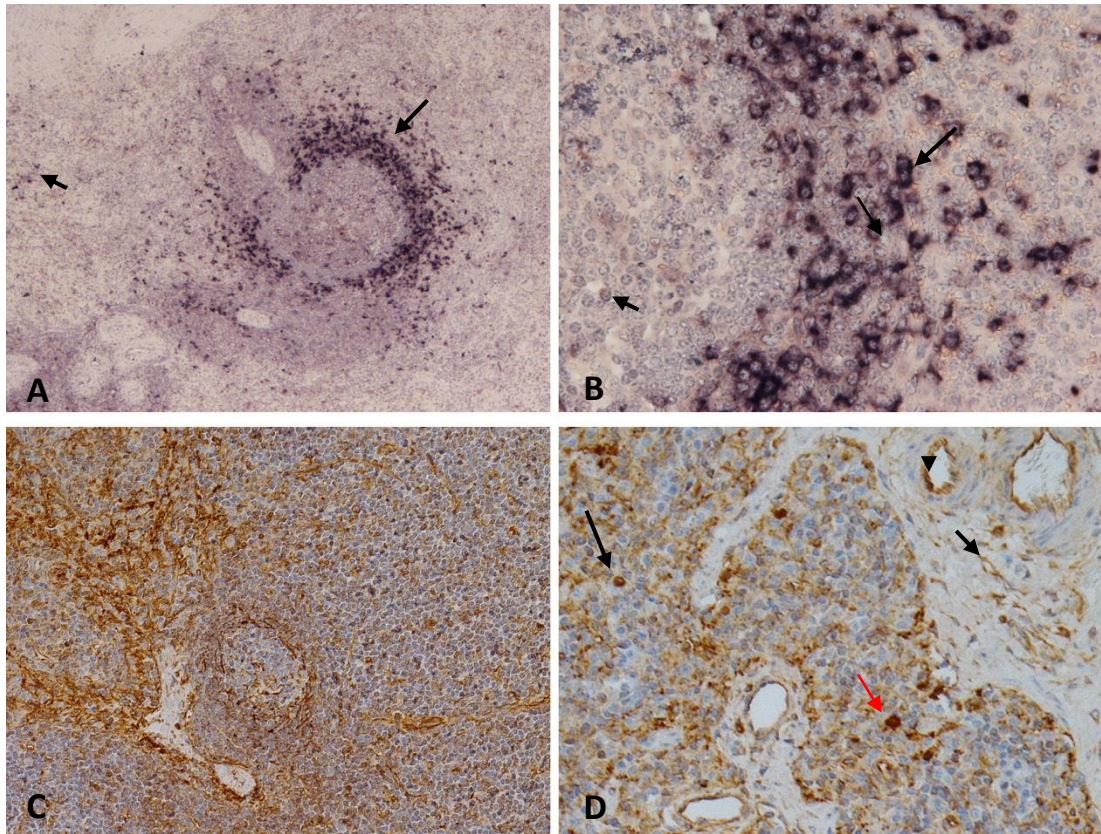


Figure 3-19. RNA-ISH and immunohistochemistry for viral Ov8 antigen. Spleen of a one year-old cow without MCF (13L-2591A) with a viral load of 3600 virus copies.

(A) RNA-ISH for ORF65, splenic white pulp, signals in a large proportion of cells at the mantle zone (long arrow) and in individual cells in the red pulp (100x). **(B)** Higher magnification of A, showing strong signals in lymphocytes at the mantle zone of the follicle (long arrow), and weaker signals in cells inside the follicle (short arrow), (400x). **(C)** IH for viral antigen (brown), showing the intense antigen expression through the section. **(D)** Higher magnification of C, antigen is expressed in individual lymphocytes (long black arrow), in a possible macrophage (red arrow), in VEC (red arrow), and in possible stromal fibroblasts (short arrow) (400x). BCIP/NBT (RNA-ISH), DAB (IH), haematoxylin counterstain.

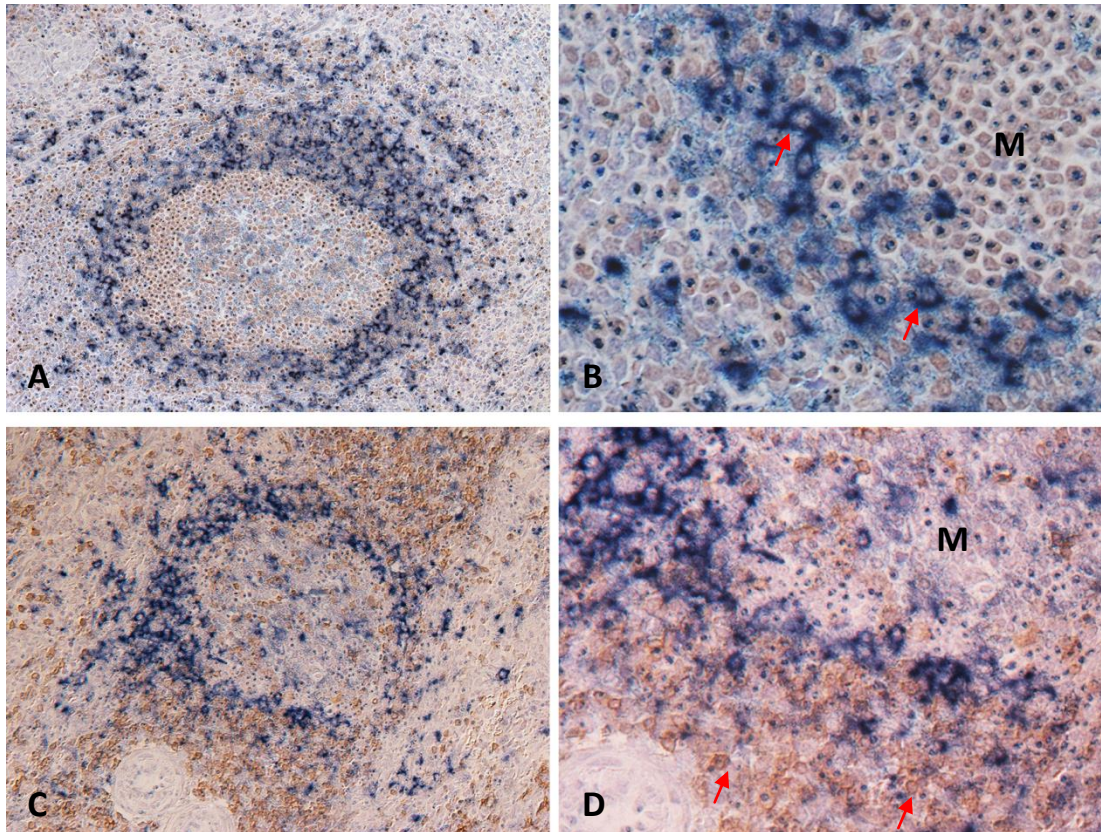


Figure 3-20. RNA-ISH and immunohistology for T and B cells. Serial sections of spleen of the one year-old cow without MCF (13L-2591A, see also Figure 3-19) with a viral load of 3600 virus copies. **(A)** Double RNA-ISH and Pax5 staining, confirming the co-localisation of viral transcripts at the mantle zone in the majority of Pax5⁺ B cells (yellowish brown), (200x). **(B)** Higher magnification of A, confirming that a proportion of PAX5⁺ B cells exhibit a positive RNA-ISH signal (arrows) at the mantle zone (M). **(C)** Double RNA-ISH and CD3 staining, showing that CD3⁺ cells (T cells, brown colour) outside the follicle, but otherwise mainly located around the follicles (200x). **(D)** Higher magnification of C, margin of the follicle (M), confirming that that the CD3⁺ T do not exhibit any ISH signal (arrows) (400x). BCIP/NBT (RNA-ISH), DAB (IH), haematoxylin counterstain.

3.2.2.5 Peripheral blood leukocytes

PBL pellets were prepared from the blood of nine clinically normal cows (collected at an abattoir) aged 13 months to two years. Six of these samples tested positive by qPCR (i.e. were viraemic) with viral DNA loads varying between 1 and 86 copies. The results are shown in Table 3-6, and also Appendix Table 2b.

Two of the qPCR-positive PBL pellets were examined by RNA-ISH and immunohistology for viral Ov8 antigen. In both pellets, viral transcripts were detected in up to 10 % of leukocytes that had the morphology of lymphocytes and/or monocytes (Figure 3-21 A). viral Ov8 antigen was weakly expressed in occasional cells with the morphology of monocytes (Figure 3-21 B). To assess the ratio of B:T cells, pellets were stained for CD3 and Pax5. This showed ratios of 2-3 % and 20-30 %, for B and T cells respectively (Figure 3-21 C,D). Based on this immunohistological staining of consecutive sections for CD3 or Pax5, it was not possible to comment on the lymphocyte subtype that carried viral transcripts.

Table 3-7. Amount of positive samples and OvHV-2 DNA loads in WBC samples of normal cows.

Cattle	Age	qPCR	ISH	IH
13L-4889 D	13 mo	-	ND	ND
13L-4889 E	13 mo	-		
13L-4889 C	18 mo	5		
13L-4889 A	19 mo	86		
13L-4889 B	19 mo	1		
Cow 1	20 mo	-		
Cow 2	2 y	32		
13L-4883 F	2 y	4	+	-
13L-4883 G	2 y	20	+	-

Abbreviations: ND: not done; "-": negative; "+": positive.

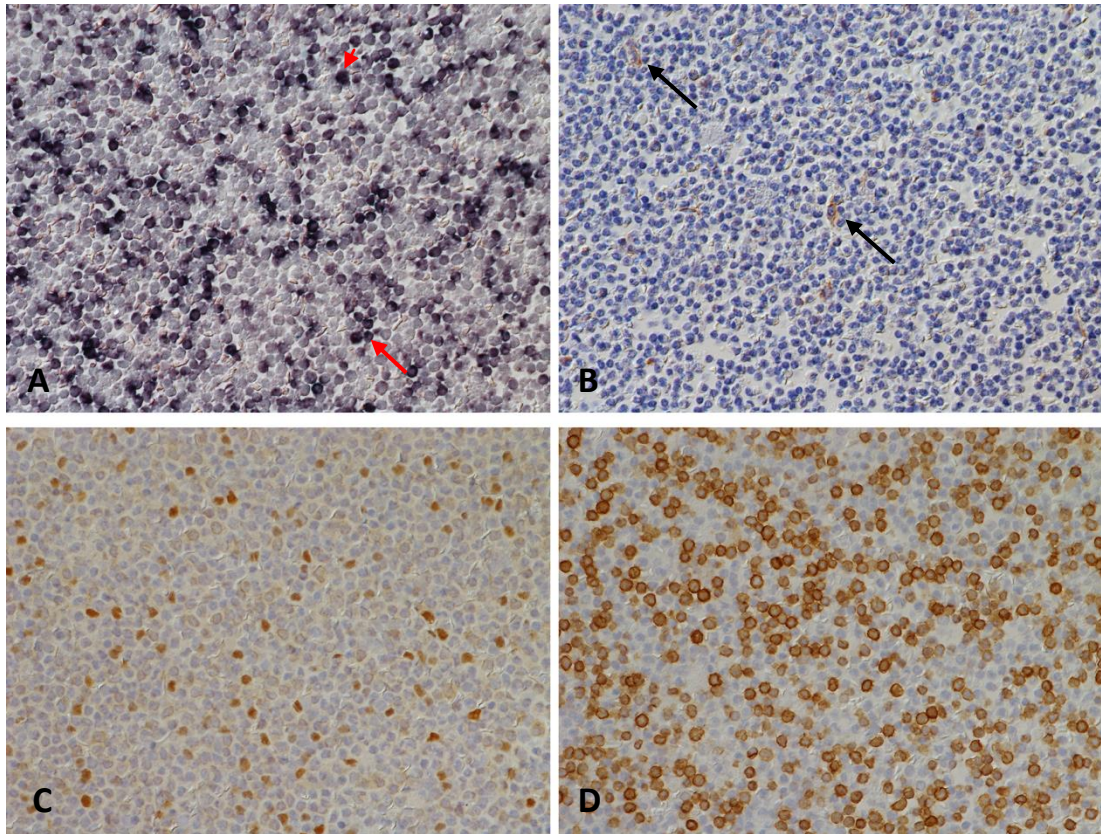


Figure 3-21. RNA-ISH and immunohistochemistry for viral Ov8 antigen and T or B cells. Pelleted peripheral blood leukocytes of a two year-old cow without MCF (13L-4883 G) with a viral load of 20 virus copies.

(A) RNA-ISH for Ov2.5. About 5-10 % of the leukocytes have ISH signals; morphemically consistent with lymphocytes (red arrow) or monocytes (red arrowheads), (200x). **(B)** IH for the viral antigen. Positive reaction in occasional monocytes (200x). **(C)** IH for Pax5, confirming that the B cells (brown) comprises approximately 2-3 % of the leukocytes count (200x). **(D)** IH for CD3, showing that the T cells (brown) comprise approximately 20-30 % of the leukocytes (200x). BCIP/NBT (RNA-ISH), DAB (IH), haematoxylin counterstain.

3.2.2.6 Uterus and placenta

In five cattle, uterus and placenta were tested by both qPCR and RNA-ISH and in one cattle, samples were additionally tested for viral antigen. In a 12-year mother (case no. S13-1452), the uterus and foetal placenta (and all other organs in both) were negative by both qPCR and RNA-ISH. In an eight year-old cow (case no. S13-1360), both the uterus and placenta had one virus DNA copy and ISH-positive. In a five-year old cow (case no. S13-1355), both the uterus and placenta were ISH-positive, while the viral DNA was only detected in uterus (56 virus copies per 100 ng genomic DNA). The mediastinal lymph node was positive by qPCR in that animal (no ISH data). This animals foetus was ISH-positive in its four organs and PCR-positive in two with 3 - 4 virus DNA copies. In another five-year old cow (case no. S13-1483), the uterus was PCR-negative, but positive for viral transcripts and antigen (also positive in its other organs), while the foetal placenta was ISH-positive and negative for viral Ov8 antigen (no qPCR data for placenta, all other organs were PCR/antigen-negative, but ISH-positive). In a younger calf, aged eight weeks (case no. S13-1434), the uterus was PCR-negative, but ISH- positive (only with ORF65 probe, Ov2.5 was negative), all other organs were PCR-negative/ISH-positive.

Generally, in uterus, the viral transcripts were found in stromal cells, glandular epithelium and vascular endothelial cells (Figure 3-22 A,B), while the viral Ov8 antigen was expressed by stromal fibroblasts but not by epithelial cells. Also in placenta, usually viral transcripts were exhibited in stromal cells and vascular endothelial cells (Figure 3-22 C,D), and in maternal placental epithelium and glandular epithelium.

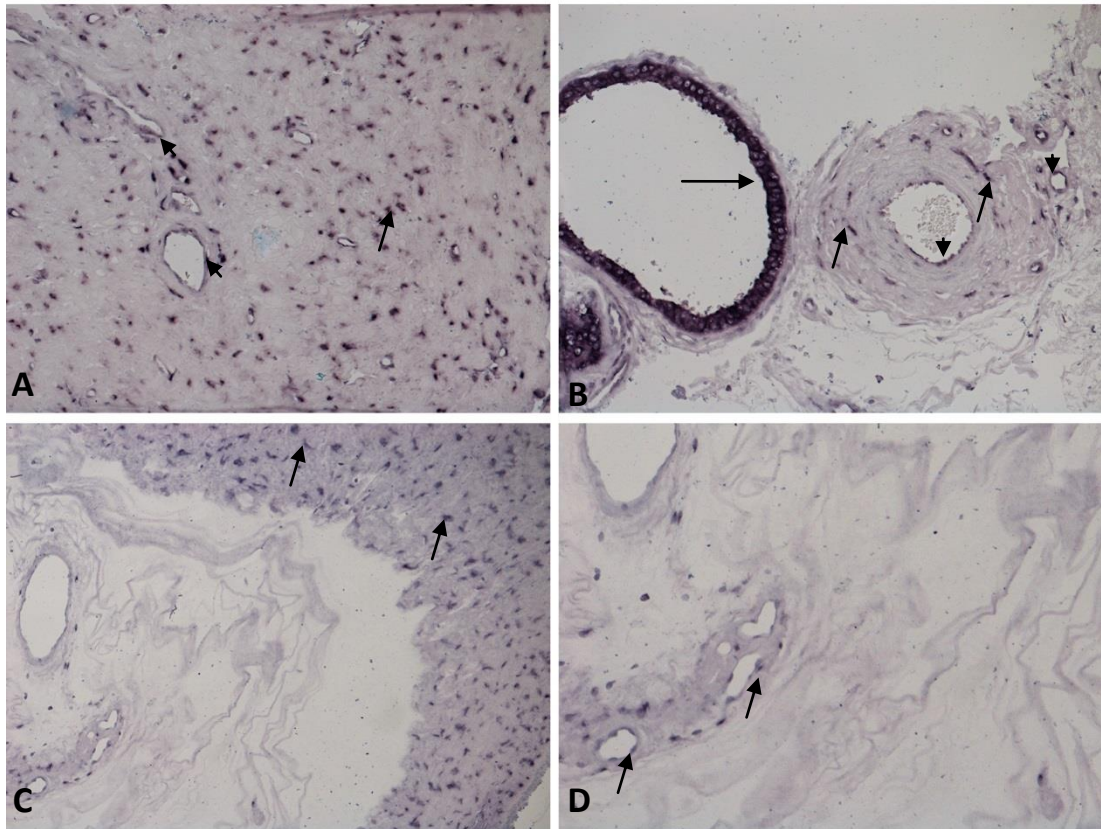


Figure 3-22. RNA-ISH in uterus and placenta. A five year-old pregnant cow without MCF (S13-1355.4) with virus loads of 58 virus copies in the uterus and zero copies in the placenta.

(A) RNA-ISH for Ov2.5, myometrium, ISH signals seen in smooth muscle cells (arrow) and VEC (short arrow), (200x). **(B)** RNA-ISH for Ov2.5 in the same cow as A, endometrium, showing very strong signals in the glandular epithelial cell (long arrow), in VEC (arrowheads) and vascular smooth muscle cells (short arrows), (200x). **(C)** RNA-ISH for Ov2.5, maternal placentome of same cattle as C, weak signals in stromal cells (arrows), (200x). **(D)** Higher magnification of E, showing and weak signals in VEC (400x). BCIP/NBT, haematoxylin counterstain.

3.2.2.7 Controls

Negative control sections served to check the specificity of the probes and reliability of the results obtained with the antisense probes. The sense probes were tested in lung, lymph node, tongue and spleen sections and did not yield any signal in any of the tissues (Figure 3-18 A-D). However, with the ORF65 sense probe, a diffuse background staining was seen in some sections.

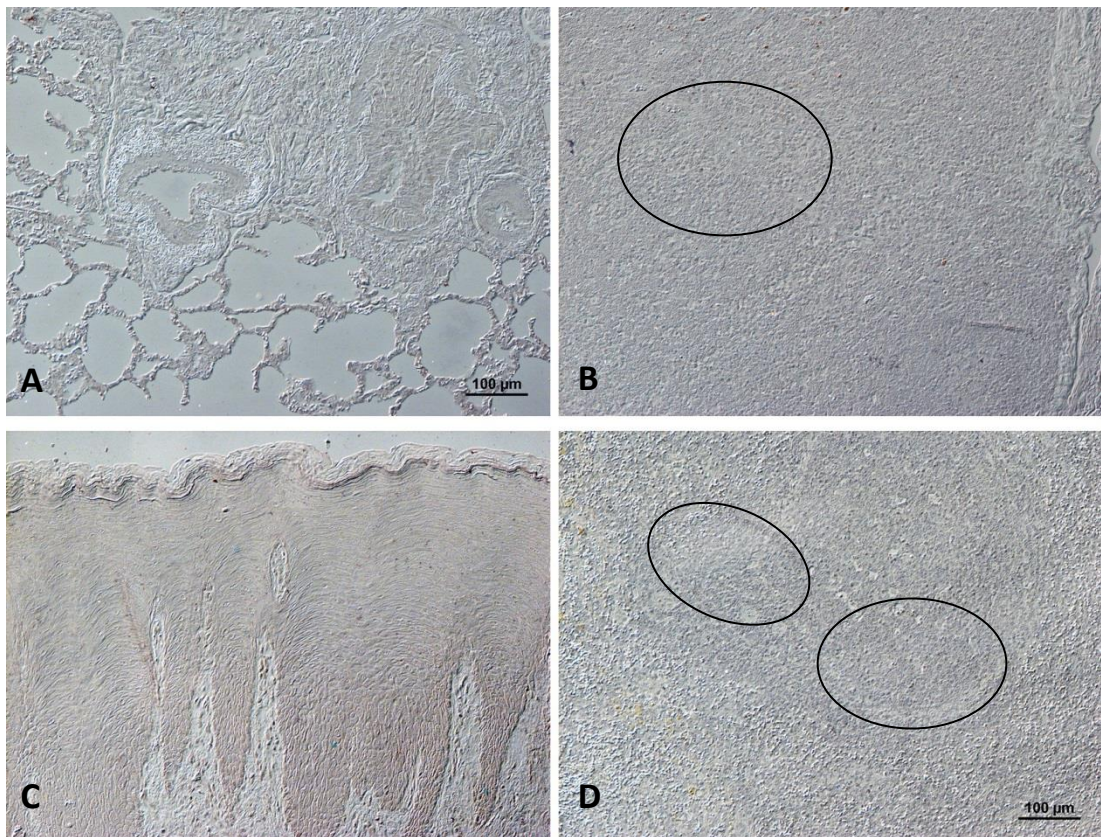


Figure 3-23. RNA-ISH negative controls. Sense probes in lung, mediastinal lymph node, tongue and spleen of a normal cow without MCF.

(A) RNA-ISH for ORF65 sense, lung of one year old cattle (13L-2591A, see also Figure 3-14 for comparing ISH signals). No ISH signals are seen in the section (100x). **(B)** RNA-ISH for Ov2.5 sense, mediastinal lymph node, of the same cattle in A, follicle is outlined by a black circle for visibility. There are no ISH signals (100x). **(C)** RNA-ISH for Ov2.5 sense, tongue of a 2.5 year old cattle (13L-2600D). No ISH signal in the epithelium (100x). **(D)** RNA-ISH for Ov2.5 sense, spleen of the same cattle in C. The white pulps are outlined by black circles for visibility, no ISH signals are observed (100x). BCIP/NB, haematoxylin counterstain.

3.3 Assessment of OvHV-2 in animals with MCF

The third group of animals examined were cattle, water buffaloes and a Javan Banteng with MCF caused by OvHV-2, confirmed by post-mortem findings, histopathological examination and PCR. From six cattle, material for OvHV-2 qPCR was available also, all other animals were only tested by RNA-ISH and, selectively, immunohistology for OvHV-2 Ov8 antigen.

3.3.1 Quantification of OvHV-2 DNA loads

Samples from six cattle and a Javan banteng with MCF, aged between 11 months and 3 years, were tested by qPCR and the viral loads were quantified (Table 3-7). In addition, the aborted foetus at eight months gestation of the MCF affected Javan Banteng was tested (Table 3-8).

In the six cattle, samples from eight different organs were tested. All samples were positive with higher virus loads than in the other animal groups, ranging between 5,227 viral copies in the small intestine to 1,360,448 in the mesenteric lymph nodes. The average value copy number was 189,225 and the median was 52,539 (Table 3-8). In the aborted foetus, spleen and liver were positive with relatively low viral loads (1 and 2 virus copies respectively). Results obtained in the individual cases is provided in Table 3 in Appendix

Table 3-8. Positive samples and OvHV-2 DNA loads in tissues of animals with MCF.

Tissue	samples ^a		OvHV-2 DNA copies			
	Total	Pos	Min ^b	Max ^c	Average	Median
Mediastinal LN	4	4	106,283	328,288	175,276	133,268
Mesenteric LN	4	4	42,029	1,360,448	414,008	126,778
Liver	1	1	12,281			
Small intestine	1	1	5,227			
Kidney	1	1	18,843			
Placenta	1	1	152,665			
Heart	1	1	16,769			
Eye	1	1	86,234			

a. Samples tested, **Total:** the number of all samples taken for that particular type of specimen. **Pos:** those tested positive. **B:** Minimum virus copies. **c:** Max: maximum virus copies. LN: lymph node.

Table 3-9. Amount positive samples and OvHV-2 DNA loads in tissues of the aborted foetus at eight-month gestation from a dam affected with MCF.

Aborted foetus samples	OvHV-2 DNA copies
Thoracic fluid	0
Spleen	2
Liver	1
Kidney	0

3.3.2 Histological observations and identification of cells harbouring OvHV-2 RNA and antigen in tissues and lesions of cattle with MCF

Tissues from 10 MCF retrospective cases were tested; in five, only tissue blocks were available for RNA-ISH and immunohistology and in the other five, tissues (partial though) were available for qPCR.

Haematoxylin and eosin stained tissue sections of lesions from MCF affected bovine were microscopically examined and the disease confirmed based on the typical histological changes (vasculitis and epithelial necrosis) (Figures 3-14 A,B, 3-25 A, 3-26 A, 3-27 A,C, 3-28 A). A wide range of samples from different organs were examined by RNA-ISH and immunohistology, to identify viral transcripts and antigen in infected cells. All tested animals with MCF were positive for RNA-ISH and immunohistology for OvHV-2 Ov8. Additionally, immunohistology was performed to highlight the infiltrating lymphocyte subtypes, i.e. B cells (CD20-positive, Pax5-positive) and T cells (CD3-positive) in lesions in animals with MCF. Both Ov2.5 and ORF65 probes yielded almost identical signal intensity and cell ranges (Figures 3-24 A,B). However, in a few tissues, one of the probes did not work, which was most likely due to the poor preservation of the tissues post mortem. Almost invariably, in most of the tested organs, viral transcript and antigens (in selected tissues) were detected in infiltrating lymphocytes, macrophages, vascular endothelial cells, epithelial cells, alveolar epithelial cells, fibroblasts and neurons. The detailed description of each MCF case result is provided in Table 3 in the Appendix.

3.3.2.1 Lung

Six lung samples were examined by RNA-ISH, in all of them histological changes were noticed and all were ISH-positive. One lung was additionally tested by immunohistology for viral Ov8 antigen and was positive. Generally, histological changes in lung included mild to moderate hyperaemia; activated VEC associated with mild to moderate perivascular lymphocytes and macrophage like cells infiltration surrounding and partly infiltrating the vessels walls. The vasculitis was mainly lymphocytic-dominated arteritis; mild to moderate multifocal of (partly) mixed cellular infiltrates peribronchial mononuclear infiltrates. There were also mild to severe oedema and alveolar haemorrhages (Figure 3-243 A,B; 3-25 A).

In the lung, there are no qPCR data to measure amount of the virus. The viral transcripts were strongly exhibited in a large proportion of infiltrating perivascular and peribronchial lymphocytes. Also in the bronchial columnar epithelial cells, vessels endothelium and in few circulating leukocytes in lumens of the vessels (Figures 3-24 C-E, 3-25 B). In addition, the viral Ov8 antigen was abundantly expressed through the section and was seen in the infiltrating and intravascular leukocytes, occasional macrophages, vascular endothelial cells, individual alveolar cells and chondrocytes in the bronchus (Figure 3-25 C,D).

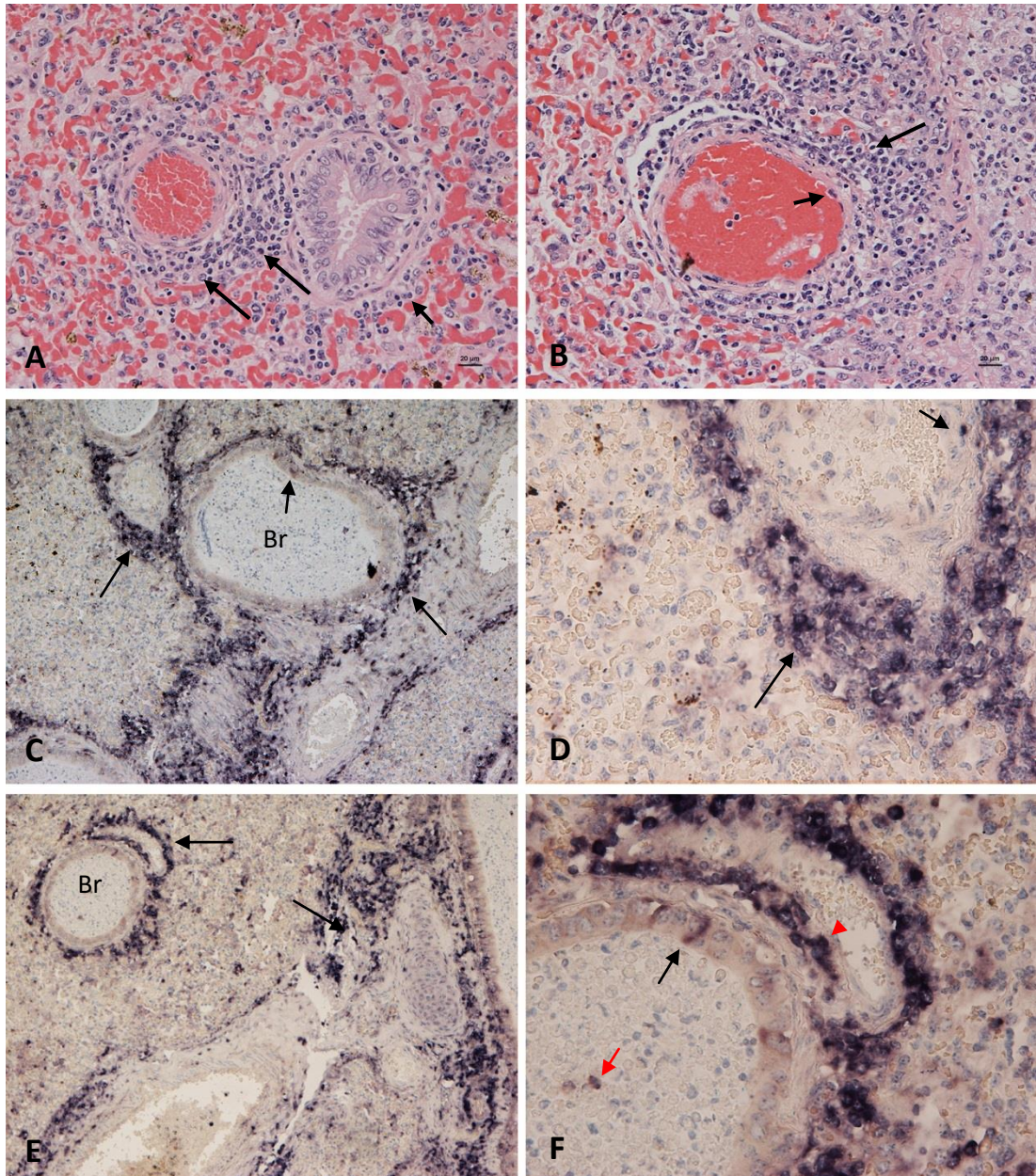


Figure 3-24. Histology and RNA-ISH of lung in an 11 month-old cow with MCF (12L-0200 B).

(A) HE, pulmonary changes showing defused hyperaemia, lymphocytes and macrophage like cells surrounding the artery (long arrows) and the bronchiole (short arrow), bar=20 μ m. **(B)** Higher magnification, showing perivascular lymphocytes (long arrow) and the activated VEC (short arrow), bar=20 μ m. **(C)** ISH for ORF65, strong signals in the infiltrating perivascular and peribronchiolar (Br) lymphocytes (arrows) and weaker signals in bronchiolar epithelial cells (100x). **(D)** Higher magnification of C, showing a proportion of infiltrating perivascular lymphocytes have ISH signals (400x). **(E)** ISH for Ov2.5, signals in similar cellular range as in ORF65 (100x). **(F)** Higher magnification of E, showing strong signals in activated VEC (arrowhead), weaker signals in columnar bronchiolar epithelial cells (black arrow) and in individual cells in the exudate in the bronchiolar lumen (red arrow), (400x). Haematoxylin and eosin (histology), BCIP/NBT (RNA-ISH), haematoxylin counterstain.

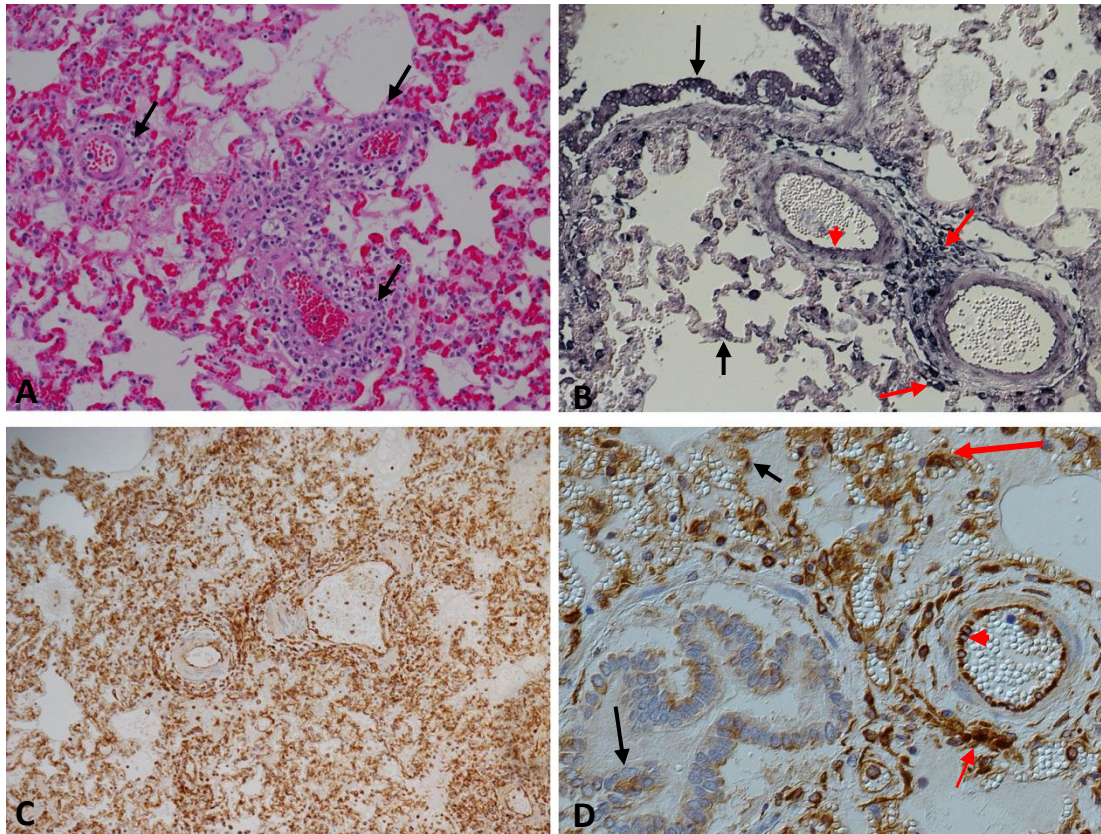


Figure 3-25. Histology, RNA-ISH and immunohistochemistry for viral Ov8 antigen in lung lesions. An 18 month-old water buffalo with MCF (S11-564.2).

(A) HE, Mild to moderate disseminated perivascular infiltrated lymphocytes (arrows), (100x). **(B)** ISH for Ov2.5, strong signals in infiltrating lymphocytes and VEC (arrowhead), respiratory epithelial cells (long black arrow), perivascular lymphocytes (long red arrow) and in alveolar cells (short black arrow), (200x). **(C)** IH for viral antigen (brown), the virus antigen is extensively expressed through most of cell types in the lung (100x). **(D)** Higher magnification of D, antigen is strongly expressed in infiltrating lymphocytes (short red arrow), possible alveolar macrophages (long red arrow), alveolar cells (short black arrow), VEC (arrowhead) and weaker expression in bronchus epithelial cells (long black arrow), (400x). Haematoxylin and eosin (histology), BCIP/NBT (RNA-ISH), DAB (IH), haematoxylin counterstain

3.3.2.2 Muzzle and alimentary tract

Only one muzzle was examined by RNA-ISH and was histologically characterised by multifocal epidermal erosions and ulcerations, mixed leukocytic subepithelial perivascular infiltration, increased macrophages and fibroblasts, melanin incontinence, and VEC activation. There are no qPCR data for muzzle, but the viral transcripts were exhibited in the hair follicles, different proportions of basal epithelial cells, epidermal gland epithelium, and in inflammatory leukocytes. In skin samples, the viral Ov8 antigen was expressed in basal cells in hair follicles, individual basal epidermis cells and in dendritic cells at the basement membrane.

The oral lesions were mild to moderate diffuse interstitial lymphocytic and macrophage infiltration. Other findings observed were similar to that in muzzle, with more extensive erosion and ulceration (Figure 3-26 A). Tongue also had similar lesions as oral mucosa, and the viral transcripts were revealed in patches of epithelial cells and signals were more intense with the inflammation, also in infiltrating leukocytes, in a few fibrocytes in interstitium, but the endothelial cells did not show viral transcripts (Figure 3-26 B).

In other parts of the alimentary tract such as small intestine, there was evidence of congestion, distortion at villi tips and focal necrosis in the villi. Moderate to marked diffuse mucosal and perivascular submucosal mixed cellular lymphocyte-dominated infiltration (Figure 3-26 C). The viral transcripts were revealed in the epithelial cells of the villi and in infiltrating leukocytes (Figure 3-26 D). The virus Ov8 antigen was expressed in a large proportion of infiltrating mucosal lymphocytes and plasma cells; and also in VECs (Figure 3-26 E).

Many other parts of digestive system, respiratory system, urinary system; and heart were tested by the RNA-ISH and a selection of immunohistology, their full details are listed in Table 3 in the Appendix.

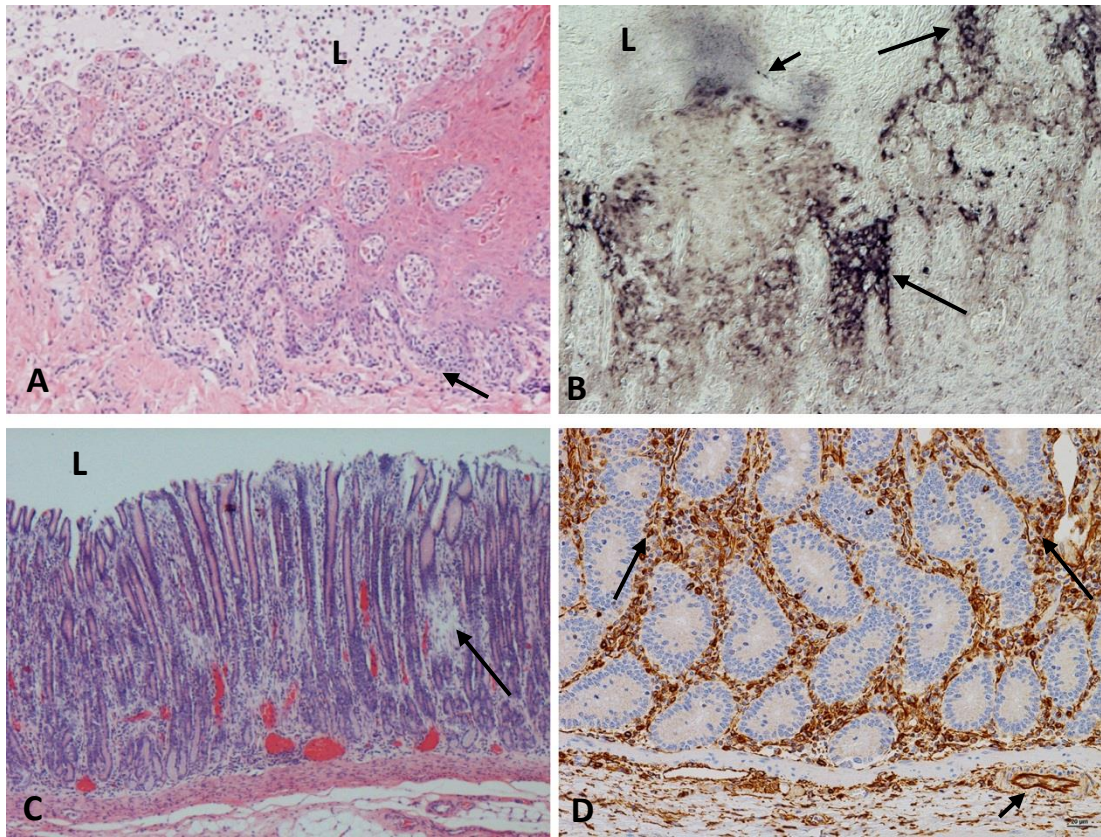


Figure 3-26. Histology and RNA-ISH and immunohistochemistry for viral Ov8 antigen in alimentary tract of cattle with MCF. Alimentary tract of an eleven-month-old cow (12L-0200) figures A-C, and an adult water buffalo (S12-0124) figure D.

(A) Oral mucosa, erosion of the mucosa with massive focal necrosis extending to submucosa, the lumen (L) is covered with inflammatory exudate (200x). **(B)** ISH for Ov2.5, tongue, strong signals in basal and outer epithelial cells (long arrows) and in individual cells in the lumen (short arrow), (200x). **(C)** Small intestine- duodenum, congestion, distortion of the villi tips and necrotic foci in the villus (long arrow), (100x). **(D)** IH for viral antigen, small intestine. Reaction in the majority of lymphocytes in mucosal infiltrates (long arrows) and in VEC (short arrow), bar=20 μ m. Haematoxylin and eosin (histology), BCIP/NBT (RNA-ISH), DAB (IH), haematoxylin counterstain.

3.3.2.3 Lymph nodes and spleen

All of the seven lymph nodes (mediastinal and mesenteric) from four bovine MCF cases examined by qPCR were positive. Only one lymph node was tested for both qPCR and RNA-ISH, another three lymph nodes (two retropharyngeal and one unknown) were tested only by RNA-ISH and were positive. The lymph nodes contained relatively higher average viral loads ranging between 175,276 and 414,008 virus DN copy numbers per 100 ng genomic DNA in mediastinal and mesenteric lymph nodes respectively. The peak viral loads among all organs was detected in mesenteric lymph node (1,360,448 viral DNA copies per 100 ng genomic DNA). Histological changes in lymph nodes were characterised by different degrees of mild to moderate sized mildly depleted secondary follicles, sinus histiocytosis (Figure 3-27 A). Viral transcripts were revealed in the cortex mainly in lymphocytes at the periphery and centre of the follicles and at the T cell zones, and also in cells in the medulla and sinuses (Figure 3-27 B). The viral Ov8 antigen was expressed in lymphocytes at the margin of follicles and in the T cell zones, in sinus FDCs and macrophages, and vascular endothelial cells (Figure 3-27 C).

Four spleen specimens were tested by RNA-ISH, one was additionally tested by immunohistology for viral antigen. The changes in the spleen were characterised by small to moderate sized secondary follicles, diffused lymphocytic (and few macrophages) infiltration, cell-poor to cell-rich red pulps (Figure 3-28 A). There are no qPCR data for spleen, but with the RNA-ISH, viral transcripts were intensively revealed in the secondary follicles and in large proportion of lymphocytes in T cell zone. Also ISH signals were seen in vascular endothelial cells and sporadic leukocytes throughout the red pulp zones (Figure 3-28 B,C). The viral Ov8 antigen was expressed in a proportion of lymphocytes in the follicles and in the T-cell zones, in FDCs, in few cells in red pulp and in the vessels endothelial cells (Figure 3-28 D).

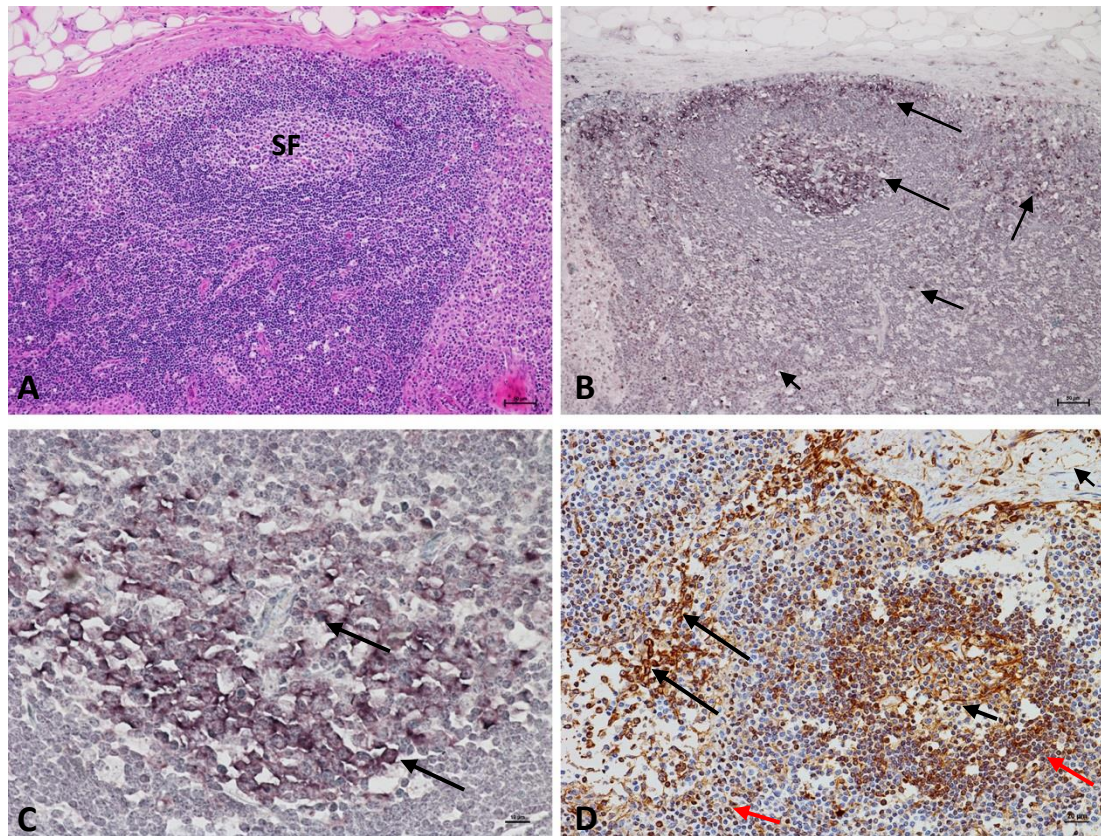


Figure 3-27. Histology, RNA-ISH and immunohistochemistry for viral Ov8 antigen in retropharyngeal lymph node lesion. From two adult water buffalos with MCF (S12-0083) figures A, B & C; and (S12-0124) figure D.

(A) HE, moderate sized mildly depleted secondary follicle (SF), moderate lymphoid hyperplasia, bar= 50 μ m. **(B)** RNA-ISH for Ov2.5, viral transcript are seen in lymphocytes at the periphery and centre (long arrows) of the cortical follicle, and a few in the T cell zones and medulla (arrowheads), bar= 50 μ m. **(C)** Higher magnification of the follicle's centre, ISH signals are seen in lymphocytes, bar= 10 μ m. **(D)** IH for viral antigen, the antigen is expressed in FDC (medium black arrow), in a few LC in follicle periphery and in T cell zones (red arrows), in possible dendritic cells or possibly macrophages in the sinuses (long black arrows), and in VEC (short black arrow), bar= 20 μ m. Haematoxylin and eosin (histology), BCIP/NBT (RNA-ISH), DAB (IH), haematoxylin counterstain.

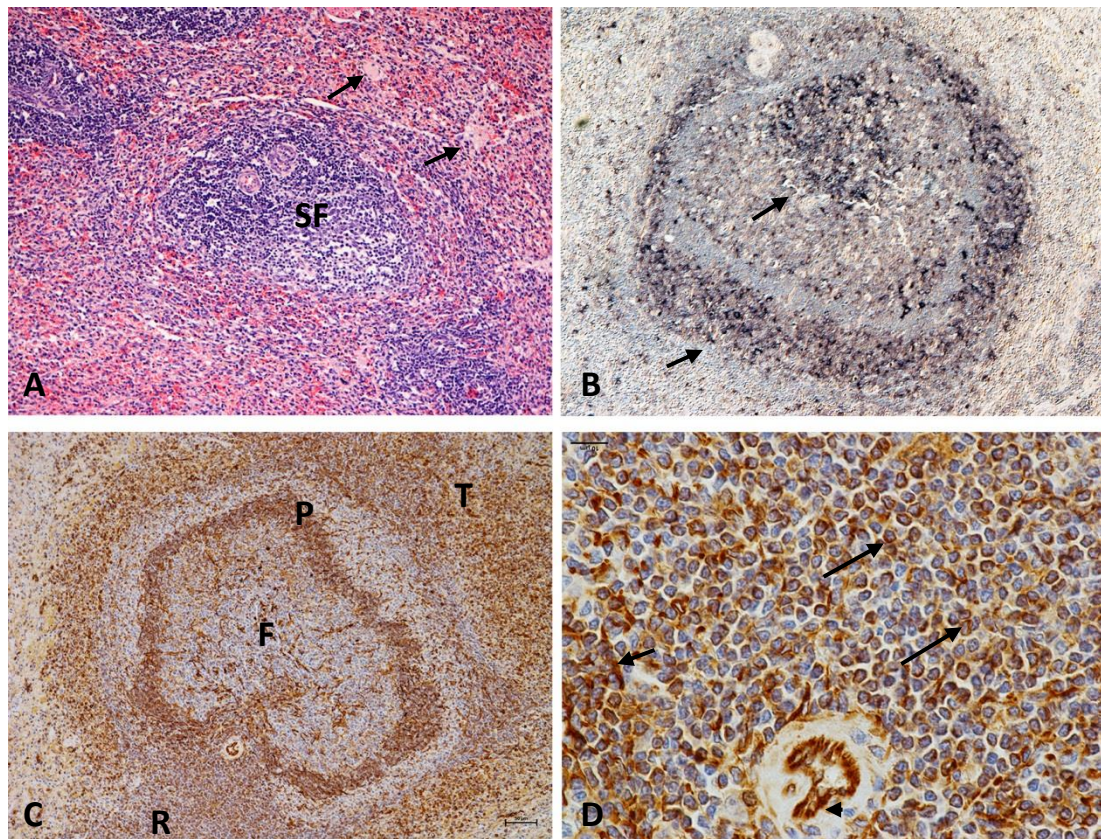


Figure 3-28. Histology, RNA-ISH and immunohistochemistry for viral Ov8 antigen in spleen lesion. An 11 month-old cow (12L-0200 B) figures A & B; and an adult water buffalo with MCF (S12-0124) figures C & D.

(A) Small secondary follicles and T cell zone, the red pulp is cell rich and there are necrotic foci (arrow), (200x). **(B)** RNA-ISH for Ov2.5, viral transcript is revealed in many lymphocytes at the follicle centre and mantle zones, and other weaker signals in individual cells interfollicular areas (200x). **(C)** IH for viral antigen, the antigen is extensively expressed in a proportion of lymphocytes in follicle (F) periphery (P) and in T cell Zones (T) and in cells in the red pulp (R), bar= 50µm. **(D)** Higher magnification of the follicle periphery of C, viral antigen is in lymphocytes (long arrows), in a possible FDC or macrophage (short arrow), and in activated VEC (arrowhead). Haematoxylin and eosin (histology); BCIP/NBT (RNA-ISH), DAB (IH) haematoxylin counterstain.

3.3.2.4 Rete mirabile and brain

The classical histopathological change unique to MCF is usually observed in the rete mirabile in the brain, which is characterised by non-purulent lymphocytic vasculitis and perivascular infiltration. A total of seven rete mirabile were examined histologically and were found to have mild to severe lymphocytic dominated vasculitis characterised by activated vessel endothelium and fibrinoid necrosis in the vessel walls and also lymphocytic infiltration around the vessel wall (Figure 3-29 A). There are no qPCR data to measure virus DNA loads in these tissues. The viral Ov2.5 and ORF65 transcripts and Ov8 antigen were seen in all samples and observed in large proportions infiltrating in lymphocytes around the vessels, in vascular activated endothelial cells, in lymphoblasts and arterial wall myocytes (Figure 3-29 B,C,E,F). In addition, the majority of infiltrated leukocytes were shown to be T cells (Figure 3-29 C).

In the brain, the architecture appeared normal, but there were congestion and perivascular cuffing with lymphocytes and macrophages with necrosis of the arterial endothelium. The viral transcripts were revealed in neurons, vascular endothelium and infiltrating lymphocytes.

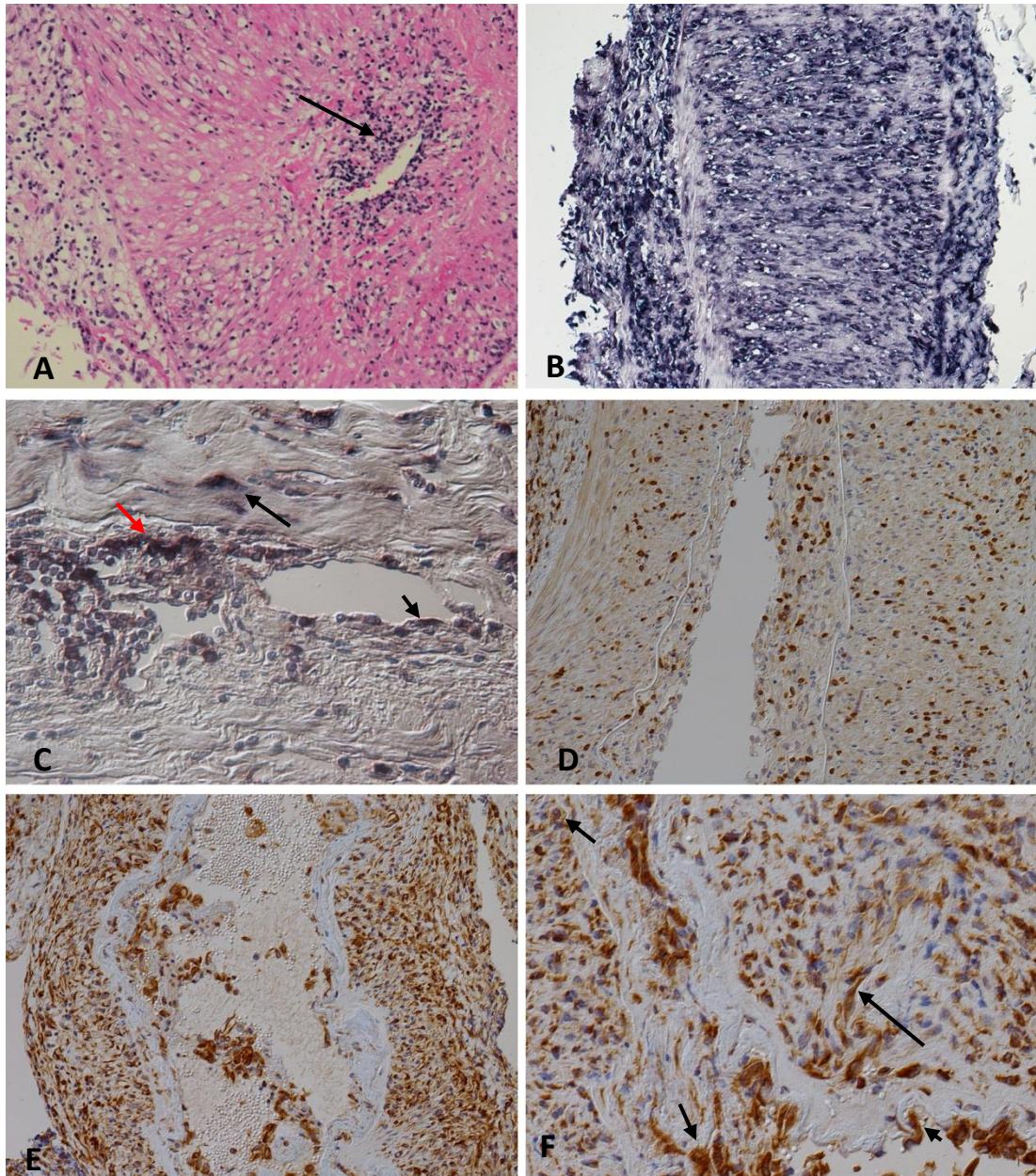


Figure 3-29. Histology, RNA-ISH and immunohistochemistry for T cells marker and viral Ov8 antigen in rete mirabile. An 18 month-old water buffalo with MCF (S11-0654).

(A) Moderate trans mural lymphocytic infiltration (arrow), (200x) **(B)** RNA-ISH for Ov2.5, extensive signals (dark blue) in most of cells through the section (200x). **(C)** Higher magnification, ISH signals are seen in infiltrating lymphocytes (red arrow), VEC (short black arrow), smooth muscle cells or probably fibroblasts (long black arrow), (400x). **(D)** IH for CD3, showing that the majority of infiltrating leukocytes are CD3⁺ T cells (brown), (200x). **(E)** IH for viral antigen (brown), the antigen is expressed in majority of cells through the section, (200x) **(F)** Higher magnification, virus antigen is expressed in infiltrating lymphocytes (medium arrows), VEC (short arrow), smooth muscle cells and probably fibroblasts (long arrow), (400x). Haematoxylin and eosin (histology); BCIP/NBT (RNA-ISH), DAB (IH), haematoxylin counterstain.

3.3.2.5 Controls

Negative control sections served to check the specificity of the probes and reliability of the results obtained with the antisense probes. The sense probes were tested in lung and other tissue sections and did not yield any signal in any of the tissues (Figure 3-30 A & B). However, with the ORF65 sense probe, a diffuse background staining was seen in some sections.

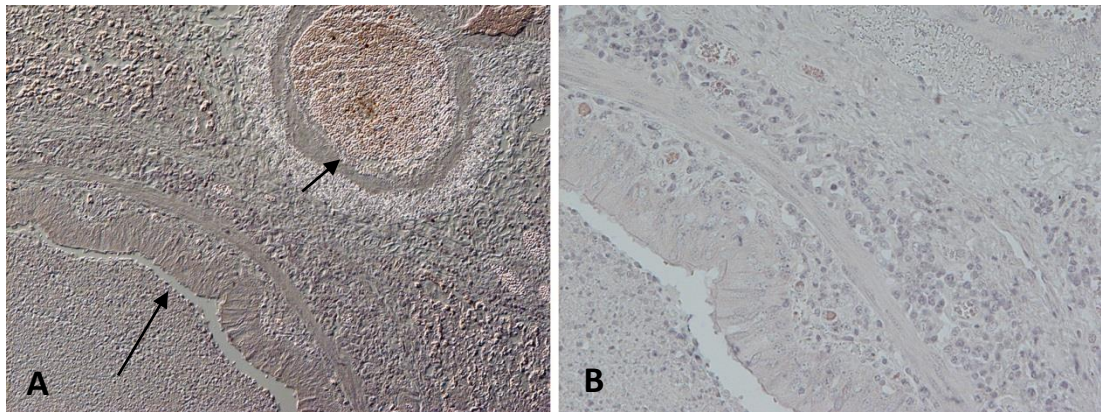


Figure 3-30. RNA-ISH negative controls for Ov2.5 sense probe in lung. An 11 month-old cow with MCF (12L-0200 B, see Figure 3-24 to compare antisense ISH signals).

(A) Lung bronchus (arrow) and an adjacent vessel (short arrow), there are no definitive ISH signals through the section (200x). **(B)** Higher magnification of A (400x). BCIP/NBT, haematoxylin counterstain.

CHAPTER FOUR: DISCUSSION

4.1 OvHV-2 infection in the reservoir host, sheep

4.1.1 The qPCR set up for quantification of OvHV-2

A highly sensitive qPCR optimisation and validation was necessary to assess OvHV-2 load in animals, especially those that have low virus loads such as sheep and cow without MCF. When both nested PCR and one step qPCR methods were evaluated, the qPCR was found to be more sensitive, time saving and simpler for quantification of the virus DNA copy numbers. The nested PCR had a number of drawbacks such as it was complicated to calculate the viral copies because the first round amplification did not always give consistent or even amplification of the standard serial dilutions; thus, using standard templates from the first round PCR did not always yield an appropriate standard curve in the qPCR. This situation probably exists when dealing with herpesviruses at low viral copies. For example, another gammaherpesvirus, BoHV-6, has low viral loads in cattle. Here too, the qPCR was shown to be more sensitive than the nested PCR (Kubiś *et al.*, 2013).

Eventually, after a series of optimisations, the one-step qPCR was enabled to detect one copy of the viral target DNA per reaction mix when tested on standards from 1 to 1×10^5 copies. The amplification plot yielded a linear standard line (Figure 3-1). The final viral DNA copy numbers were obtained after normalisation, using the mammalian housekeeping gene 12S RNA. Our TaqMan qPCR setup that amplifies the OvHV-2 ORF63 gene has a lower detection limit than previously published qPCR protocols that were used to measure the OvHV-2 DNA loads in animals. These protocols were used to amplify conserved MCFV DNA polymerase (DPOL) gene of a minimum detection limit of 50 virus copies (Cunha *et al.*, 2009), or targeted OvHV-2 ORF63 tegument gene with a minimum detection limit of 10 virus copies (Hüssy *et al.*, 2001). This makes the protocol developed in this study a useful tool to monitor the OvHV-2 loads and also for diagnostic purposes for SA-MCF.

4.1.2 Amounts of OvHV-2 in sheep

Sheep are the known natural host of OvHV-2 and are infected without developing MCF under natural conditions, as reviewed in chapter one. In sheep, different tissues were found to harbour OvHV-2 (Hüssy *et al.*, 2002). In our study, a number of randomly-selected naturally-infected sheep were tested by the qPCR, and the

quantification data showed high variability in OvHV-2 DNA copies in the examined sheep population (Table 3-2). However, virus loads were variable and generally low, the overall median value was 5.5 copies per 100 ng genomic DNA. This very high variability could be due to a number of factors: first, the samples were taken from a heterogeneous population of animals at different ages; each individual was infected at an unknown time point and could have been exposed to variable initial viral doses. This is in contrast to a scientific project where age and viral inoculum dose can be monitored. Secondly, the nature of the short episodic shedding pattern of OvHV-2 in sheep that especially happens between the ages of about 5 – 9 months. The viral loads generally subside after the age of nine months (Li *et al.*, 2001a; Li *et al.*, 2004). Sampling might not have coincided with the episodes of shedding except probably for few young sheep, which possibly were shedding the virus. The detection of viral transcripts (Ov2.5 and ORF65) and Ov8 antigen (by RNA *in situ* hybridisation [RNA-ISH] and immunohistology [IH] for viral antigen) yielded more consistent results than the qPCR. This is because of the larger anatomical size and the number of cells examined proportion of cells exhibiting viral mRNA in the section. Since only very small portions of tissues (approximately 1 x 2 x 1 mm) were used for DNA extraction and qPCR compared to the large area (generally approximately 1 x 2 cm) which were used for RNA-ISH or immunohistology.

In the 28 sheep tested by qPCR, only two animals had relatively very high viral loads in their organs, meaning a possible viral shedding status, while all the others were relatively much lower, indicating a latent status infection. Evaluation of the results of detecting OvHV-2 DNA by qPCR in sheep according to ascending age (Table 3-1), indicated that from 7 - 11 month-age, the viral DNA was readily detectible and that animals tend to be positive for viral DNA in most if not all of their organs. Although not as many samples were tested, the viral quantification results from sheep peripheral blood leukocytes (PBLs) showed that all blood samples (n=5) were positive with low virus loads. This can make blood a favourable and easy to collect sample to test for OvHV-2 presence in sheep over the nasopharyngeal swabs where only three of six swabs were positive.

4.1.3 Localisation of OvHV-2 infected cells in sheep

It has been previously shown that sheep can shed OvHV-2 (Li *et al.*, 2004), but details about the cells that can support the virus replication are not well described. Because of the unavailability of an *in vitro* system to support OvHV-2 propagation, it was not known which cell types can support either lytic and latent phases of OvHV-2. It is suggested that transmission of OvHV-2 from persistently-infected sheep to naive sheep is through nasal secretion that can contain high numbers of the virus (Li *et al.*, 2004). Hence, respiratory airways are an important location to study the mechanism of OvHV-2 infection. Using fluorescence immunohistological techniques for capsid protein (ORF25, lytic cycle product), it was shown that respiratory alveolar epithelial cells are lytically infected in experimentally infected sheep early during infection (Taus *et al.*, 2010). This indicated that those cells were lytically infected, not latently.

In our study approach, we have used RNA probes to latent and lytic genes transcripts (Ov2.5 and ORF65 respectively) to identify cells that support either infection cycles of OvHV-2. A precise *in vivo* detection technique to test a wider range of tissues was needed to provide information about *in situ* virus localisation and whether the infection is lytic or latent. We developed and validated riboprobes for RNA-ISH to detect and localise OvHV-2 latent and lytic transcripts in the cells that transcribe those viral genes. In addition to the RNA-ISH, an immunohistological technique was applied to detect a viral glycoprotein (a lytic cycle protein, product of OvHV-2 Ov8 gene), using a specific polyclonal antibody. As a result, it was shown that a wide range of cells in which both transcripts were found at the same time, suggesting either a lytic or abortive lytic infection. Additionally the viral antigen was found in same cell types such as RNA-ISH and additionally in other cells in sheep. Generally, by the RNA-ISH, in respiratory and alimentary tract, lymphoid tissues, and reproductive organs, infected cells were detected as the following cells types: B lymphocytes, macrophages, mucosal and glandular epithelial cells, type II alveolar cells, and vascular endothelial cells (VEC). While the viral antigen, was found in follicular dendritic cells, macrophages, and epithelial cells, VEC and fibroblasts (especially in tissues where the viral loads were high in the last two cell types). This difference in transcription/expression by the OvHV-2 in lung and other organs

indicates that OvHV-2 biology is not the same in different cell types as it transcribes genes in certain cells and expresses Ov8 protein (and possibly other proteins) in specific cell types. This implies that in cells where the Ov8 protein was seen, may support virus replication as it is a structural protein (putative glycoprotein). On the other hand, according to Ov8 staining results, it was noticed that the type of infected cells was rather viral load dependant, i.e. in tissues with low virus load, the Ov8 antigen was only seen in FDC (figure 3-4 A), while with high virus loads the antigen was found in many cell types such as epithelial cells, VEC, fibroblasts, macrophages and FDC (Figure 3-5 C,D).

In addition to the previous data of Taus *et al.*, 2010, besides the alveolar type II cells, we have identified a wider range cell types that are targeted by and may support OvHV-2 replication such as other epithelial airway cells, VECs and lymphocytes (Figure 3-2 A,B). Additionally, Ov8 antigen was expressed in scattered individual type II pneumocytes and peribronchial fibroblasts (Figure 3-4 B,C,D). In sheep, BALT are well developed (after one month of age) and have a follicle-like compartment containing a secondary follicle consisting of aggregates of B cells, few CD4⁺ T cells, macrophages and dendritic cells and outside of follicles are T cells (*reviewed in* (Liebler-Tenorio & Pabst, 2006)). The RNA-ISH signals (Ov2.5 and ORF65) were observed as a distinct pattern of clusters of positive cells within the BALT follicle and then we have confirmed that these clusters are predominantly B-lymphocytes (Figure 3-3 A-F). The epithelial cell infection by OvHV-2 in sheep lung resemble that of other gammaherpesviruses such as the murine herpesvirus 68 (MHV-68) in mice lungs (Stewart *et al.*, 1998).

In the tongue, viral transcripts were observed in superficial epithelial cells, salivary gland epithelial cells and VEC in the submucosa (Figure 3-5 A,B) and ov8 antigen was found in sub-epithelial VECs and in outer epithelial cells (Figure 3-5 C,D). Similarly, in the muzzle, viral transcripts were revealed in epidermal cells, glandular epithelium and hair follicles epithelial cells (Figure 3-5 E), the ov8 antigen was expressed in (epi)dermal basal epithelial cells (Figure 3-5 F) where those cells are actively proliferating, and in macrophages. Infection in tongue and muzzle suggests that contact transmission is another important route of OvHV-2 transmission, especially when dams lick neonate lambs face and the tongue and

muzzle come in contact with the neonate. This may explain finding of the viral transcripts in very young lambs (the five weeks-old [Figure 3-5 A] and the three month-old).

In lymphoid organs such as the lymph nodes, the viral transcripts were seen mainly in lymphocytes in cortical follicles, and in few interfollicular cells (Figures 3-6 A-F), and in the spleen specifically and predominantly at the mantle zones (Figure 3-9 A,B). In both organs, the infected lymphocytes were confirmed to be B cells (Figure 3-7 A-D, 3-9 A-D). The ov8 antigen was specifically found in a proportion of macrophages and FDCs (Figure 3-8 A,B, 3-9 E,F). Types of infected cells in these two organs is again similar to tropism of MHV-68 to B cells and macrophages (Sunil-Chandra *et al.*, 1992). Macrophages and FDCs cells may also support latent OvHV-2 infection, as in case of MHV-68 in mice (Flano *et al.*, 2000). However only two samples of thymus in sheep was tested, but they were negative for viral DNA, transcripts or antigen in sheep.

Mantle zones are composed of mainly mature B cells located at the periphery of the follicle; these cells are responsible for antibody production and are long-lived cells. Thus the OvHV-2 possibly infects B cells to ensure its persistence as is the case with EBV where infected B cells are activated to produce specific immunoglobulin and become memory B cells thus avoiding apoptosis (Longnecker *et al.*, 2013). In terms of the cell types infection, OvHV-2 may be partially similar to KSHV (HHV-8) which shows latent infection in B cells and endothelial cells (Blossom & Damania, 2013). Although, the virus may be in a latent state in B cells owing to the detection of the Ov2.5 (latent and lytic transcript), the ORF65 (lytic transcript) was also found, but the Ov8 antigen was not expressed. The lack of viral Ov8 antigen in B cells implies that those cells may not support a replicative cycle for the virus but only transcribe some lytic mRNAs in a form of abortive infection.

Finding of the Ov8 antigen in FDC and possibly in macrophages, as these cells are phagocytes, implies they could have phagocytized infected cellular debris or possibly virus particles. The ISH signals were also seen in cells that were possibly macrophages, but because of the harsh preparation of tissue section for RNA-ISH and diffusion of colour signals may not be according to the cell morphology, this makes it difficult to morphologically distinguish between macrophages and FDC

based on RNA-ISH staining. However, OvHV-2 may not replicate in macrophages, but it infects and partially transcribe and express its genes (Ov8 in our case), as the virus might have been transmitted to macrophages from infected epithelial cells, endothelial cells or the B cells by phagocytosis.

Finding OvHV-2 transcripts (and DNA) in PBLs (Figure 3-10 A) extends another previous finding (Hüssy *et al.*, 2002) that suggested peripheral blood leukocytes play a role in the systemic spread of OvHV-2 to all body tissues. However, the PBL subtypes were not confirmed in our study, but morphologically, infected cells were consistent with lymphocytes or probably monocytes. In a previous study it was shown by FACS (Fluorescence-activated cell sorting) that CD2⁺/CD4⁺ T cells and monocytes were positive for viral DNA (Meier-Trummer *et al.*, 2010). Nevertheless, in our study we showed that B cells were virus-positive in tissues, but in the PBL pellet section. A comparison of the ratio of ISH-positive cells to stained CD3⁺ or Pax-5⁺ lymphocytes, revealed no possible comment which cell is the ISH-positive lymphocyte or monocytes.

Although viral transcripts or antigen were not found in the uterus, but the viral transcripts and antigen were found in the placental epithelium (Figure 3-11 A) and the stromal fibroblasts and in VEC, (Figure 3-11 B), giving rise to the possibility of intrauterine foetal infection through the placenta.

Generally, results in sheep fit with a model of respiratory spread of OvHV-2. The virus first infects alveolar cells; then it is picked by pulmonary macrophages by phagocytosis of the infected cell. Macrophages, as APC, then traffic the virus to BALT where B cells become infected and then from their the virus spreads systemically.

4.2. OvHV-2 infection in cows without MCF

4.2.1 Detection and amounts of OvHV-2 DNA

Domestic cattle (cows) are one of the susceptible hosts of MCF viruses worldwide, and the disease has been reported since the 1800s (Werner & Hugh, 2008). PCR screening to detect OvHV-2 infection in cattle populations has been done previously in a few studies. This is in contrast to naturally-infected sheep populations that were extensively surveyed and OvHV-2 has been detected and the shedding patterns

has been analysed (Hüssy *et al.*, 2002; Hüssy *et al.*, 2001). In a survey, dairy cattle were periodically examined by PCR (and ELISA for MCFVs) for existence of OvHV-2 DNA. The study has shown that more than half (17 of 30 [57%]) of the population had intermittent positive PCR results (at least in one sample over the 18 months study time) in nasal and ocular swabs, blood and milk, and none of those cattle had clinical signs of MCF (Powers *et al.*, 2005). In our study, we have examined random heterogeneous populations of either clinically-normal cows (including fetuses) obtained from an abattoir in north England or cows submitted for a diagnostic post mortem examination to the Institute of Veterinary Pathology, Vetsuisse Faculty, University of Zurich, Switzerland. Surprisingly, we have shown that a large proportion of cattle harboured OvHV-2 DNA, viral transcripts (Ov2.5 and ORF65) and Ov8 glycoprotein antigen in most of their organs. The qPCR test results showed that overall 26 of 39 (67%) cattle harboured viral DNA in least one organ. Later we have extended these results by finding viral transcripts and antigen by RNA-ISH and immunohistology. In addition, six of nine (66.7 %) cattle where only PBL was tested, were positive by qPCR.

Positively-tested cattle showed similar OvHV-2 DNA loads as sheep which were highly variable, but generally lower compared to those in sheep, and less positive organs per individual bovine animal compared to sheep (Table 3-4). Also interestingly, three of seven fetuses (43 %) were positive for viral DNA by qPCR. The results in fetuses suggest trans-uterine (vertical) virus transmission. In the qPCR-positive cattle, the virus DNA copies were very low and variable between one and 156 copies per 100 ng genomic DNA, except for two cases which had relatively high viral DNA copy numbers. This implies that that majority of cattle possibly have subclinical viral infection, while detection of such a high viral load in the two cases (a stillborn calf and a one year-old cow), suggests a replicative viral infection, otherwise without development of MCF. The variability in viral DNA copies in cattle can be due to the same reasons discussed in qPCR data in sheep (see 4.1.2), which are: first, patchy distributio of viral-infectedcells and the those areas may not have been taken for qPCR, and secondly, the nature of low virus copes in these animals.

In this study, it was unexpected to detect OvHV-2 DNA in such a high frequency of cattle that were not showing any evident clinical signs or lesions of

MCF. The PCR data does not give information about the localisation of the virus in cells in tissues or whether the animal is shedding virus, so we used RNA-ISH and IH for that purpose.

4.2.2 Localisation of OvHV-2 infected cells in cows without MCF

Domestic cattle are considered a susceptible host for OvHV-2 infection and they are thought to develop clinical signs of MCF when infected with the OvHV-2. In addition to the qPCR where 67 % of animals were positive, more animals (approximately 90 %) were shown to be positive by RNA-ISH. Only four cows were negative (three foetuses and one mature animal) of the 42 tested cattle.

Either Ov2.5 and ORF65 transcripts and Ov8 antigen were always invariably found in a certain range of cell types, including epithelial cells, VEC, lymphocytes, macrophages, dendritic cells, and sometimes fibroblasts and smooth muscle cells. This is the first time that cattle have been shown to be subclinically infected with OvHV-2, in a similar way to that of sheep.

Lung is likely the primary organ for OvHV-2 entry, infection and replication of the virus in cattle, as suggested in other studies (Taus *et al.*, 2006). In our study, in a cattle population, 36 % of the lungs were qPCR-positive and even more was positive by RNA-ISH. The ISH signal intensity was variable, and generally did not correspond with the virus DNA copies in a particular tissue. In some samples the lung were positive by RNA-ISH and negative by qPCR. This could be because of the relatively patchy viral localisation in lung tissues and the low proportion of OvHV-2-infected cells. But with the RNA-ISH, a larger area of tissue is examined and picking of viral transcripts is much more likely possible. In cattle lungs, the pattern of infection was similar to sheep lungs. Thus, the infected cells (Ov2.5 and ORF65 transcripts and Ov8 antigen) were epithelial cells (alveolar type II cells, bronchiolar columnar epithelial cells); VEC; BAL leukocytes (lymphocytes, macrophages and dendritic cells); and pulmonary macrophages (Figures 3-13 A-F, 3-14 A-D). However, very occasional B cells were detected in the lung BAL and they were the infected lymphocyte type (ISH/Pax5-positive), while the stained T cells were not infected (CD3-positive/ISH-negative) (Figure 3-14 A-D).

The type OvHV-2-infected cell types in cattle lung is partially similar to what is found in sheep where alveolar cells were infected (Taus *et al.*, 2010). In addition, the type of infected cells in cattle lung (epithelial and endothelial cells and macrophages) resembles that of other gammaherpesviruses such as MHV-68 in mice and EBV (Stewart *et al.*, 1998; Sunil-Chandra *et al.*, 1992; Weck *et al.*, 1999). Finding infected interstitial and alveolar macrophages, indicates either the virus is probably picked by these cells from virus-infected alveolar cells by phagocytosis, or they are infected by free virions. Then the virus may replicate and spread from these cells to neighbour cells then to B lymphocytes then to systemic lymphocytes through blood as we have seen PBL ISH-positive leukocytes (Figure 3-21 A).

Ov2.5/ORF65 transcripts and Ov8 antigen were also found in cattle tongue (and muzzle) in VECs cells, mucosal leukocytes and especially in glandular epithelial cells and in mucosal epithelial cells (Figure 3-15 A-D). An interesting observation of the RNA-ISH in tongue, was that generally the Ov2.5 transcripts were in basal epithelial cells (Figure 3-15 A,B), while the ORF65 transcripts were in more superficial epithelial cells. An explanation of this is that the virus may have a lytic abortive infection of more superficial cells, as these cells normally slough off and may release the virus. While Ov2.5 RNA was generally detected in basal cells, suggestive of latent infection. This finding in tongue explains the detection of the virus in early age calves, as the dam licks the neonate calf after parturition. Horizontal transmission to other cows in a farm can possibly occur by contamination of the grass or water trough with oral secretions of OvHV-2 infected cow during pasturing or drinking.

In lymph nodes and spleen, ORF65 and Ov2.5 transcripts and Ov8 antigen were found in lymphocytes, macrophages, dendritic cells and VECs (Figure 3-16 A-D, 3-18 A-F, -19 A-D) in the periphery and in the centre follicle in lymph nodes, and at the mantle zones in spleen. In both organs, B cells were infected by the virus (Figure 3-17, 3-20 A,B). These might be the latently infected B cells by OvHV-2, similar to other gammaherpesviruses such as Epstein Bar virus (Falk & Ernberg, 1993) and Kaposi's sarcoma-associated herpesvirus (Chen & Lagunoff, 2005).

Virus DNA was also found in PBL in normal cattle. This is in agreement with a previous study where OvHV-2 DNA found in blood samples from healthy dairy cattle

without MCF at different intervals during a 20 months study (Powers *et al.*, 2005). Our results also showed the infected cell types, a considerable proportion of circulating leukocytes with the morphology of lymphocytes and monocytes (Figure 3-21 A) transcribe viral genes (Ov2.5/ORF65), and a few sporadic cells (possible monocytes) were expressing viral Ov8 antigen (Figure 3-21 B). Thus, the virus infects a proportion of cells, but it may not efficiently replicate in all infected cells. The virus can possibly replicate in circulating monocytes but not in B cells according to the results in PBL. Comparing the ratio of ISH-positive cells to stained lymphocytes types (B cells 2-3 %, T cells 20-30 %) in the PBL sections (Figure 3-21 C,D), implies that either monocytes are the majority of cells containing viral transcripts, or there is a possibility that a proportion of T cells are infected in the PBLs, unlike lymph nodes, spleen or lung. The proportion of PBL positive samples (66 %) makes blood a favourable and easy-to-collect sample for diagnostic purposes to detect OvHV-2 subclinical infection in healthy cattle.

As in the qPCR tests, Ov2.5/ORF65 transcripts were found in uterus and placenta in a range of cells including stromal cells, glandular epithelium and VECs (Figure 3-22 A-D). Presence of OvHV-2 (and owing to that a proportion of the foetuses were found to be infected) in these organs indicate possible trans-uterine virus transmission from uterus to placenta and then to the foetus. However, it is not known yet whether this infection causes any harm to the foetus or not. In humans, intrauterine foetal infection with herpes simplex virus (HSV), CMV and EBV are rare, but when happens it may cause severe diseases and different foetal malformations (*reviewed in* (Avgil & Ornoy, 2006)).

High viral loads were detected in a stillborn foetus (case no. S13-1438), this was interesting as the calf was aborted because of a non-infectious, but otherwise unknown cause. There could be a relationship between the stillborn and the high OvHV-2 loads. The death also could be related to other gammaherpesviruses that were previously isolated from aborted calves such bovine herpesvirus 6 (BoHV-6) (Gagnon *et al.*, 2010). Viral DNA was not readily detected by the qPCR in early gestation in foetal calves, however they were positive by RNA-ISH. From the results of foetuses, it is possible that a proportion of calves are born as OvHV-2-infected, or they could have picked the virus soon after birth when licked by infected dams (via

the tongue and muzzle). These results can possibly explain why calves at a very young ages (five-weeks old) were reported to have MCF in other studies (Abu Elzein *et al.*, 2003; Luvizotto *et al.*, 2010).

4.3 OvHV-2 in cattle with MCF

4.3.1 Amounts of OvHV-2 DNA in SA-MCF

Cattle with clinical MCF were examined by qPCR to assess the OvHV-2 DNA loads. It was noticed that there were significantly higher OvHV-2 DNA copies (generally about three logs higher) (Table 3-8) compared to sheep or cows without MCF. Generally, lymph nodes had the highest viral loads with an average of 5.9×10^5 virus copies, while in non-lymphoid tissues the average was 4.8×10^3 . Thus the viral load is approximately 100 times more in lymphoid organs than in other non-lymphoid tissues. This result suggests that lymphocytes have the highest OvHV-2 loads, as they are the main component in lymph nodes.

In contrast to the dam with MCF with a very high virus DNA copies (a Javan Banteng, case no. 14L-1075), very low viral load (1-2 virus copies) was found in the aborted foetus (case no. 14L-1075L). This qPCR result in the foetus can be interpreted as either contamination with maternal material or there could be an intrauterine virus transmission and infection.

4.3.2 Localisation of OvHV-2 infected cells in animals with MCF

MCF tissue sections were histologically examined for classical MCF pathological changes, and available tissues were examined by qPCR for virus presence and measuring viral DNA loads. Tissue sections were tested by RNA-ISH for Ov2.5/ORF65 transcripts and by immunohistology for viral Ov8 protein to observe the distribution and nature of infected cells in the MCF. The characteristic histological changes were confirmative of MCF such as lymphoid proliferation, vasculitis and epithelial necrosis (Liggitt & DeMartini, 1980a; b) in examined tissue sections (Figures 3-24 A,B, 3-25 A, 3-26 A, 3-27 A, 3-28 A).

In tissues tested from animals with MCF, the Ov2.5 and ORF65 transcripts and Ov8 antigen were found in a large proportion of infiltrating lymphocytes, macrophages, necrotic epithelial cells and in the normal and activated VECs

especially in arteries. For example in lungs, strong expression of both viral transcripts and Ov8 antigen were shown in airways epithelial cells (alveolar cells and bronchus epithelial cells), perivascular and peribronchiolar infiltrated lymphocytes and macrophages, circulating leukocytes inside the vessels lumens and VECs (Figure 3-24 C-E, 3-25 B). In the alimentary tract, viral transcripts and antigen were found in necrotic epithelial cells, infiltrating lymphocytes through the submucosa (Figure 3-26 B,D). In the lymph nodes and spleen, infected cells were mainly proliferating lymphocytes at the periphery and centre of follicles and in many cells in the T cell zones, macrophages and VECs (Figure 3-27 B,C,D; 3-28 B,C,D).

FDC were found to be positive by immunohistology for Ov8 antigen, this is probably because those cells uptake the virus particles as antigen presenting cells, or they could be infected. MCF is characterized by non-purulent vasculitis in rete mirabile in brain (Brown *et al.*, 2007). In the rete mirabile which is a blood vessel-rich tissue, we demonstrated viral transcripts and antigen in infiltrated perivascular lymphocytes and in VECs, also in lymphoblasts and arterial wall myocytes (Figure 3-29 B,C,E,F). In addition, the majority of infiltrated leukocytes in rete mirabile were T lymphocytes (Figure 3-29 D). These infiltrated leukocytes were confirmed to be CD8⁺ T cells in previous studies (Nelson *et al.*, 2010; Simon *et al.*, 2003). In addition to CD8⁺ T cells, a proportion of the infiltrating lymphocyte phenotype were shown to be CD43⁺ T cells, which proliferate *in situ* in non-lymphoid tissues (Schock & Reid, 1996), these cells bind to endothelium that may contribute to the vasculitis in MCF.

4.3.3 General discussion of OvHV-2 infection in sheep, cows without MCF and cattle with MCF

Unlike other gammaherpesviruses that have a relatively a narrow host range, OvHV-2 has a wider range of hosts and can infect and induce disease in sheep (Li *et al.*, 2005b), goats (Jacobsen *et al.*, 2007), cattle (Reid *et al.*, 1986), bison (Berezowski *et al.*, 2005), deer (Audigé *et al.*, 2001), swine (Albini *et al.*, 2003b), and also experimentally in rabbits and hamsters (Buxton & Reid, 1980; Reid *et al.*, 1986) and sometimes rarely in other species such as horses (Costa *et al.*, 2009b). The susceptibility of all of these species is not the same, some are more liable to

infection such as bison and others are more resistant such as domestic European cattle (Li *et al.*, 2014).

In our study, in both sheep and cattle without MCF, OvHV-2 was shown to infect almost the same cell types, namely epithelial cells in mucosa and glands of muzzle, tongue and bronchial airways, alveolar cells and reproductive organs (in female), VECs in most of the organs, and in antigen presenting cells (B cells, macrophages and dendritic cells) in BALT, lymph nodes and spleen. However, these cells were not always infected in a given tissue. Detection of OvHV-2 DNA; ORF65/Ov2.5 transcripts; and Ov8 antigen in the absence of the clinical signs of MCF in such a high ratio in cattle populations, is an evidence that cattle are potential carriers of OvHV-2 similar to sheep. However, OvHV-2 is seemingly less adapted to cattle, and from detecting the very low viral loads, cattle are assumed to be latently lifelong infected with OvHV-2. This since it is known that once a gammaherpesvirus infects a host, it either induces disease (and sometimes death) or lifelong infection of the host (Pellett & Roizman, 2013).

The difference in OvHV-2 infection between sheep and cattle, is that in sheep, generally more tissues/organs per individual animal harboured the virus and the viral titres were relatively higher than in cattle. However, OvHV-2 subclinical infection in MCF-susceptible species were also previously suspected. Antibodies to OvHV-2 were detected in the blood of domestic cattle, bison, elk and deer breeds (Benetka *et al.*, 2009; Frölich *et al.*, 1998; Li *et al.*, 1996; Powers *et al.*, 2005). Although, finding antibodies does not always mean a subclinical infection, as it can also be due to recovered disease. In our study, according to the RNA-ISH and Ov8 antigen results, OvHV-2 infection in domestic cattle is a mix of latent and lytic infection, and since there is no MCF evidence, the animal is considered as subclinically infected, similar to sheep.

In a recent study, a proportion of clinically normal white-tailed deer were tested positive (72 %) for OvHV-2 DNA in their PBL (Palmer *et al.*, 2013), this is a similar ratio to that found in cattle (67 %) in our study. However, the infection incidence is higher than that (as high as 90 %) as shown by RNA-ISH. This is because that a low proportion of cells are infected (as shown by th RNA-ISH) that makes it difficult to find viral DNA, but the *in situ* hybridisation technique examines a big

tissue area (generally 1 cm x 1 cm) and the chance of encountering virus infected cells is much higher. Other studies support the idea that cattle can normally harbour OvHV-2. This is when the viral DNA was identified in clinically healthy cattle blood, lymph nodes and spleen (Kojouri *et al.*, 2009; Yazici *et al.*, 2006) and in American bison (Sausker & Dyer, 2002). In addition to OvHV-2 DNA, 15-A monoclonal antibody was used to detect MCFVs seroprevalence in cattle population, it was found that about 15% were positive (Yeşilbağ, 2007), meaning that animals were exposed to one of the MCF viruses.

Sheep are carriers and they shed the OvHV-2 through their nasal and lacrimal discharges (Li *et al.*, 1998; Li *et al.*, 2004). However, we have not collected nasal swabs from cattle, but it is possible that cattle shed cell-free virions since tongue and respiratory epithelium were shown to contain OvHV-2 DNA, transcripts and protein. To detect shedding virus by cattle, PCR survey for nasal and oral swabs can be done in the field.

An interesting point should be commented on that probably contribute the nature of OvHV-2 shedding and may underlie the higher viral loads in sheep lung, is the BALT structure difference between sheep and cattle. The BALT structural organisation is well developed in sheep, having secondary follicles that contain many B cells. We showed that the majority of these B cells are infected by OvHV-2. Hence there are many cells harbouring the virus, consequently viral shedding is most likely to happen. In cattle, BALT has no such secondary follicle structure and/or it is less frequent in number through the lung (Anderson *et al.*, 1986). Thus, in cattle lung, there are less B cells (that can play role as OvHV-2 reservoir) and consequently less proportion of infected cells. This can probably imply why we have observed cattle lung generally had lower viral loads and were less frequently positive compared to sheep. Additionally, it can be seen from the qPCR data in cattle lung that after one year-age until two year-age, the OvHV-2 DNA is more readily detectable. This could be related to (the increasing number of) developing BALT and eventually B cells. After that age (one year), the cattle BALT fully develops at the age of 18 months, then the number of these starts to decline (Anderson *et al.*, 1986). Apart from more infected B cells, the rest of infected cell types in the lung of cattle were similar to sheep.

As mentioned, the range of infected cell types in sheep and cattle without MCF were very similar. Interestingly the infected cells types in clinical MCF is almost the same as in cattle without MCF. In this regard, in addition to the histopathological changes, there are two key differences between normal cattle (non-MCF) and cattle with MCF. First, in MCF there is significant increase (more than three logs) in OvHV-2 DNA copy numbers (about 189×10^3 viral DNA copies on average among all organs) compared to those in normal cattle. This is evidence that the number of viruses (i.e. own virus) acts an important role in the development of the clinical signs of MCF, as there is a sharp increase in viral copy numbers in MCF. Secondly, the difference between the infected lymphocyte subtype, B cells are infected in normal cattle, while in clinical MCF, the infected lymphocyte type is predominantly T cells (mainly CD8⁺) (Nelson *et al.*, 2010; Simon *et al.*, 2003).

It was shown in experimental AIHV-1 induced MCF in rabbits, that the CD8⁺ T cells start to proliferate without the virus being detectable in blood by PCR, but viral loads sharply increase few days before death (Dewals *et al.*, 2008). Combining this finding and showing that epithelial cells, VEC and B cells and macrophages (or dendritic cells) are infected in normal cattle (in our study), leads to a possible explanation for the induction of MCF. Which is that by an unknown mechanism (possibly by macrophages or other APCs) the virus switches from B cells to T cells. Then the virus latently infects T cell, transform those cells to cytotoxic T cells and deregulates their normal cell cycle leading to proliferation. Later, these cells attack the other infected epithelial and endothelial cells, causing the epithelial and endothelial cells necrosis in tissues. During this time the virus starts to replicate efficiently in these proliferating cells. A possible similar situation to that of OvHV-2 is EBV, as EBV normally infects B cells latently, but was seen to be associated with T/NK cells lymphoproliferative disorders that lead to organ complications and death in human, (Kimura *et al.*, 2012).

Species susceptibility to MCF is another aspect in the pathogenesis. It is shown that different species have different susceptibilities to MCF when inoculated by doses of OvHV-2 (Taus *et al.*, 2006; Taus *et al.*, 2005). Sheep were observed to be highly resistant to MCF (Li *et al.*, 2005b), however they can develop MCF-like symptoms when challenged with very high OvHV-2 doses. Cows, especially

European domestic cattle breeds are relatively resistant to OvHV-2-MCF. Hence, it seems that the immune system keeps the viral load in control to prevent the disease. In addition to classical fatal MCF, cattle can have non-fatal chronic, recovered or transient skin inflammation forms of MCF (Munday *et al.*, 2008; O'Toole *et al.*, 1997). Similarly, skin conditions caused by OvHV-2 were also reported in sheep (Pritchard *et al.*, 2008). These different MCF manifestations can be determined by many factors including initial viral dose exposure by the animal, such as low viral loads found in our study. Being exposed to low OvHV-2 loads can be the norm in cattle. Possibly only sporadic animals who are exposed to high virus doses succumb MCF. This may explain the sporadic nature of MCF among cattle herds (cows). Another possible reason of MCF induction is when the balance of viral copies is breached in the host's body due to any stimuli such as being immunocompromised, stress or infection by other pathogens.

The OvHV-2 is the only known agent associated with the disease in the case of SA-MCF. There is a far possibility that other gammaherpesviruses, especially those which are endemic in cattle contribute in the pathogenesis of MC. Gammaherpesviruses are known for encoding lymphoproliferative latent proteins that transform or dysregulate lymphocytes. An example of such a virus is BoHV-6 (Collins *et al.*, 2000). In addition to that, the caprine herpesvirus 2 (CpHV-2) DNA that was detected beside the OvHV-2 DNA in cervid SA-MCF cases (Vikøren *et al.*, 2006). It is still not known whether these viruses contribute in inducing MCF in naturally occurring diseases, but it is worthy that material from MCF cases to be tested for other bovine gammaherpesviruses (even those not known to induce MCF) such as CpHV-2, BHV-6, BHV-4.

4.4 Conclusions

In this study, the specific cells types in organs and tissues targeted by OvHV-2 in sheep was determined. Namely epithelial and endothelial cells of respiratory and alimentary tract and reproductive system, B lymphocytes and macrophages in lung, lymph nodes and spleen. Cattle are considered as the susceptible host for OvHV-2, we have shown that a large proportion of cattle infected without clinical signs of MCF, and are subclinically infected similar to sheep, and the same range of cell

types are targeted by the virus. A lower proportion of cattle are infected compared to sheep and generally the viral DNA loads are lower in cattle. From these findings and previous studies, cattle are considered as hidden carriers for OvHV-2 and they probably shed the virus in their oral secretions, owing to finding of viral DNA, transcripts and protein in their tongue, muzzle and airways epithelium. Beside mature cattle, we have found viral transcripts and DNA in tissues from cattle foetuses, indicating a possible sexual or intrauterine virus transmission. In addition, to observe the infected cell types in disease cases, material from MCF affected animals were examined and it was found that the same type of cells are infected as in cattle without MCF. These observations show that cattle are a possible reservoirs for OvHV-2. Based on this vaccination only in cattle may not be ideal in controlling of MCF, as a large number of cattle were found to be subclinically infected by the virus, but more study is needed to explain factors that contribute to pathogenesis of MCF.

APPENDIX

References

APPENDIX. Detailed results of qPCRs, histological findings, OvHV-2 RNA-ISH and immunohistology of individual animals

Table 1. List of sheep tested for OvHV-2 infection by qPCR, RNA-ISH and immunohistology.

A. List of sheep of which organs were tested, including information on the post mortem diagnosis (necropsied cases), histological features and results of RNA-ISH and immunohistology for OHV-2. Animals are listed on the order of age, from foetus onwards.

Case number; Sex/age	Origin	Block	Tissue	qPCR	Histological findings, results of OvHV-2 RNA-ISH and IH	Relevant post mortem diagnoses
S13-1453 F, 14 y [Mother]	CH	2	Mediastinal LN	6	HE: Moderate cortical and medullary sinusoidal haemosiderosis OV2.5: Signal in very few cells in sinuses and follicles OVHV-2 IH: Weak reaction in some macrophages and FDC in the follicles	Traumatic injury to front leg (open wound).
			Spleen	ND	HE: Marked diffuse hyperaemia, small follicles and T cell zones. OvHV-2 IH: Weak reaction in some macrophages and FDC in follicles.	
		3	Muzzle	0	HE: Mild dermal perivascular and periadnexal LC (LC) infiltration OV2.5: Negative	
			Nasopharynx	0	HE: Mild multifocal to coalescing subepithelial LC infiltration. OV2.5: Negative OvHV-2 IH: Reaction in subepithelial and interstitial fibroblasts in glands, and in occasional VEC	
			Placentome	2	HE: Moderate diffuse stromal oedema OV2.5: Weak signal in part of the maternal placental epithelium OvHV-2 IH: Reaction in stroma (fibroblasts) and VEC, the epithelium is negative	
S13-1453 Foetus (Crown rump length [CRL] 17.5 cm) neonate	CH	4	Spleen	0	HE: No formed follicles, marked diffuse hyperaemia OV2.5: Negative OvHV-2 IH: Faint reaction possibly in some stromal fibroblasts	
			Thymus	0	HE: NHAIR; OVHV-2 IH: Negative	
			Lung	0	HE: NHAIR, apart from foetal atelectasis OvHV-2 IH: Negative	
			Placenta	3	HE: NHAIR; OVHV-2 IH: Negative	
S13-1353 M, 5 we	CH	6	Lung	0	HE: NHAIR, apart from moderate diffused hyperaemia OV2.5: Negative; ORF65: Negative	Clostridial enterotoxaemia (Cl. perfringens type D) with catarrhal enteritis and “pulpy kidneys”; endoparasitosis
			Thymus	0	HE: NHAIR; OV2.5: Negative; ORF65: Negative	
			Spleen	0	HE: Inconspicuous follicles and T cell zones OV2.5: Negative; ORF65: Negative	
			Mediastinal LN	0	HE: NHAIR, apart from moderately depleted follicles OV2.5: Negative; ORF65: Negative	
		7	Tongue	0	HE: NHAIR	

References

					OV2.5: Signals in some epithelial cells in the papillae ORF65: Similar to OV2.5	
			Nares	0	HE: Mild multifocal subepidermal LC infiltration OV2.5: Negative; ORF65: Negative	
			Turbinate	0	OV2.5: Negative; ORF65: negative	
			Conchae	0	OV2.5: Negative; ORF65: Negative	
S14-0017 F, 3 mo	CH	1	Lung	0	HE: NHAIR OV2.5: Signals in a proportion of perivascular LC, REC and VEC	Clostridial enterotoxaemia (C; Perfringens type A or C) with catarrhal enteritis; severe endoparasitosis
			Spleen	0	HE: Inconspicuous follicles and T cell zones OV2.5: Signals in occasional LC in follicles and T cell zones and in red pulp, in VEC and fibroblasts around vessels	
			Mediastinal LN	0	HE: Moderate follicular depletion; small parasite granuloma OV2.5: Signals in a few LC in follicles and T cell zones, in VEC and arterial SMC	
		2	Uterus	0	HE: NHAIR OV2.5: Negative	
			Muzzle	1	HE: NHAIR OV2.5: Focal area with signals in squamous epithelial cells	
			Nares	0	HE: Mild multifocal subepithelial lymphocyte infiltration and lymphatic follicle formation OV2.5: Negative	
13L-2593 7 mo	UK	A	Lung	4	HE: Mild multifocal perivascular and peribronchiolar LC infiltration OV2.5: Signals as focal reaction; in proportion of perivascular and peribronchiolar LC and VEC (arteries), in occasional REC and cells in alveolar wall (possibly macrophages), in one area also intravenous LC ORF65: Similar to OV2.5 CD3, PAX-5 IH: T and B cells in similar proportions	Not applicable (tissues collected at the abattoir).
			Mediastinal LN	2	HE: Moderately sized secondary follicles; acute sinus haemorrhage OV2.5: Relatively numerous LC in follicles (also possibly TBM), occasional LC in paracortex and medullary strands (CD3, PAX-5) ORF65: Very similar to Ov2.5	
			Nasopharynx	215	HE: NHAIR (with lymphatic tissue/tonsil) OV2.5: Signals in some epithelial cells in salivary gland, LC surrounding ducts (CD3, PAX-5 IH: T and B cells in similar proportions), VEC; lymphatic tissue: see the mediastinal LN ORF65: Similar, but also signal in glandular duct epithelial cells	
			Retrophar. LN	ND	HE: Mild follicular depletion OV2.5: Relatively numerous LC in follicles (also possibly TBM), occasional LC in paracortex and medullary strands ORF65: Very similar to Ov2.5	
13L-2595 7 mo	UK	B	Lung	5	HE: Mild BALT formation; mild multifocal acute alveolar haemorrhage OV2.5: Signals in some perivascular LC and some REC and glandular epithelial cells, VEC and SMC (arteries), and chondrocytes ORF65: Very similar to OV2.5, but less intense in epithelium and cartilage	Not applicable (tissues collected at the abattoir).
			Mediastinal LN	2	HE: Moderately sized, mildly depleted secondary follicles OV2.5: Signals in relatively numerous LC in follicles (also TBM) and in occasional cells in paracortex and medullary strands	

References

					<p>ORF65: Very similar to OV2.5, but more intense (higher number of cells)</p> <p>HE: Moderately sized, mildly depleted secondary follicles</p> <p>Ov2.5: Signals in submucosal lymphocytic aggregations, VEC (arteries) and in intravascular leukocytes</p>	
		A	Nasopharynx	2		
			Retrophar. LN	ND	<p>HE: Moderate sized, mildly depleted secondary follicles</p> <p>OV2.5: See mediastinal LN</p>	
13L-2594 7 mo	UK	A	Lung	45	<p>HE: Mild to moderate BALT formation; mild multifocal acute alveolar haemorrhage</p> <p>OV2.5: Numerous perivascular and peribronchiolar LC (also in follicle) (CD3, PAX-5: T and B cells in similar proportions) and most REC and glandular epithelial cells, occasional VEC (arteries), chondrocytes (weak), arterial SMC, fibroblasts; also in some individual cells in alveolar wall</p> <p>ORF65: Very similar to OV2.5</p> <p>OVHV-2 IH: Reaction in macrophages/FDC in BALT, scattered individual type II pneumocytes, occasional chondrocytes and peribronchial fibroblasts.</p>	Not applicable (tissues collected at the abattoir).
					Mediastinal LN	
		B	Nasopharynx	22	<p>HE: Moderate mixed cellular subepithelial infiltration with several apoptotic cells (probably neutrophils and LC)</p> <p>Ov2.5: Variably intense, partially strong signal in all squamous epithelial cell layers, in LC in follicles (mainly PAX-5 positive B cells), proportion of LC in subepithelial infiltrates (mainly CD3 positive T cells), in occasional salivary gland epithelial cells; strong signals in ductal epithelial cells and VEC</p> <p>ORF65: Similar to OV2.5, but far less intense in epithelium</p> <p>OvHV-2 IH: Epithelium is negative, but reaction in some apoptotic bodies in subepithelial infiltrates, and probably occasional FDC</p>	
			Retrophar. LN	ND	<p>HE: Relatively large secondary follicles with numerous mitotic cells</p> <p>OV2.5: Signals in relatively numerous LC in follicles (also TBM?) (CD3, PAX-5: indicate signals are in B cells), occasional LC in paracortex and medullary strands and occasional intravascular LC.</p> <p>ORF65: Similar to OV2.5</p> <p>OvHV-2 IH: Reaction in moderate number of cells in follicles (macrophages/FDC) and along sinuses, also in apoptotic cells</p>	
13L-2592B 8 mo	UK	B	Lung	18	<p>HE: Moderate to marked BALT formation (with secondary follicle formation)</p> <p>OV2.5: Signals in several LC in BALT follicles; occasional signal in REC, VEC and occasional individual alveolar cells and intravascular LC</p> <p>ORF65: Similar to OV2.5, but less intense</p> <p>OV2.5 plus PAX-5: Proportion of B cells in follicle in BALT exhibit RNA-ISH signal</p> <p>OV2.5 plus CD3: T cells without RNA-ISH signal</p> <p>OvHV-2 IH: Overall relatively strong reaction in VEC, type I and II pneumocytes, macrophages, PIM and macrophages/FDC in follicle-like structures in BALT (30-40%); some leukocytes infiltrating the bronchial epithelium are also positive (REC are negative), also reaction in desquamated alveolar macrophages in alveolar and bronchiolar lumen</p>	Not applicable (tissues collected at the abattoir).
					Mediastinal LN	

References

					<p>ORF65: Similar to OV2.5, but less intense OV2.5 plus PAX-5: Proportion of B cells in follicle exhibit RNA-ISH signal OV2.5 plus CD3: T cells without RNA-ISH signal OVHV-2 IH: Strong reaction in macrophages/FDC in follicles, also in DC in sinuses and in medullary cords, in proportion of macrophages and LC in medulla (?) and in VEC</p>	
			Spleen	2	<p>HE: Moderately sized secondary follicles and T cell zones OV2.5: Signals in numerous LC (and TBM) in follicles; several LC in red pulp ORF65: Similar to Ov2.5 OV2.5 plus PAX-5: proportion of B cells in follicle exhibit RNA-ISH signal OV2.5 plus CD3: T cells without RNA-ISH signal OVHV-2 IH: Strong reaction in macrophages/FDC in follicles, also in macrophages in red pulp and in VEC</p>	
13L-2592C 8 mo	UK	C	Lung	4200	<p>HE: Moderate to marked BALT formation (with secondary follicles) OV2.5: Signals in some LC in BALT follicles, but generally very weak signal in centre of section, at margin very similar to 13L-2592B (BALT LC, REC, alveolar epithelial cells) ORF65: Very weak signals</p>	Not applicable (tissues collected at the abattoir).
			Mediastinal LN	3200	<p>HE: Large, mildly depleted secondary follicles Ov2.5, ORF65: See 13L-2592B, but weaker signals</p>	
			Spleen	3300	ND	
13L-2592A 8 mo	UK	A	Lung	1	<p>HE: Mild BALT formation; mildly increased interstitial cellularity ORF65: Numerous LC in BALT, some REC and glandular epithelial cells; VEC (arteries), occasional intravascular LC, some individual alveolar cells; OV2.5 plus PAX-5: Proportion of B cells in follicle in BALT exhibit RNA-ISH signal OV2.5 plus CD3: T cells without RNA-ISH signal</p>	Not applicable (tissues collected at the abattoir).
			Mediastinal LN	0	<p>HE: Relatively large secondary follicles with numerous TMB OV2.5 Signals in relatively numerous LC (and TBM) in follicles (CD3, PAX-5: indicate signals are in B cells), occasional LC in paracortex and medullary strands and occasional intravascular LC; VEC (arteries), the section is half destroyed. ORF65: Similar to Ov2.5 but less intense OV2.5 plus PAX-5: Proportion of cells in follicle in BALT exhibit RNA-ISH signal, but generally very weak reaction for PAX-5 OV2.5 plus CD3: T cells without RNA-ISH signal</p>	
			Submand. LN	ND	<p>HE: Moderately sized follicles with several TMB OV2.5: Signals in relatively numerous LC (and TBM) in follicles (CD3, PAX-5: indicate signals are in B cells), occasional LC in paracortex and medullary strands and occasional intravascular LC; VEC (arteries), the section is half destroyed. ORF65: Similar to Ov2.5 but less intense OV2.5 plus PAX-5: Proportion of B cells in follicle in BALT exhibit RNA-ISH signal, but generally very weak reaction for PAX-5 OV2.5 plus CD3: T cells without RNA-ISH signal</p>	
13L-4220 10 mo	UK	A	Mediastinal LN	229	<p>HE: Moderately sized secondary follicles with several TMB OV2.5: Signals in a few to some LC in follicles, rare LC in cords</p>	Not applicable (tissues collected at the abattoir).
			Spleen	276	<p>HE: Moderately sized secondary follicles with several TMB, moderately sized T cell zones OV2.5: Very weak signal in some LC in follicles</p>	
		B	Muzzle	648	<p>HE: mild multifocal dermal LC infiltration, focal serocellular crust formation with neutrophils; neutrophils also in underlying dermis</p>	

References

			Tongue	97	OV2.5: Signal in one small area (with focal inflammatory infiltrate), in a few LC and epithelial cells HE: NHAIR OV2.5: Signals in a few patches of epithelial cells (stratum spinosum)	
		C	Lung	143	HE: Moderate multifocal acute alveolar haemorrhage OV2.5: negative	
13L-4220 10 mo	UK	D	Lung	23	HE: moderate to marked BALT formation with secondary follicles OV2.5: some LC in BALT, occasional glandular epithelial cells (overall weak signal) OVHV-2 IH: Overall strong reaction in VEC, type I and II pneumocytes, macrophages, PIM and a proportion of leukocytes in the BALT (30-40%); some leukocytes infiltrating the bronchial epithelium are also positive (REC are negative), also reaction in desquamated alveolar macrophages in bronchiolar lumen, and in cartilage.	Not applicable (tissues collected at the abattoir).
			Mediastinal LN	61	HE: Moderately sized, mildly depleted secondary follicles with numerous TBM Very weak signal in some LC in follicles OV2.5: Generally weak signals in a few to some LC in follicles OVHV-2 IH: Overall strong reaction, in macrophages (and FDC) in follicles and in macrophages in sinuses, also VEC	
		E	Tongue	667	HE: Focal mild mixed cellular (macrophages, LC, plasma cells, neutrophils) subepithelial infiltration OV2.5: Weak signal, patchy in aggregates of epithelial cells (stratum spinosum) and in a few cells (possibly epithelial cells and LC) beneath these. OVHV-2 IH: Overall strong reaction, in VEC and in infiltrating leukocytes and fibrocytes/fibroblasts beneath the epithelium	
		Spleen	36	HE: Moderately sized secondary follicles with TBM, moderately sized T cell zones OV2.5: Very weak patchy signal in some LC in follicles. OVHV-2 IH: Overall strong reaction in macrophages (and FDC) in follicles and in macrophages in red pulp, in a few LC and in VEC		
13L-4218 11 mo	UK	A	Lung	15	HE: Mild BALT formation OV2.5: Occasional weak signal in LC, VEC (subpleural vessels) ORF65: Negative	Not applicable (tissues collected at the abattoir).
			Nasopharynx	4	HE: Mild focal, predominantly mononuclear (LC, plasma cells, macrophages and some neutrophils) infiltration OV2.5: Section is overexposed. Focal patchy area of signal in all epithelial cells and in underlying LC (at edge of section) ORF65: Similarly overexposed, similar signals	
		B	Mediastinal LN	27	HE: Moderately sized secondary follicles OV2.5: weak signal in some LC in follicles ORF65: Very similar to OV2.5	
			Spleen	9	HE: Moderately sized follicles and T cell zones OV2.5: Very weak patchy signal in some LC in follicles ORF65: Very similar to OV2.5	
		C	Muzzle (epidermis)	56	HE: Moderate multifocal periadnexal LC infiltration OV2.5: Signal mainly in hair follicle (and shaft) epithelial cells, not in epidermis, some infiltrating LC and some VEC ORF65: very similar to OV2.5, but more extensive signal (also in epidermis) OVHV-2 IH: Some fibroblasts and macrophages in the dermis; epithelial cells. neg.	
			Epididymis	32	HE: NHAIR, apart from mild diffuse stromal oedema OV2.5: Signals in ductal epithelial cells, VEC and arterial SMC, fibroblasts OVHV-2 IH: Weak reaction in stromal fibroblasts	

References

13L-4218 11 mo	UK	D	Lung	57	<p>HE: Mild to moderate BALT formation with small follicles, focal interstitial fibrosis and mixed cellular alveolar infiltration</p> <p>OV2.5: Signals in a few LC in BALT</p> <p>ORF65: Signals in a few type II pneumocytes in focal area with inflammation</p> <p>OVHV-2 IH: Reaction in occasional macrophage and apoptotic cell in alveolar septa and around bronchioles, occasional VEC, occasional type II pneumocytes and macrophages/DC in BALT, some desquamated alveolar macrophages/type II pneumocytes, weak reaction in chondrocytes</p>	Not applicable (tissues collected at the abattoir).
			Mediastinal LN	344	<p>HE: Large follicles with numerous TBM in particular in deeper cortex</p> <p>ORF65: Weak signals; a few large cells in follicles (probably macrophages and some LC)</p> <p>OVHV-2 IH: Moderate number of cells in follicles mainly in deeper cortex (macrophages and/or FDC) and a few macrophages in medulla along sinuses</p>	
		E	Tongue	222	<p>HE: Mild multifocal subepithelial perivascular LC infiltration</p> <p>OV2.5: Signals in very few individual epithelial cells</p> <p>ORF65: Negative</p> <p>OVHV-2 IH: Reaction in occasional subepithelial fibroblasts and some vessels with very weak reaction in VEC; epithelial cells are negative</p>	
			Spleen	108	ND	
			Muzzle	290	<p>HE: Mild multifocal dermal and periadnexal LC infiltration; mildly to moderate pigmented basal cell layer</p> <p>OV2.5: Variably intense signal in hair follicle epithelial cells (not related to pigmentation); very occasional infiltrating LC</p> <p>ORF65: Similar to OV2.5, but weaker signal</p> <p>OVHV-2 IH: Strong reaction in relatively numerous cells in basal epidermal layer, some reaction in dermal fibroblasts</p>	
13L-4219 11 mo	UK	A	Mediastinal LN	556	<p>HE: Moderately sized follicles</p> <p>OV2.5: : Weak signals; a few large cells in follicles (probably macrophages and some LC)</p>	Not applicable (tissues collected at the abattoir).
			Spleen	24	<p>HE: Moderately sized secondary follicles and T cell zones</p> <p>OV2.5: Very weak patchy signal in some LC in follicles</p>	
		B	Tongue	345	<p>HE: Moderate subepithelial LC infiltration</p> <p>OV2.5: No definite signals</p>	
			Muzzle	5	<p>HE: Moderate dermal/periadnexal LC and fibroblast infiltration</p> <p>OV2.5: Signal mainly in hair follicle (and shaft) epithelium, no definite signal in epidermis; generally weak signals</p>	
		C	Lung	131	<p>HE: Mild to moderate BALT formation with secondary follicle formation; mild increased interstitial cellularity; focal alveolar macrophage desquamation</p> <p>OV2.5: Signals in some LC in follicle-like BALT aggregates; in some glandular epithelial cells and REC, focal area with a few positive desquamated alveolar cells (probably macrophages); generally weak signal</p>	
13L-4219 11 mo	UK	D	Lung	136	<p>HE: Mild to moderate BALT formation with secondary follicle formation; mild increased interstitial cellularity; focal alveolar macrophage desquamation</p> <p>OV2.5: Signals in some LC in follicle-like BALT aggregates; some glandular epithelial cells (and REC), focal area with a few positive desquamate alveolar cells (macrophages?); generally weak signal</p>	Not applicable (tissues collected at the abattoir).
			Mediastinal LN	122	<p>HE: Moderately sized secondary follicles with some TBM</p> <p>OV2.5: Weak patchy signal in some LC in follicles</p>	
		E	Muzzle	251	<p>HE: Mild multifocal dermal/periadnexal LC infiltration, small hair granuloma</p> <p>OV2.5: Signals in mucosa and skin (some hair follicle epithelial cells, some (superficial) squamous epithelial cells, but</p>	

References

			Tongue	2	generally weak signal HE: Mild multifocal subepithelial LC infiltration OV2.5: Signal in (superficial) squamous epithelial cells, but generally weak signal	
		F	Testis	12	HE: NHAIR, active spermatogenesis OV2.5: Weak signal in epididymal ductal epithelial cells and in focal areas of germinative cells	
			Spleen	5	Tissue not on section	
S13-1516 3.5 year	CH	3	Lung	61	ND	Pulmonary adenomatosis and fibrinous pneumonia (isolation of Mannheimia haemolytica)
			Mediastinal LN	2	ND	
			Spleen	1	ND	
		4	Tongue	1	ND	
			Uterus	0	ND	
			Muzzle	0	ND	
			Turbinate	0	ND	

Abbreviations:

BALT: bronchus associated lymphoid tissue; CH: animals submitted for a diagnostic post mortem examination to the Institute of Veterinary Pathology, Vetsuisse Faculty, University of Zurich, Switzerland; F: female; FDC: follicular dendritic cells; HE: haematoxylin and eosin stain; IH: immunohistology; LC: lymphocyte; LN: lymph node; ND: not done; M: male; mo: month; NHAIR: no histological abnormality is recognised; PIM: pulmonary intravascular macrophages; REC: respiratory epithelial cells; SMC: smooth muscle cells; Retrophar.: retropharyngeal; Submand.: submandibular; TBM: tingible body macrophages; UK: healthy animals slaughtered in abattoir in the UK; VEC: vascular endothelial cells; we: week; y: year.

References

1B. List of sheep of which only white blood cells (WBCs) tested, including information on the histological features and results of RNA-ISH and immunohistology for OHV-2. Relevant post mortem diagnoses is not applicable as blood collected at the abattoir.

Case number/age	Origin	Block	qPCR	Histological findings, results of OvHV-2 RNA-ISH and IH
13L-4883 A 9 mo	UK	A	6	HE: Blood cell pellet with leukocytes embedded in fibrin with a few erythrocytes OV2.5: Large proportion of cells with signal (30-40%), morphology consistent with LC ORF65: Similar to OV2.5 PAX-5: Approximately 30% of cells positive (B cells) CD3: Approximately 50% of cells positive (T cells) OVHV-2 IH: Negative
13L-4883 B 9 mo	UK	B	5	Ov2.5: Large proportion of cells with signal (>50%), morphology consistent with LC (very small pellet with only few leukocytes) Pax5: B cells comprise 10-20 % of the nucleated cells. CD3: Approximately 50% of nucleated cells positive (T cells) OVHV-2 IH: Negative (very small pellet with very few leukocytes)
13L-4883 C 9 mo	UK	C	9	HE: Blood cell pellet with leukocytes mixed with fibrin and erythrocytes ISH, IH: ND
13L-4883 D 9 mo	UK	D	8	HE: Blood pellet of fibrin, with focal are with embedded leukocytes and erythrocytes ISH, IH: ND
13L-4883 E 9 mo	UK	E	5	HE: Blood cell pellet with leukocytes mixed with fibrin and erythrocytes ISH, IH: ND

Abbreviations:

HE: haematoxylin and eosin stain; IH: immunohistology; LC: lymphocyte; mo: month; y: year.

1C. List of sheep, of which nasopharyngeal swabs were taken at an abattoir in the UK for OvHV-2 qPCR only.

Sample	Age	qPCR
Swab 1	8 month	6
Swab 2	8 month	297
Swab 3	8 month	0
Swab 4	8 month	0
Swab 5	8 month	0
Swab 6	8 month	14

Table 2. List of cattle without clinical MCF tested for OvHV-2 infection by qPCR, RNA-ISH and immunohistology.

References

2A. List of cows without clinical MCF, of which organs were tested, including information on the post mortem diagnosis (necropsied cases), histological features and results of RNA-ISH and immunohistology for OHV-2. Animals are listed on the order of age, from foetus onwards.

Case number and age	Origin	Block	Organs	qPCR	Histological findings, results of OvHV-2 RNA-ISH and IH	Relevant post- mortem diagnoses
S13-1350 Aborted Foetus (crown- rump length [CRL] 54 cm; 6 mo)	CH	7	Lung	0	HE: Complete foetal atelectasis, otherwise NHAIR Ov2.5: Negative	Abortion (non- infectious, cause unknown)
			Thymus	0	HE: NHAIR, Ov2.5: Negative	
			Spleen	0	HE: NHAIR, Ov2.5: Negative	
			Mediastinal LN	0	HE: NHAIR, Ov2.5: Negative	
S13-1448 Stillborn calf (CRL 81 cm) 8 mo	CH	16	Lung	0	HE: Complete foetal atelectasis , otherwise NHAIR Ov2.5: Signals in VEC (capillaries) and some RES	Stillbirth (non-infectious, cause unknown)
			Thymus	0	HE: NHAIR; Ov2.5: negative	
			Spleen	0	HE: NHAIR Ov2.5: Signals in VEC (capillaries) in capsule and red pulp	
			Mediastinal LN	0	HE: NHAIR; Ov2.5: negative	
S13-1355 Mother, 5 y	CH	2	Lung	0	HE: NHAIR	Chronic active ulcerative abomasitis with melaena
			Spleen	0	HE: NHAIR	
		3	Mediastinal LN	1	HE: NHAIR, apart from relatively small secondary follicles	
			Nasopharynx	2	HE: NHAIR	
		4	Placenta	0	HE: NHAIR Ov2.5: Weak signal in stromal cells	
			Uterus	56	HE: Mild mixed cellular diffuse subepithelial infiltration Ov2.5: Signal in glandular epithelial cells, VEC and in smooth muscle cells of the wall	
S13-1355 Foetus (CRL 72 cm) 8 mo	CH	5	Lung	0	HE: Complete foetal atelectasis: NHAIR; Ov2.5: Signals in occasional individual type II pneumocyte and alveolar macrophages	
			Thymus	0	HE: NHAIR Ov2.5: Weak signals in a few cells (probably LCs) in the medulla	
			Spleen	4	HE: NHAIR Ov2.5: Signals in a moderate number of random cells in red pulp (possibly macrophages)	
			Mediastinal LN	3	HE: NHAIR Ov2.5: Signals in a small number of cells (macrophages and LC) mainly in cortex, also in and around the follicles	
S13-1483 Mother, 5 y	CH	6	Lung	10	HE: NHAIR Ov2.5: Negative OvHV-2 IH: Individual positive cells (fibroblasts, macrophages) in interstitium	Aspiration pneumonia

References

			Spleen	5	HE: Relatively small, mildly depleted follicles, small T cell zones; numerous apoptotic cells in the red pulp, mild haemosiderosis Ov2.5: Negative OvHV-2 IH: FDC in follicles, numerous pos. apoptotic/autolytic cells are positive.		
			Conchae	0	HE: Autolytic tissue; NHAIR Ov2.5: Negative; OvHV-2 IH: Negative		
			7	Tongue	16		HE: NHAIR; Ov2.5: Negative; OvHV-2 IH: Negative
			Nasopharynx	49	HE: NHAIR, apart from slight subepithelial perivascular LC infiltration; mild pigmentation of basal epithelial cells. Ov2.5: Negative OvHV-2 IH: Negative, apart from reaction in basal epithelial cells (pigmentation?)		
			Uterus	0	HE: NHAIR Ov2.5: Signals in the majority of epithelial cells OvHV-2 IH: Epithelial cells negative, reaction in stromal fibroblasts		
S13-1483 Foetus, 8 mo		8	Turbinate	ND	HE: NHAIR; Ov2.5: Negative; OvHV-2IH: negative		
			Lung	0	HE: Complete foetal atelectasis, NHAIR; Ov2.5: Negative; OvHV-2 IH: Negative		
			Thymus	0	HE: NHAIR; Ov2.5: Negative; OvHV-2 IH: Negative		
			Spleen	0	HE: NHAIR Ov2.5: Signals in relatively numerous cells (in red pulp and in follicles (LC, macrophages?) OvHV-2 IH: Negative		
			Mediastinal LN	0	HE: NHAIR Ov2.5: Signals in few individual cells (LC?, macrophages?) in and around follicles in cortex OvHV-2 IH: Some positive FDC and macrophages outside follicles		
Placenta	ND	HE: NHAIR Ov2.5: Distinct signal in stromal fibroblasts, VEC and epithelial cells OvHV-2 IH: Negative					
S13-1452 Mother, 12 y	CH	1	Lung	0	HE: NHAIR; Ov2.5: Negative	Perforation of reticulum due to foreign body, with fibrinosuppurative peritonitis	
			Spleen	0	HE: Moderate hyperaemia, inconspicuous follicles and T cell zones Ov2.5: Negative		
			Mediastinal LN	0	HE: NHAIR, apart from relatively small secondary follicles Ov2.5: Negative		
		2	Uterus	0	HE: NHAIR; Ov2.5: Negative		
			Nasopharynx	0	ND		
S13-1452 Foetus (CRL 101 cm) 8 mo		3	Lung	0	HE: NHAIR; Ov2.5: Negative		
			Thymus	0	HE: NHAIR; Ov2.5: Negative		
			Spleen	0	HE: NHAIR; Ov2.5: Negative		
			Mediastinal LN	0	HE: NHAIR, apart from focal extensive sinus haemorrhage Ov2.5: Negative		

References

			Placenta	0	HE: NHAIR; Ov2.5: Negative	
S13-1360 Mother, 8 y	CH	1	Lung	0	HE: NHAIR, apart from mild diffuse alv. emphysema and slight BALT formation Ov2.5: Signals in individual cells in BALT (possible LC) ORF65: similar to Ov2.5	Abscess in glutaecal muscle
			Spleen	0	HE: Moderately sized, mildly depleted secondary follicles and T cell zones, mild haemosiderosis Ov2.5: Very numerous cells (LC) in follicular mantle zones ORF65: Similar, but less intense signal and fewer cells	
		2	Mediastinal LN	0	HE: Moderately sized and moderately depleted secondary follicles Ov2.5: Strong signal in LC in follicular mantle zone ORF65: Very similar to Ov2.5	
			Buccal Mucosa	0	HE: NHAIR, apart from subepithelial mononuclear infiltration (LC, plasma cells) Ov2.5: Signal in proportion of infiltrating LC ORF65: Simila to Ov2.5	
			Tongue	1	HE: NHAIR Ov2.5: Signals in small patchy area of epithelial cells ORF65: Similar to Ov2.5	
			Nasopharynx	3	HE: NHAIR; Ov2.5: Negative; ORF65: Negative	
		3	Turbinates	0	ND	
			Uterus	1	HE: NHAIR; Ov2.5: Signal in proportion of glandular epithelial cells, patchy signals in the surface epithelium ORF65: similar to Ov2.5, but very weak	
S13-1360 Foetus (CRL 66 cm) 7 mo	CH	4	Placenta	1	HE: NHAIR Ov2.5: Signal in trophoblasts and stromal cells ORF65: Similar to Ov2.5, but very weak	
			Lung	0	HE: Complete foetal atelectasis; NHAIR Ov2.5: Negative; ORF65: Negative	
			Thymus	0	HE: NHAIR; Ov2.5: Negative; ORF65: Negative	
			Spleen	0	HE: NHAIR; Ov2.5: Negative ORF65: Signals in occasional cells in early follicles	
H04-981G Aborted foetus, 9 mo	UK	G	Mediastinal LN	1	HE: NHAIR Ov2.5: Signals in individual cells (LC) in follicles ORF65: Similar as Ov2.5	
			BM	ND	HE: NHAIR; Ov2.5: Negative; ORF65: Negative; OvHV-2 IH: Negative	
			Spleen	ND	HE: NHAIR; Ov2.5: Negative; ORF65: Negative; OvHV-2 IH: Negative	
			Thymus	ND	HE: NHAIR; Ov2.5: Negative; ORF65: Negative; OvHV-2 IH: Negative	
		K	LN.	ND	HE: NHAIR; Ov2.5: Negative; ORF65: Negative OvHV-2 IH: Negative apart from very faint reaction in fibroblasts and reaction in nerve fibres	
			Heart	ND	HE: NHAIR; Ov2.5: Negative; ORF65: Negative; OvHV-2 IH: Negative	
			Lung	ND	HE: NHAIR; Ov2.5: Negative; ORF65: Negative; OvHV-2 IH: Negative	
Liver	ND	HE: NHAIR; Ov2.5: Negative; ORF65: Negative; OvHV-2 IH: Negative				

References

			Muscle	ND	HE: NHAIR; Ov2.5: Negative; ORF65: Negative; OvHV-2 IH: Negative	
S13-1438 Stillborn calf (CRL 76 cm) 9m	CH	1	Lung	6	HE: NHAIR; Ov2.5: Negative ORF65: Focal area of signals in bronchiolar epithelium and VEC OvHV-2 IH: Negative	Sillbirth (non-infectious, cause unknown)
			Thymus	5	HE: Relatively cell poor, multifocal acute haemorrhages Ov2.5: Signals in few LC at the periphery of the section, in individual macrophages ORF65: Similar to Ov2.5, but more intense OvHV-2 IH: Negative	
			Spleen	25	HE: NHAIR Ov2.5: Signals in sporadic cells in white pulp, a few LC in follicles with weak signals ORF65: Similar to Ov2.5 and more intense OvHV-2 IH: Scattered individual positive macrophages in red pulp	
			Mediastinal LN	6300	HE: Small and cell poor cortex and paracortex Ov2.5: Strong signals in sporadic LC in cortex and medulla, in a few LC in follicles, weak and occasional individual cells in medulla (possibly macrophages). ORF65: Similar to Ov2.5, but more intense OvHV-2 IH: Negative	
S13-1418 M, 1 we	CH	4	Lung	0	HE: NHAIR; Ov2.5: Negative	Severe catarrhal enteritis (Escherichia coli)
			Thymus	0	HE: Numerous disseminated apoptotic cells Ov2.5: Negative	
			Spleen	0	HE: NHAIR; Ov2.5: Negative	
			Mediastinal LN	0	HE: Cell poor, depleted follicles Ov2.5: Negative	
		5	Tongue	0	HE: Mild interstitial oedema Ov2.5: Basal epithelial cells and patchy in papillary epithelial cell aggregates ORF65: Similar to Ov2.5	
			Nasopharynx	0	HE: NHAIR Ov2.5: Some signal in glandular epithelial cells ORF65: Similar to Ov2.5	
			Nasal epithelium	0	HE: Moderate subepithelial mononuclear infiltration Ov2.5: Weak signal in basal epithelial cells ORF65: Similar to Ov2.5, but also signals in squamous epithelium of inflamed glandular ducts	
S13-1329 10 d	CH	6	Spleen	0	HE: Depleted follicles, small T cell zones Ov2.5: Occasional cells in follicles with weak signals ORF65: Similar as Ov2.5 OvHV-2 IH: Negative	Fibrinosuppurative and necrotising pneumonia, pleuritis, peri- and epicarditis, suppurative leptomeningitis and necrotising encephalitis (Trueperella pyogenes)
			Thymus	0	HE: NHAIR; Ov2.5: Negative; OvHV-2 IH: Negative	
			Mediastinal LN	0	HE: Moderate follicular depletion Ov2.5: Signals in LC in follicles and some individual LC in medulla OvHV-2 IH: Weak reaction in individual FDC/macrophages in follicles	

References

			Nasopharynx	0	HE: NHAIR; Ov2.5, ORF65: Negative OvHV-2 IH: Weak reaction in stromal fibroblasts	
			Tongue	0	HE: NHAIR Ov2.5: Strong signal in epithelial cells in taste buds ORF65: Similar to Ov2.5 OvHV-2 IH: Weak reaction in stromal fibroblasts	
		7	Lung	0	HE: Poorly aerated, otherwise NHAIR Ov2.5: Negative; ORF65: Negative; OvHV-2 IH: Negative	
			Nares	4	HE: NHAIR Ov2.5: Negative ORF65: Weak signals in some glandular epithelial cells and in some surface epithelial cells OvHV-2 IH: Weak reaction in stromal fibroblasts	
S13-1377 F, 5 we	CH	1	Lung	0	HE: Increased interstitial cellularity Ov2.5: Negative	Abomasal ulcer with perforation and fibrinous peritonitis, aspiration pneumonia
			Thymus	0	HE: Moderate to marked involution Ov2.5: Negative	
			Spleen	0	HE: Moderate follicular depletion Ov2.5: Negative	
			Mediastinal LN	0	HE: Moderate follicular depletion Ov2.5: Negative	
		2	Tongue	0	HE: NHAIR; Ov2.5: Negative	
			Nasopharynx	0	HE: Focal subepithelial LC accumulation (follicle like) Ov2.5: Signal in several cells in a follicle like LC aggregates	
S13-1419 M, 7 we	CH	6	Lung	156	HE: NHAIR; Ov2.5: Negative ORF65: Variably intense signal in REC (bronchioles) OvHV-2 IH: A few positive PIM	Severe ulcerative abomasitis
			Thymus	0	HE: Numerous disseminated apoptotic cells Ov2.5: Negative; ORF65: Negative; OvHV-2 IH: Negative	
			Spleen	417	HE: Small follicles and T cell zones Ov2.5: Very weak signal of occasional cells ORF65: Individual cells in follicles and in T cell zones OvHV-2 IH: Weak reaction in FDC/macrophages in follicles, scattered positive macrophages in red pulp	
			Mediastinal LN	129	HE: Markedly depleted cortex and paracortex Ov2.5: Signals in a few individual cells in follicles ORF65: Similar as Ov2.5 OvHV-2 IH: Weak reaction in FDC/macrophages in follicles	
			7	Tongue	12	

References

					<p>ORF65: Similar to Ov2.5 and stronger OvHV-2 IH: Weak reaction in stromal fibroblasts and VEC</p>	
			Nasopharynx	12	<p>HE: Multifocal mononuclear subepithelial infiltration Ov2.5: Signal in most of mucosal epithelial cells. ORF65: Similar to Ov2.5 and stronger OvHV-2 IH: Weak reaction in stromal fibroblasts and VEC</p>	
			Turbinate	50	<p>HE: Focal mononuclear infiltration of glands beneath epidermis Ov2.5: Signal in epidermal basal cells and hair follicle epithelium. ORF65: Similar to Ov2.5 OvHV-2 IH: Weak reaction in stromal fibroblasts and VEC</p>	
S13-1373 M, 7 we	CH	5	Lung	1	<p>HE: Mild BALT formation and leukocytostasis OV2.5: Signals in a few cells (LC, macrophages) in BALT and in occasional REC.</p>	Fibrinosuppurative pleuritis and pericarditis, suppurative bronchopneumonia (Mannheimia haemolytica)
			Spleen	1	<p>HE: Small and mildly depleted secondary follicles, small T cell zones OV2.5: Signals in a few cells in follicles (possibly macrophages)</p>	
			Thymus	0	<p>HE: Mild involution, numerous disseminated apoptotic cells in the cortex OV2.5: Negative</p>	
			Mediastinal LN	0	<p>HE: Moderately sized, mildly depleted secondary follicles OV2.5: Several cells within and outside follicles (possible macrophages)</p>	
		6	Tongue	1	<p>HE: NHAIR OV2.5: Signal in patches of epithelial cells in taste buds (and occasional basal epithelial cells) with variably intense signal</p>	
		Nares	ND	<p>HE: NHAIR OV2.5: Signals in glandular epithelial cells and weak signal in many surface epithelial cells</p>		
		Nasopharynx	ND	<p>HE: subepithelial lymphatic follicles OV2.5: Signals in LC in follicles and in overlying epithelium</p>		
S13-1434 F, 8 we	CH	5	Lung	0	<p>HE: NHAIR Ov2.5: Signals in scattered type II pneumocytes ORF65: Similar, and more intense and more positive cells</p>	Severe rumenal acidosis
			Thymus	0	<p>HE: NHAIR; Ov2.5: Negative; ORF65: Negative</p>	
			Spleen	0	<p>HE: Small follicles and T cell zone Ov2.5: Signals in some cells in follicles and red pulp ORF65: Similar</p>	
			Mediastinal LN	0	<p>HE: Moderately cellular cortex and paracortex Ov2.5: Some cells in cortex with signal ORF65: Relatively numerous cells in medulla, similar in cortex</p>	
		6	Tongue	0	<p>HE: NHAIR Ov2.5: Signals in a few patches of 3-4 epithelial cells in taste buds ORF65: Similar as Ov2.5, but more extensive</p>	
		Uterus	0	<p>HE: NHAIR OV2.5: Negative ORF65: Signals in glandular and surface epithelial cells</p>		

References

			Nasopharynx	0	HE: NHAIR Ov2.5: Signals in patches of positive epithelial cells ORF65: Similar as Ov2.5, but more extensive	
			Turbinate (Nares)	0	HE: Mild subepithelial mixed cellular infiltration Ov2.5: Signals in a few infiltrating cells ORF65: stronger signals in infiltrating cells, glandular epithelial cells and patches of epithelial cells	
S13-1460 F, 13 we	CH	6	Thymus	0	HE: Moderate involution Ov2.5: Negative	Bronchointerstitial pneumonia (BRSV infection), abomasal ulcera
			Spleen	0	HE: Small follicles and T cell zones Ov2.5: Negative	
			Mediastinal LN	0	HE: Mildly depleted cortex and paracortex Ov2.5: Negative	
			Lung	0	HE: NHAIR; Ov2.5: Negative	
		7	Tongue	0	HE: NHAIR; Ov2.5: Signals in patches of epithelial cells in taste buds	
		Nasopharynx	0	HE: NHAIR; Ov2.5: Negative		
			Turbinate (Nares)	0	HE: NHAIR; Ov2.5: Negative	
S13-1459 F, 18 we	CH	6	Thymus	0	HE: marked involution Ov2.5: Negative	Severe bronchointerstitial pneumonia (BRSV infection), abomasal ulcera
			Spleen	0	HE: Inconspicuous white pulp Ov2.5: Signals in individual cells in follicles and medulla	
			Mediastinal LN	3	HE: Marked depletion Ov2.5: Signals in cells in white pulp	
		7	Lung	ND	HE: Mild BALT and desquamated epithelial changes Ov2.5: Negative	
		Tongue	ND	HE: NHAIR; Ov2.5: Negative		
		Nasopharynx	ND	HE: NHAIR; Ov2.5: Negative		
		Turbinate	ND	HE: NHAIR; Ov2.5: Negative		
S13-1352 F, 8 mo	CH	7	Tongue	0	HE: NHAIR Ov2.5: Signals in patches of basal epithelial cells	Severe emaciation, suppurative tracheobronchitis with intralesional fungi (aspergillosis)
			Nasopharynx	0	HE: Mild focal subepithelial mixed cellular infiltration Ov2.5: Signals in some infiltrating cells and some glandular epithelial cells	
		8	Lung	0	HE: NHAIR; Ov2.5: Signals in a proportion of REC	
		Mediastinal LN	0	HE: inconspicuous cortex and paracortex Ov2.5: Signals in numerous cells in cortex and paracortex and VEC		
		Spleen	0	HE: NHAIR Ov2.5: Signals in some cells in follicles and red pulp		
		Turbinate	0	HE: NHAIR Ov2.5: Signals in surface and glandular epithelial cells, chondrocytes and VEC		

References

13L-2591 1 y	UK	A	Spleen	3600	<p>HE: Moderate sized secondary follicles, small T cell zones Ov2.5: Signals mainly follicular mantle zone LC, outside the follicle and some in red pulp ORF65: Similar as Ov2.5 , but more intense signal OvHV2-IH: Reaction in FDC, macrophages in red pulp, VEC OV2.5 and CD3: Positive cells are CD3 negative OV2.5 and PAX-5: Positive cells are PAX5- positive</p>	Not applicable (tissues collected at the abattoir)
			Submandib. LN	6500	<p>HE: Moderate sized secondary follicles Ov2.5: Signals in LC in outer mantle zone ORF65: Similar as OV2.5, but more intense signal; also some cells in medulla OvHV2-IH: FDC, macrophages in sinuses and medulla, and VEC OV2.5 and CD3: Positive cells are CD3 negative OV2.5 and PAX-5: Positive cells are PAX5- positive</p>	
		B	Lung:	5800	<p>HE: NHAIR Ov2.5: Weak signals in endothelial cells within few arteries throughout the lung section ORF65: Negative OV2.5 and CD3: Positive cells are CD3 negative. OV2.5 and PAX-5: Positive cells are PAX5- positive at the BALT</p>	
			Tongue	3900	<p>HE: NHAIR Ov2.5: Strong signals in most epithelial cells particularly in the basal cells ORF65: Signals in one piece of the tongue there is very few signals in sporadic macrophage beside a couple of epithelial cells in the mucosa. In the other bit of the tongue there are moderate signal in the mucosal deep layer epithelial cells</p>	
13L-4893 18 mo	UK	A	DRG	0	HE: NHAIR; Ov2.5: Negative	Not applicable (tissues collected at the abattoir)
			Cerebellum	2	<p>HE:NHAIR Ov2.5: Signal in neurons and Purkinje cells ORF65: Similar as ov2.5 OVHV-2 IH: Negative</p>	
		B	Hippocampus	0	<p>Ov2.5: Signals in neurons ORF65: similar to Ov2.5</p>	
	Pituitary gland	0	Ov2.5: Negative; ORF65: Negative			
13L-2599 19 mo	UK	A	Lung	5	<p>HE: Mild to moderate BALT formation, slight interstitial fibrosis Ov2.5: Signals in several cells (LC and some macrophages) in BALT; individual leukocytes between bronchial epithelial cells; VEC; disseminated individual type II alveolar cells and possibly alveolar macrophages ORF65: Similar reaction in BALT, otherwise far less intense</p>	Not applicable (tissues collected at the abattoir)
			B	Mediastinal LN	0	
			Bronchial LN	2	<p>HE: Relatively large secondary follicles Ov2.5:signals mainly in LC in outer mantle zone and possibly a few macrophages ORF65: Similar as Ov2.5, but less intense</p>	
13L-2596 20 mo	UK	A	Spleen	9	<p>HE: moderate sized secondary follicles and T cell zones, mild haemosiderosis Ov2.5;ORF65: sections is overexposed, cannot be assessed OvHV2-IH: Reaction in FDC</p>	Not applicable (tissues collected at the abattoir)

References

			Bronchial LN	ND	HE: Relatively large secondary follicles with numerous apoptotic LC and TBM, and mild sinus histiocytosis Ov2.5;ORF65: Sections is overexposed, cannot be assessed OvHV2-IH: Reaction in FDC	
		C	Lung	8	HE: NHAIR Ov2.5: Signals in occasional type II pneumocytes ORF65: No clear signal	
			Mediastinal LN	14	HE: Moderate sized secondary follicles Ov2.5: Signals in occasional mantle zone LC and sinus macrophages ORF65: Similar, but more positive cells than Ov2.5	
13L-2597 20 mo	UK	A	Lung	7	HE: Mild BALT formation and increased interstitial cellularity Ov2.5: Signals in the REC (bronchioles) and type II pneumocytes and VEC	Not applicable (tissues collected at the abattoir)
		B	Spleen	3	Ov2.5, ORF65: Hardly any signal	
			Bronchial LN	2	Ov2.5, ORF65: Hardly any signal	
13L-2598 20 mo	UK	A	Lung	0	Ov2.5: Signal in respiratory epithelium (bronchiole), VEC, some LC in the BALT and the infiltrating between epithelial cells of bronchiolar, alveolar epithelial cells (possibly type II), and in arterial SMC ORF65: Similar as Ov2.5, but far less intense	Not applicable (tissues collected at the abattoir)
			Spleen	5	Ov2.5: Signals in LC in follicular mantle zone, also proportion in red pulp ORF65: Similar, but far less intense	
		B	Bronchial LN	0	Ov2.5: Dirty; signals in some LC in follicular mantle zone ORF65: Similar as Ov2.5	
13L-4213 2 y	UK	A	Lung	0	HE,ISH,IH: ND	Not applicable (tissues collected at the abattoir)
		B	Tongue	0	HE: NHAIR, apart from slight perivascular lymphoplasma-cellular subepithelial infiltration Ov2.5: Patchy signal in basal epithelial cells; and in in salivary gland	
			Muzzle	16	HE: NHAIR, apart from focal glandular lympho-plasmacellular infiltration Ov2.5: Signals in a few cells possibly macrophages in the dermis, most fibrocytes, salivary gland epithelial cells, and VEC in some vessels	
		C	Mediastinal LN	6	HE: Relatively large secondary follicles with numerous apoptotic cells Ov2.5: Signals in a few LC disseminated in the follicles and outside, and a few macrophages in follicles	
			Spleen	4	HE: Moderate sized secondary follicles Ov2.5: Signals in a few LC in follicular mantle zone and a few macrophages in the centre	
13L-4213 2 y	UK	D	Lung	1	HE: Mild BALT formation; Ov2.5: Negative	Not applicable (tissues collected at the abattoir)
			Tongue	4	HE: NHAIR, apart from mild multifocal subepithelial LC accumulations Ov2.5: Negative	
		F	Mediastinal LN	0	HE: moderate sized secondary follicles with several apoptotic cells. Ov2.5: Negative	
			Spleen	0	HE: relatively small secondary follicles Ov2.5: Signals in a few LC in follicular mantle zone and occasional macrophages in centre of follicles	
13L-4214 2 y	UK	A	Lung	0	HE: Mild BALT formation, mild alveolar emphysema Ov2.5: Signals in a few to several macrophages and LC in BALT	Not applicable (tissues collected at the abattoir)
			Tongue	0	HE: Mild multifocal subepithelial plasma cells-dominated, partly mixed infiltration, small subepithelial lymph follicle	

References

					aggregate Ov2.5: One edge area with positive epithelial cells; some LC and macrophages in subepithelial infiltrates	
		B	Mediastinal LN	0	HE: Relatively small secondary follicles Ov2.5: Signals in some follicles with positive LC in mantle zone and a few in centres (also possibly macrophages)	
			Spleen	0	HE: Small follicles; Ov2.5: Negative	
13L-4214 2 y	UK	C	Mediastinal LN	1	HE,ISH,IH: ND	Not applicable (tissues collected at the abattoir)
			Spleen	1	HE,ISH,IH: ND	
		D	Tongue	0	HE,ISH,IH: ND	
			Muzzle	2	HE,ISH,IH: ND	
		E	Lung	0	HE,ISH,IH: ND	
13L-4215 2 y	UK	A	Lung	8	HE: NHAIR Ov2.5: Signals in endothelial cells of small to medium sized arteries, very occ. pneumocytes ORF65: Negative OvHV2-IH: Very weak reaction in endothelial cells of some arteries, and occasional cells in interstitium and pneumocytes	Not applicable (tissues collected at the abattoir)
			Mediastinal LN	9	HE: Large secondary follicles; moderate haemosiderosis Ov2.5: Patchy signals in some follicles with LC in mantle zones, a few cells in centre ORF65: Similar as Ov2.5 OvHV2-IH: Weak reaction in a few FDC	
		B	Tongue	2	HE: NHAIR Ov2.5: Negative ORF65: Patchy signals in the basal epithelial cells	
			Trachea	3	HE: Moderate mononuclear to mixed cellular subepithelial infiltration Ov2.5: Signals in REC, VEC, infiltrating LC and glandular epithelium ORF65: Similar, but less extensive	
			Aorta	1	HE: NHAIR Ov2.5: Signals in endothelial cells of vasa vasorum ORF65: Signals in the fibrocytes of media	
		13L-4215 2 y	UK	C	Lung	
Mediastinal LN	0				HE: Moderate sized secondary follicles Ov2.5: Signals in a few LC in follicular mantle zones and occasional macrophages in centre	
D	Tongue			0	HE: Mild multifocal mixed cellular subepithelial perivascular infiltration Ov2.5: Patchy, unfocused signals in (supra) basal epithelial cells ORF65: Relatively weak signals in mainly mid layers of the epithelium	
	Spleen			1	Not on section	
13L-4216 2 y	UK	A	Lung	0	HE: Moderate BALT formation Ov2.5: Signals in some LC in BALT and around bronchiolar epithelium	Not applicable (tissues collected at the abattoir)
			Mediastinal LN	0	HE: Moderate BALT formation Ov2.5: Weak signal in a few mantle zone LC and possibly macrophages in centre	

References

		B	Spleen	0	HE: Small follicles and T cell zones; cell rich red pulp Ov2.5: Negative	
			Aorta	0	HE: NHAIR; Ov2.5: Negative	
		C	Muzzle	0	HE: mild multifocal subepithelial perivascular LC infiltration Ov2.5: Negative	
			Tongue	0	HE: NHAIR; Ov2.5: Negative	
13L-4216 2 y	UK	D	Spleen	5	HE: Relatively small follicles, cell rich red pulp Ov2.5: Signals in very few cells in follicular mantle zones	Not applicable (tissues collected at the abattoir)
		E	Trachea	2	HE: Mild to moderate diffuse subepithelial LC-dominated infiltration Ov2.5: Signals in numerous subepithelial LC ORF65: Similar, with slightly more intense signals	
13L-4217 2 y	UK	A	Lung	0	HE: Acute focal suppurative pneumonia Ov2.5: Signals in some LC and a few arteries endothelial cells	Not applicable (tissues collected at the abattoir)
			Tongue	0	HE: Several aggregates of large sec. follicles (small LNs) Ov2.5: Signals in a few LC in follicles	
		B	Mediastinal LN	0	HE: Large secondary follicles Ov2.5: Signals in a few LC (and possible macrophages) in the follicles	
			Spleen	0	HE: Relatively small follicles, cell rich red pulp Ov2.5: Signals in several LC in mantle zone	
13L-4221 2 y	UK	A	Mammary LN	1	HE: Relatively small secondary follicles Ov2.5: Signal in several mantle zone LC and macrophages in follicle centres ORF65: Similar to ov2.5	Not applicable (tissues collected at the abattoir)
13L-2600A,B 2.5 y	UK	A	Lung	13	HE: Slight BALT formation and interstitial fibrosis Ov2.5: Signals in arterial smooth muscle cells and endothelial cells; in type II pneumocytes, and in LC in BALT ORF65: Negative	Not applicable (tissues collected at the abattoir)
			Mediastinal LN	0	HE: Relatively large secondary follicles Ov2.5: Probably some macrophages in follicle centres and LC outside have signals ORF65: Signals in a few macrophages in follicle centres and LC outside	
		B	Tongue	0	HE: Focal subepithelial and perivascular LC aggregates Ov2.5: Signal in superficial epithelial cells in mucosa ORF65: Very weak signals in epithelial cells	
			Spleen	0	HE: Moderate sized secondary follicles and T cell zones Ov2.5: Signals in a few macrophages in follicles ORF65: Similar as Ov2.5	
13L-2600C,D 2.5 y	UK	C	Lung	3	HE: Mild interstitial fibrosis and arterial media hypertrophy, diffuse mild alveolar emphysema Ov2.5: Signals in arterial SMC, bronchiolar epithelium, some LC ORF65: Signals in arterial endothelial cells, a few LC in the BALT	Not applicable (tissues collected at the abattoir)
			Mediastinal LN	0	HE,ISH,IH: ND	

References

		D	Tongue	8	HE: NHAIR, apart from focal subepithelial LC aggregate Ov2.5: Rare signals in LC in subepithelial infiltrates ORF65: Patchy area with some positive epithelial cells, and some macrophages in subepithelial infiltrates	
			Spleen	0	HE: Relatively small secondary follicles Ov2.5: Signals in a few macrophages in follicles ORF65: similar as Ov2.5	
S13-1515 F, 3 y	CH	2	Lung	0	HE,ISH,IH: ND	Vertebral fracture (S1 and S2)
			Spleen	0	HE,ISH,IH: ND	
			Mediastinal LN	0	HE,ISH,IH: ND	
		3	Tongue	6	HE,ISH,IH: ND	
			Nasopharynx	0	HE,ISH,IH: ND	
			Turbinate	0	HE,ISH,IH: ND	
Uterus	0	HE,ISH,IH: ND				
S13-1425 F, 4 y	CH	5	Lung	0	HE: Mild multifocal neutrophilic infiltration and necrosis Ov2.5: Negative	Severe suppurative bronchopneumonia (Trueperella pyogenes)
			Mediastinal LN	0	HE: No distinct follicles; Ov2.5: Negative	
			Spleen	0	HE: Very small secondary follicles and T cell zones Ov2.5: Negative	
		6	Tongue	0	HE: NHAIR Ov2.5: Signals in basal epithelial cells, subepithelial fibrocytes	
			Nasopharynx	0	HE: Mild diffuse subepithelial infiltration (possibly LC and plasma cells) Ov2.5: Signals in a few infiltrating LC	
			Turbinate	0	HE: Mild, predominantly mononuclear subepithelial infiltration Ov2.5: Signals in several LC, VEC, and in glandular epithelial cells	

Abbreviations:

BALT: bronchus associated lymphoid tissue; CH: animals submitted for a diagnostic post mortem examination to the Institute of Veterinary Pathology, Vetsuisse Faculty, University of Zurich, Switzerland; CRL: crown-rump length; F: female; FDC: follicular dendritic cells; HE: haematoxylin and eosin stain; IH: immunohistology; LC: lymphocyte; LN: lymph node; ND: not done; M: male; mo: month; NHAIR: no histological abnormality is recognised; PIM: pulmonary intravascular macrophages; REC: respiratory epithelial cells; SMC: smooth muscle cells; Submand.: submandibular; TBM: tingible body macrophages; UK: healthy animals slaughtered in abattoir in the UK; VEC: vascular endothelial cells; we: week; y: year.

References

2B. List of cows without MCF, of which white blood cells were tested, including information on the histological features and results of RNA-ISH and immunohistology for OHV-2. Relevant post mortem diagnosis is not applicable as blood collected at the abattoir in the UK. Animals are listed on the order of age.

Case number/age	Block	qPCR	Histological findings, results of OvHV-2 RNA-ISH and IH
13L-4889 13 mo	D	0	Cells were destroyed during WBC isolation, no definitive signals are seen
13L-4889 18 mo	E	0	
13L-4889 18 mo	C	5	
13L-4889 19 mo	A	86	
13L-4889 19 mo	B	1	
Cattle 1 20 mo		0	qPCR Only, leukocytes were not pelleted
Cattle 2 2 y		32	
13L-4883 2 y	F	4	HE: Predominantly neutrophils, with fewer monocytes and LC Ov2.5 (section 1): Signals in up to 10% of cells, morphology of LC and (lesser) monocytes. PAX-5 (2): Few B cells approximately (1-2%) CD3 (3): Approximately 20-30% of cells ORF65 (4): Similar to Ov2.5 OVHV-2 IH: Reaction in occasional monocytes with very weak reaction.
13L-4883 2 y	G	20	HE: predominantly neutrophils, with fewer monocytes and LC Ov2.5 (section 1): signals in >10% of cells, morphology of LC and (lesser) monocytes PAX-5 (2): few B cells approximately (2-3%) CD3 (3): approximately -30% of cells ORF65 (4): similar, but lower no. of cells. OVHV-2 IH: occasional monocytes with weak reaction.

Abbreviations: HE: haematoxylin and eosin stain; IH: immunohistology; LC: lymphocyte; mo: month; WBC: white blood cells; y; year.

References

Table 3. List of cattle with MCF tested by qPCR, information on histological features and results of RNA-ISH and immunohistochemistry for OHV-2.

Case number Sex, age	Origin	Block	Tissue	qPCR	Histological findings, results of OvHV-2 RNA-ISH and IH
12L-0200 cow 11 mo	UK	A	Muzzle	ND	HE: Focal extensive epidermal erosion, mixed cellular subepithelial perivascular infiltration, an increase in macrophages and fibroblasts, melanin incontinence, and VECs activation. OV2.5: Signals in hair follicle and epithelial, in activated VEC and probably in some leukocytes ORF65: Occasional leukocyte (no epithelium on slide)
			Oral mucosa	ND	HE: Mild to moderate diffuse interstitial LC and macrophage infiltration, else similar to muzzle with more extensive erosion and ulceration OV2.5: There are no signals in squamous epithelial cells, but signals in cells directly beneath epidermis; salivary gland and in a few infiltrating cells
		B	Lung	ND	HE: moderate hyperaemia, vessels with activated VEC and LC and macrophage like cells surrounding and partly infiltrating the walls; moderate multifocal, partly mixed cellular infiltrates, also peribronchial mononuclear infiltrates OV2.5: Signals in large proportion of infiltrating leukocytes; vessels: endothelial cells are negative, rare in intravascular LC ORF65: Generally weak signal in bronchus, glandular epithelial cells (negative with OV2.5) and in REC
		C	Tongue	ND	HE: Multifocal erosion and LC serration, with subepithelial mononuclear infiltration, often perivascular, and with vasculitis OV2.5: Signals in patches of epithelial cells (more intense with inflammation, also infiltrating leukocytes; a few fibrocytes in interstitium, VECs are negative ORF65: Signals are less intense, but similar
			Rumen	ND	HE: Multifocal erosion and ulceration, mild but almost diffuse mononuclear subepithelial infiltration OV2.5: Signals in epithelial cells patches (more intense with inflammation, also in infiltrating leukocytes, in some intravascular leukocytes, in some vessels also endothelial cells (close to inflammation) ORF65: Similar, but partly less intense
		D	Spleen	ND	HE: Small secondary follicles and T cell zones, relatively cell rich red pulp, there are activated endothelial cells. OV2.5: Signals seem to be in LC only in (centre and) periphery of follicles, and a few in red pulp ORF65: Similar signals, but substantially fewer cells and lower intensity.
			Heart	ND	HE: Focal interstitial LC-dominated perivascular infiltration OV2.5: Signals in infiltrating leukocytes ORF65: Similar signals, but substantially fewer cells and lower intensity
			Abomasum	ND	HE: Moderate mucosal and submucosal perivascular LC-dominated infiltration OV2.5: Signals in infiltrating leukocytes in (sub)mucosa, in neurons in submucosal and myenteric plexus structures, some signal in basal epithelium, in occasional in VECs ORF65: Similar signals, but substantially lower number of cells and lower intensity
		E	Small intestine	ND	HE: Moderate to marked diffuse mucosal and perivascular submucosal LC-dominated infiltration; some dilated crypts OV2.5: Infiltrating leukocytes in (sub)mucosa, neurons in submucosal and myenteric plexus structures, crypt epithelium; endothelial cells in some arteries in muscular layer ORF65: Similar, but far less extensive signal
			Large intestine	ND	HE: Moderate to marked diffuse mucosal and perivascular submucosal LC-dominated infiltration OV2.5: Signals in infiltrating leukocytes in (sub)mucosa , crypt epithelium, LC in gut associated lymphoid tissues (GALT) ; ORF65: Negative
			Mediastinal LN	ND	HE: Mildly depleted secondary follicles

References

					<p>OV2.5: Signals in LC mainly in; periphery of follicles; numerous in medulla and some in sinuses. ORF65: Similar, but far less cells</p>
		G	Kidney	ND	<p>HE: Moderate multifocal interstitial, partly perivascular and arterial infiltration OV2.5: Signals in infiltrating LC, individual or groups of tubular epithelial cells in medulla. ORF65: Similar, but a bit less intense</p>
			Liver	ND	<p>HE: Moderate diffuse portal LC-dominated infiltration (partly vasculitis), multifocal areas of hepatocyte necrosis and haemorrhage OV2.5: Signals in infiltrating LC ORF65: Similar to Ov2.5</p>
			Small intestine	ND	<p>HE: Mild to moderate diffuse mucosal LC-dominated infiltration OV2.5: Signals in infiltrating LC, in epithelial cells, in neurons in plexus structures, and in some arterial endothelial cells. ORF65: Signals in epithelial cells and LC, but far less cells and less intense</p>
		H	Hippo-campus	ND	<p>HE: Slight to mild multifocal perivascular LC-dominated cuffs in brain stem OV2.5: No definitive signal but possibly a few neurons in cortex ORF65: similar to Ov2.5</p>
		J	Cerebellum	ND	<p>HE: Slight to mild multifocal perivascular LC-dominated cuffs, mainly in white matter and leptomeninges OV2.5: Negative ORF65: Signals in neurons in all layers in cerebellum, VEC, large neurons in brainstem, infiltrating LC and in some glial cells CD3: There are many T cells PAX-5: No B cells detected</p>
S11-0564 Water buffalo F, 1.5 y	CH	01	Lip	ND	<p>HE: Focal ulceration and deep (perivascular and extending into muscle layers) LC dominated infiltration with evidence of fibroblasts (chronic vascular lesions) OV2.5: Signals in patchy basal epithelial cell accumulations, proportion of LC (and possibly fibroblasts) in infiltrates underneath epithelial, VEC are negative ORF65: Epithelial signals are similar, but slightly more extensive than OV2.5, signals in infiltrating cells and also in fibroblasts, and in endothelial cells of some vessels</p>
			Trachea	ND	<p>HE: Marked hyperaemia and (peri)vascular and glandular LC infiltration OV2.5: Very weak signals in infiltrating LC ORF65: Strong signal in infiltrating LC and in VEC</p>
			Lung	ND	<p>HE: Mild to moderate disseminated perivascular LC infiltrations OV2.5: Signals in a proportion of infiltrating LC ORF65: Signals in the most of infiltrating LC and occasional weak signal in REC</p>
		02	Lung	ND	<p>HE: Multifocal perivascular LC infiltration OVHV-2 IH: Reaction in infiltrating and intravascular LC, VEC, alveolar cells (probably macrophages), also individual REC and chondrocytes OV2.5: Weak signals in infiltrating LC ORF65: Section is overstrained, but signal distribution almost identical to viral antigen expression.</p>
		03	Rete mirabile	ND	<p>HE: moderate LC-dominated vasculitis OvHV-2 IH: reaction in endothelial cells, SMC (scattered and weak), in relatively small proportion of infiltrating LC, proportion of intravascular LC, neurons or probably myelin sheath in nerves OV2.5: Signals in a proportion of infiltrating LC ORF65: Signals in a large proportion of infiltrating LC and some VEC</p>
S11-1232	CH	08	Rete mirabile	ND	<p>HE: Severe LC-dominated vasculitis with fibrinoid endothelial cells necrosis of vessel wall</p>

References

Water buffalo F, 2.5 y					<p>OVHV-2 IH: Reaction in a proportion of infiltrating LC, VEC, fibroblasts, arterial SMC</p> <p>OV2.5: Signal in a proportion of infiltrating LC (relatively weak signals)</p> <p>ORF65: Overexposed; signals pattern almost dentical to protein expression</p> <p>CD3: Large proportion of infiltrating LC</p> <p>CD20 (B cells): Few proportion of infiltrating LC</p>
S11-1353 Water buffalo F, 2 y	CH	01	Kidney	ND	<p>HE: Multifocal chronic hyperplastic arteritis and moderate interstitial LC infiltration</p> <p>OV2.5: Signals in a large proportion of infiltrating LC, in interstitium, some in arterial walls; epithelial cells of some tubules, sometimes in VEC</p> <p>ORF65: Similar, but slightly more intense</p>
		02	Urinary bladder	ND	<p>HE: Chronic follicular cystitis (large subepithelial LC aggregates), oedema, haemorrhage, and Clostridia growth</p> <p>OV2.5: Signals in a proportion of LC in infiltrates, and in occasional epithelial cells</p> <p>ORF65: Signals in majority of LC infiltrates, and in VEC</p>
			Muscle	ND	<p>HE: interstitial haemorrhage, and Clostridia growth</p> <p>OV2.5: Negative</p> <p>ORF65: Individual interstitial cells have signals</p>
			Lung	ND	<p>HE: autolytic changes, alveolar haemorrhage, oedema and bacterial aggregates</p> <p>OV2.5: negative</p> <p>ORF65: negative, but possible weak signal in a few individual LC</p>
		04	Rete mirabile	ND	<p>HE: NHAIR, apart from a few LC in arterial walls and perivascular</p> <p>OV2.5: signals in endothelial cells in some vessels, a few cells (possible LC) in arterial walls</p> <p>ORF65: signals arterial SMC and endothelial cells, and in infiltrating cells in arterial walls</p>
			Basal ganglia	ND	<p>HE: NHAIR</p> <p>OV2.5: Signals in neurons</p> <p>ORF65: Similar as Ov2.5</p>
			Spleen	ND	<p>HE: LC depletion, mild haemosiderosis</p> <p>OV2.5: Signals in hardly any follicles (may be a few of the LC arranged in a loose follicle-like structure), moderate number of cells in red pulp (probably LC, plasma cell and macrophages), in endothelial cells of some vessels in capsule</p> <p>ORF65: Signals in the cells in follicle like structure, numerous cells in red pulp (s. OV2.5), VEC in spleen and splenic capsule</p>
S12-0083 Water buffalo F, adult	CH	08	Retrophar. LN	ND	<p>HE: Mildly depleted secondary follicles, moderate sinus histiocytosis</p> <p>OV2.5: Signals in LC in follicle centres and in medulla</p> <p>ORF65: see OV2.5</p>
			Basal ganglia and rete mirabile	ND	<p>HE: Mild multifocal (peri)vascular LC and plasma cell-dominated mixed cellular infiltrates</p> <p>OV2.5: Signals in VEC, neurons, intravascular and infiltrating LC and fibroblasts</p> <p>ORF65: see OV2.5</p>
		09	Rete mirabile	ND	<p>HE: Marked LC-dominated arteritis with fibrinoid necrosis of vessel wall, LC-dominated perivascular infiltrate</p> <p>OV2.5: Weak, small proportion of infiltrating cells</p> <p>ORF65: Section is overexposed and overdigested. Signals in arterial endothelial cells, fibroblasts, LC, and in some arterial SMC</p>
			Large intestine	ND	<p>HE: Autolytic changes, moderate LC-dominated vasculitis in muscularis</p> <p>OV2.5: Signals in a proportion of infiltrating LC in mucosa</p> <p>ORF65: Section is overexposed and overdigested. Signals in epithelial cells, VEC; LC in mucosa and submucosal infiltrates (vasculitis)</p>
		12	Lung	ND	<p>HE: mild LC-dominated arteritis; severe subacute pneumonia</p> <p>OV2.5: Signals in a substantial proportion of infiltrating LC</p>

References

		14	Kidney	ND	ORF65: majority of infiltrating LC, VEC, in occasional REC and glandular epithelial cells (bronchi, bronchioles) HE: moderate multifocal interstitial LC and plasma cell infiltration, in medulla there is mild vasculitis OV2.5: Signals in s proportion of infiltrating LC, rare medullary tubular epithelial cell ORF65: Signals in a large proportion of infiltrating LC; in medulla numerous tubules with positive epithelial cells
S12-0124 Water buffalo M, adult	CH	8	Rete mirabile	ND	HE: Very mild LC and plasma cell associated arteritis OVHV-2 IH: Reaction in endothelial cells, arterial SMC, infiltrating LC, fibroblasts OV2.5: Signals in a few infiltrating cells and arterial SMC (weak) ORF65: See IH
			Pituitary gland	ND	HE: NHAIR OvHV-2: Reaction in VEC OV2.5: Negative
			Spleen	ND	HE: Moderate sized, moderate depleted follicles, mild haemosiderosis, relatively cell poor red pulp OVHV-2 IH: Reaction in endothelial cells, in a proportion of LC in follicle periphery and in T cell Zones, FDC, proportion of cells (probably LC) in red pulp and in vessels OV2.5: Signal in very rare LC In follicle ORF65: Signal in LC in follicle centre and mantle zone and outside the follicles, in endothelial cells, and some cells in red pulp
			Small intestine	ND	HE: Moderate diffuse mucosal LC and PC infiltration OVHV-2 IH: Reaction in a proportion of LC and plasma cells in mucosal infiltrates and VEC OV2.5,ORF65: Negative
		9	Pituitary gland	ND	HE: NHAIR OVHV-2 IH: Reaction in cells in adenohypophysis, probably basket cells; and in VEC
		10	Retrophar. LN	ND	HE: Moderate sized, moderate depleted follicles and T cell zones, mild sinus histiocytosis OVHV-2 IH: Reaction in FDC, a few LC in follicle periphery and in T cell zones, DC in sinuses (and probably a few macrophages), and in VEC
18-C20-4-10 cow 11 mo (moderate autolytic changes)	UK		Kidney	ND	HE: Focal interstitial LC-dominated (perivascular) infiltration OV2.5: Signal in a portion of infiltrating LC, very rare signal in tubular epithelial cells OVH2-IH: Selective finely granular staining in tubular epithelial cells ORF65: Section is overexposed and overdigested. Signals in a proportion of tubules epithelial cells, VEC, some cells in glomeruli
			Small intestine	ND	HE: Moderate diffuse mucosal LC infiltration; mildly depleted Peyer's patches Ov2.5: Weak in some infiltrating LC, Peyer's patches negative OVH2-IH: Very weak signal in some DC in Peyer's patches ORF65: Section is overexposed and overdigested. Signals in epithelial cells, VEC, fibroblasts, and in infiltrating LC
			Abomasum	ND	HE: Mild diffuse mucosal LC infiltration ; OVH2-IH: Negative Ov2.5: Signals in large proportion of parietal cells ORF65: Section is overexposed and overdigested. Signals in parietal cells
			Mediastinal LN	328288	HE, ISH, IH: ND
			Mesenteric LN	1360448	HE, ISH, IH: ND
18-C21-4-10 cow 11 mo	UK		Kidney	ND	HE: Moderate multifocal interstitial, partly perivascular and arterial infiltration OV2.5: Section appears overdigested. There is no signal. ORF6: Signals in epithelial cells of some tubule and some infiltrating LC
			Abomasum	ND	HE: Mild diffuse mucosal LC infiltration

References

(moderate autolytic changes)					OV2.5: Section appears to be overdigested. Signal are in parietal cells and probably in some infiltrating LC in mucosa ORF65: Section is overexposed and overdigested. Signals in parietal cells and VEC
			Mediastinal LN	106283	No block was available
			Mesenteric LN	99619	No block was available
18-C22-4-10 cow 2 y (marked autolytic changes)	UK	1	Abomasum	ND	HE: Mild diffuse mucosal LC infiltration OvH2-IH: Reaction in a few infiltrating LC OV2.5: Weak signal in basal parietal cells ORF65: Section is overexposed and overdigested. Signals in parietal cells, VEC and SMC, LC
			Skin	ND	HE: Focal epidermal necrosis (early ulceration), marked perivascular LC dominated infiltration in upper dermis (with vasculitis) OvH2-IH: Reaction in basal cells in hair follicles, a few individual basal epidermal epithelial cells and DC along basement membrane OV2.5: Signals in basal cells in hair follicles ORF65: Section is overexposed and overdigested. Signals in epidermal, glandular and hair follicle epithelial cells, in VEC and LC
			Trachea	ND	HE: Focal epithelial loss (erosion/ulceration) and marked submucosal, predominantly perivascular LC infiltration (and vasculitis) OvH2-IH: Negative (loss of antigenicity is likely, as section was cut several weeks earlier) OV2.5: Signals in epithelial cells ORF65: Section is overexposed and overdigested. Signals in epithelial cells, VEC, LC and fibroblasts
		2	Kidney	ND	HE: Moderate multifocal interstitial, partly perivascular and arterial infiltration OvH2-IH: Selected finely granular staining in tubular epithelial cells Ov2.5: Negative ORF65: Section is overexposed and overdigested. strong signal in proportion of tubules (epithelial cells) and occasional cells in glomeruli
			Mesenteric LN	153938	HE: Severe autolytic changes OvH2-IH: Cannot be assessed, as reaction of post mortem infiltrating clostridia OV2.5: Signals in a few LC between follicles ORF65: Section is overexposed and overdigested. Signals cannot be assessed
			Mediastinal LN	111536	No block was available
14L-1074 Javan Banteng 3 y	UK	A	Eye	86234	HE: Multifocal LC-dominated arteritis in uvea, iris and cornea, chronic keratitis with neovascularisation, mild mixed cellular infiltration and keratocyte proliferation Ov2.5: Focal signals in few parts of the section, mainly some LC in keratitis, a few keratocyte ORF65: Similar as Ov2.5
			Lip	ND	HE: Submucosal LC dominated perivascular infiltration and arteritis, and infiltration of glands (with glandular atrophy and focal necrosis) Ov2.5: Signals in some basal and glandular epithelial cells and in few LC ORF65: Similar, but more intense than Ov2.5
			LN (?)	42029	HE: Large and mildly depleted secondary follicles, cell rich T cell zones Ov2.5: Signals in a few LC in centre and surrounding follicles, also LC in medulla ORF65: Similar with weaker signals than Ov2.5
		C	Small Intestine	5227	HE: Moderate mixed cellular mucosal infiltration; distinct Peyer's patches Ov2.5: Signals in occasional LC ORF65: More numerous LC and part of epithelial cells have signals
		D	Adrenal Gland	ND	HE: Marked LC-dominated arteritis around adrenal gland Ov2.5: Some LC in vascular infiltrates

References

				ORF65: Similar as Ov2.5
		Lung	ND	HE: Moderate multifocal LC-dominated arteritis, mild BALT Ov2.5: Signals in a proportion of infiltrating LC, also occasional alveolar epithelial cell and VEC ORF65: Similar as Ov2.5
		Lip	ND	HE: Focal erosion, moderate subepithelial LC-dominated infiltration and arteritis Ov2.5: Signals in epithelial cells in epidermis, in mucosa and hair follicles, in numerous LC and VEC ORF65: Similar as Ov2.5
	E	Small intestine	ND	HE: Moderate to marked mixed cellular mucosal infiltration, distinct Peyer's patches Ov2.5: Signals in some LC in mucosa (around crypts) and some signal in crypt epithelial cells, a few LC in the follicles of the Peyer's patches, also in neurons in plexus structures. ORF65: Similar as Ov2.5
	F	Kidney	18843	HE: Moderate multifocal interstitial, partly perivascular and arterial infiltration (arteritis) Ov2.5: Signals in relatively numerous LC in infiltrates, occasional tubular epithelial cell ORF65: Similar, but stronger and more positive cells
		Pharynx	94217	HE: Focal ulceration, marked perivascular LC-dominated infiltration, particularly in glands. Tonsil with moderate sized secondary follicles. Ov2.5: Partly basal, partly all epithelial cell layers, some LC in infiltrates, glandular duct epithelial, LC in follicles ORF65: Similar, but stronger and more positive cells in particular in association with ulceration
		Spleen	ND	HE: Moderate hyperaemia, small follicles and T cell zones Ov2.5: Signals in some LC disseminated in red pulp, and in some cells in white pulp ORF65: Similar, but stronger and more pos. cells
	G	Heart	16769	HE: LC-dominated arteritis and interstitial perivascular infiltrates. Ov2.5: Signals in a proportion of LC in arteritis and in interstitial infiltrates, and in some VEC ORF65: Similar to Ov2.5, but less intense
	H	Liver	12281	HE: disseminated mild portal LC-dominated infiltration. Ov2.5: Signals in very few LC in infiltrates ORF65: Similar to Ov2.5
		Uterus	ND	HE: Mixed cellular endometritis; LC-dominated arteritis and perivascular infiltrates in wall Ov2.5: The endometrium mainly negative, but signal in several LC in vessel associated infiltrates ORF65: Similar signals, but VEC are also positive.
		placenta	152665	No block was available
14L-1075 L Foetus of 14L-1074 9 mo	UK	Spleen	2	HE, ISH, IH: ND
		Liver	1	HE, ISH, IH: ND
		Kidney	0	HE, ISH, IH: ND
		Thoracic fluid	0	Not applicable

Abbreviations:

BALT: bronchus associated lymphoid tissue; CH: animals submitted for a diagnostic post mortem examination to the Institute of Veterinary Pathology, Vetsuisse Faculty, University of Zurich, Switzerland; F: female; DC: Dendritic cells; FDC: follicular dendritic cells; HE: haematoxylin and eosin stain; IH: immunohistology; LC: lymphocyte; LN: lymph node; ND: not done; M: male; mo: month; NHAIR: no histological abnormality is recognised; PIM: pulmonary intravascular macrophages; REC: respiratory epithelial cells; SMC: smooth muscle cells; Retrophar.: Retropharyngeal; Submand.: submandibular; TBM: tingible body macrophages; UK: healthy animals slaughtered in abattoir in the UK; VEC: vascular endothelial cells; we: week; y: year.

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