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Tracing back roots: unravelling feline enteric coronavirus pathogenesis to combat feline infectious peritonitis

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Dissertation submitted in fulfilment of the requirements for the degree of Doctor in Veterinary Sciences (PhD)

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Dutch translation of the title:
Terug naar de bron: het ontrafelen van de pathogenese van het felien enterisch coronavirus in de strijd tegen feliene infectieuze peritonitis

Desmarets L. (2015). Tracing back roots: unravelling feline enteric coronavirus pathogenesis to combat feline infectious peritonitis. Dissertation, Ghent University, Belgium.

ISBN 978-90-5864-407-7

Lowiese Desmarets was supported by the Research Foundation-Flanders (FWO-Vlaanderen).

“Do not go where the path may lead, go instead where there is no path and leave a trail.”

Ralph Waldo Emerson

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List of abbreviations

ACE2	Angiotensin converting enzyme 2
ADCC	Antibody-dependent cell-mediated cytotoxicity
ADCML	Antibody-dependent complement mediated lysis
ADEI	Antibody-dependent enhancement of infectivity
AEBSF	4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride
AEC	Amino-ethylcarbazole
AGP	Alpha1-acid glycoprotein
APN	Aminopeptidase N
BCoV	Bovine coronavirus
BMDM	Bone marrow-derived macrophages
BSM	Bovine submaxillary mucin
CEACAM	Carcinoembryonic antigen-cell adhesion molecule
CCoV	Canine coronavirus
CoV	Coronavirus
CPE	Cytopathogenic effect
C-RBD	C-terminal receptor binding domain
CrFK cells	Crandell feline kidney cells
CS	Cleavage site
DC-SIGN	Dendritic cell-specific ICAM-3 grabbing nonintegrin
DC-SIGNR	DC-SIGN related
DPP4	Dipeptidyl peptidase 4
E	Envelope
E64d	(2S,3S)-trans epoxysuccinyl-L-Leucylamido-3-methylbutane ethyl ester
EMA	Ethidium monoazide bromide
ER	Endoplasmic reticulum
ERGIC	ER-to-Golgi intermediate compartment
FBS	Foetal bovine serum
FCoV	Feline coronavirus
Fcwf cells	Felis catus whole fetus cells
FECV	Feline enteric coronavirus
FIP	Feline infectious peritonitis
FIPV	Feline infectious peritonitis virus

FITC	Fluorescein isothiocyanate
FW	Forward
HCoV	Human coronavirus
HE	Haemagglutinin esterase
HEV	Haemagglutinating encephalomyelitis virus
HR	Heptad repeat
hTERT	Human telomerase reverse transcriptase
IBV	Infectious bronchitis virus
ICAM	Intercellular adhesion molecule
IFN	Interferon
IL	Interleukin
L-SIGN	Live/lymph node specific ICAM-3 grabbing nonintegrin
M	Membrane
M.o.i.	Multiplicity of infection
N	Nucleocapsid
NA	Neuraminidase
Neu5Ac	N-acetylneuraminic acid
$Neu5,9Ac_2$	N-acetyl-9-O-acetylneuraminic acid
Neu5Gc	N-glycolylneuraminic acid
NK	Natural killer
N-RBD	N-terminal receptor binding domain
Nsp	Non-structural protein
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGM	Porcine gastric mucin
PHEV	Porcine haemagglutinating encephalomyelitis virus
P.i.	Post inoculation
PRCV	Porcine respiratory coronavirus
RBD	Receptor binding domain
RT	Room temperature
RTC	Replication transcription complex
RV	Reverse

S	Spike
SARS	Severe acute respiratory syndrome
SPF	Specific pathogen free
SV40	Simian virus 40
$TCID_{50}$	50% tissue culture infectious dose
TGEV	Transmissible gastroenteritis virus
Th	T helper
TMPRSS2	Transmembrane protease serine 2
TRS	Transcription regulatory sequence
UTR	Untranslated region
VCAM	Vascular cell adhesion molecule
VEGF	Vascular endothelial growth factor

Introduction

1.1 FELINE CORONAVIRUSES

In the sixties, a new emerging and fatal disease entity was described in cats, classically characterized by a diffuse, fibrinous and granulomatous peritonitis in the presence of ascites (Holzworth, 1963; Wolfe & Griesemer, 1966). In 1970, the aetiological agent causing this devastating feline infectious peritonitis (FIP) was discovered to be a coronavirus, designated feline infectious peritonitis virus (FIPV) (Ward, 1970). Only in 1981, it was found that many healthy cats shed a harmless coronavirus in their faeces (Pedersen *et al.*, 1981). This feline enteric coronavirus (FECV) was morphologically and antigenically indistinguishable from FIPV and explained the inconsistency seen between the high feline coronavirus (FCoV) seroprevalence and the infrequent occurrence of FIP. Moreover, with the discovery that FIPV was a virulent mutant of FECV (Vennema *et al.*, 1998), it became rapidly clear that this 'harmless' virus was the root of all problems. Despite many attempts to combat FIP in the last 5 decades, preventive and curative tools are still lacking, and FECV is still residing in virtually all multi-cat environments, where FIP has remained one of the most feared infectious causes of death in cats due to its enormous financial and emotional impact.

1.1.1 Classification and nomenclature

Feline coronaviruses belong to the family of the *Coronaviridae*, which together with the family of the *Arteriviridae, Roniviridae,* and *Mesoniviridae* are grouped within the order of the *Nidovirales*. Within the *Coronaviridae* family, feline coronaviruses are classified together with canine coronaviruses (CCoV), transmissible gastroenteritis virus (TGEV), and porcine respiratory coronavirus (PRCV) in the *Alphacoronavirus 1* species of the *Alphacoronavirus* genus (Table 1.1) (ICTV; King *et al.*, 2012). The close antigenic relationship between FCoVs, CCoVs, TGEV and PRCV, and their potential to cross species barriers, have led to the classification of those viruses as one species, and all these viruses can induce infections in cats, though most of them are asymptomatic (Barlough *et al.*, 1984; Reynolds & Garwes, 1979; Stoddart *et al.*, 1988a; Woods & Pedersen, 1979). However, at least one CCoV isolate has been shown to induce FIP upon systemic inoculation of cats (McArdle *et al.*, 1992).

FCoVs are associated with both harmless enteric and fatal systemic diseases in cats. Based on this difference in pathogenicity, they are classified as either the enteritis-inducing FECV or the FIP-inducing FIPV. Based on antigenic and genetic differences in their spike proteins, each pathotype is further divided into two serotypes (Table 1.2) (Fiscus & Teramoto, 1987a, b; Hohdatsu *et al.*, 1991a). Worldwide, the majority of all strains (both FECVs and FIPVs)

are serotype I viruses (Addie *et al.*, 2003; Benetka *et al.*, 2004; Hohdatsu *et al.*, 1992; Kummrow *et al.*, 2005; Lin *et al.*, 2009; Vennema, 1999). Serotype II viruses arise by double recombination events between serotype I FCoVs and CCoVs and bear spike and parts of the adjacent genes that are of canine origin (Herrewegh *et al.*, 1998; Lin *et al.*, 2013). Despite their low prevalence, most *in vitro* studies have been performed with serotype II strains, as these viruses are more easily cultivable compared to the serotype I strains (Dewerchin *et al.*, 2005; McKeirnan *et al.*, 1987; Rottier *et al.*, 2005; Stoddart & Scott, 1989).

Table 1.1. Overview of the *Coronaviridae* family.

Subfamily	Genus	Species	Subspecies
Coronavirinae	*Alpha-coronavirus*	*Alphacoronavirus 1*	Canine coronavirus type I
			Canine coronavirus type II
			Feline coronavirus type I
			Feline coronavirus type II
			Porcine respiratory coronavirus
			Transmissible gastroenteritis virus
		Alphacoronavirus 2[a]	Ferret enteric coronavirus
			Ferret systemic coronavirus
			Mink coronavirus
		Human coronavirus 229E	
		Human coronavirus NL63	
		Porcine epidemic diarrhoea virus	
		Miniopterus bat coronavirus 1	Miniopterus bat coronavirus 1A
			Miniopterus bat coronavirus 1B
		Miniopterus bat coronavirus HKU8	
		Rhinolophus bat coronavirus HKU2	
		Scotophilus bat coronavirus 512	
	Beta-coronavirus	*Betacoronavirus 1*	Bovine coronavirus
			Human coronavirus OC43
			Equine coronavirus
			Human enteric coronavirus
			Porcine haemagglutinating encephalomyelitis virus
			Canine respiratory coronavirus
		Human coronavirus HKU1	
		Murine coronavirus	Murine hepatitis virus
			Puffinosis coronavirus
			Rat coronavirus
		Pipistrellus bat coronavirus HKU5	
		Rousettus bat coronavirus HKU9	
		Severe acute respiratory syndrome-related coronavirus	Human SARS coronavirus
			Rhinolophus bat coronaviruses
			Palm civet coronaviruses
			Chinese ferret badger coronavirus
		Tylonycteris bat coronavirus HKU4	
		Middle East respiratory syndrome virus	
	Gamma-coronavirus	*Avian coronavirus*	Infectious bronchitis virus
			Pheasant coronavirus
			Duck coronavirus
			Pigeon coronavirus
			Goose coronavirus
			Turkey coronavirus
		Cetacean coronavirus[b]	Beluga whale coronavirus SW1
			Bottlenose dolphin CoV HKU22

	Delta-coronavirus	*Bulbul coronavirus HKU11*
		Thrush coronavirus HKU12
		Munia coronavirus HKU13
		Porcine coronavirus HKU15[c]
		White-eye coronavirus HKU16[c]
		Sparrow coronavirus HKU17[c]
		Magpie robin coronavirus HKU18[c]
		Night heron coronavirus HKU19[c]
		Wigeon coronavirus HKU20[c]
		Common moorhen coronavirus HKU21[c]
Torovirinae	*Bafinivirus*	*White bream virus*
	Torovirus	*Bovine torovirus*
		Equine torovirus
		Human torovirus
		Porcine torovirus

[a] This species has been proposed by (Vlasova *et al.*, 2011)
[b] This species has been proposed by (Woo *et al.*, 2014)
[c] (Woo *et al.*, 2012)

Table 1.2. Characteristics of the FCoV serotypes.

Pathotype	Serotype	Characteristics	Frequently used strains
FECV	I	▪ High prevalence[a] ▪ Poor *in vitro* growth: no *in vitro* cultivable strains available; currently propagated by cat-to-cat passage	UCD RM
	II	▪ Low prevalence ▪ *In vitro* cultivable (?)	WSU 79-1683[b]
FIPV	I	▪ High prevalence[c] ▪ Poor growth in cell culture: few *in vitro* passaged strains available, but most show signs of attenuation	Black (TN406)[d] UCD-1
	II	▪ Low prevalence[e] ▪ *In vitro* cultivable	WSU 79-1146 DF2 Nor15

[a] Serotype I FECVs account for 79-98% of all FCoV infections in healthy cats (Addie *et al.*, 2003; Duarte *et al.*, 2009; Hohdatsu *et al.*, 1992; Kummrow *et al.*, 2005).
[b] FCoV strain WSU 79-1683 is an enteritis-inducing strain, but is believed to rather be an avirulent FIPV than a real FECV, as this strain has genetic hallmarks of FIPVs. So far, no other serotype II enteric strain has been cultivated.
[c] Serotype I FIPVs account for 69-89% of all FIP cases (Benetka *et al.*, 2004; Duarte *et al.*, 2009; Hohdatsu *et al.*, 1992).
[d] The abundantly used high passage Black strain has been shown to be completely attenuated by cell culture propagation, as it does no longer induce FIP upon inoculation (Tekes *et al.*, 2012).
[e] Recently described in an epizootic outbreak of FIP (Wang *et al.*, 2013).

1.1.2 Virus characteristics

Coronaviruses are roughly spherical particles measuring 80-160 nm in diameter. The helical nucleocapsid, comprising the positive single stranded RNA associated with the nucleocapsid (N) proteins, is surrounded by a lipid membrane, the viral envelope. In the viral envelope, 3 structural proteins are embedded, the spike (S), membrane (M) and envelope (E) proteins (Figure 1.1).

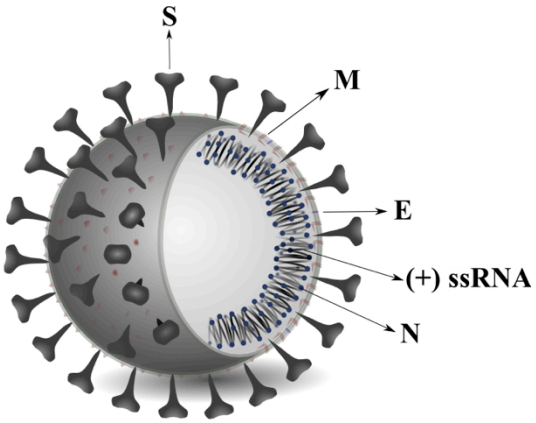


Figure 1.1. FCoV virion. Coronaviruses contain a positive, single stranded RNA (+ ssRNA), complexed with nucleocapsid (N) proteins. This helical nucleocapsid is surrounded by an envelope in which 3 structural proteins are embedded, the spike (S), the membrane (M), and the envelope (E) protein.

1.1.2.1 Genome organization

FCoVs have a single-stranded, positive-sense polyadenylated RNA genome of around 29 kilobases (Figure 1.2). This positive single stranded RNA directly serves as mRNA for the generation of the viral replicative proteins via translation of open reading frame (ORF)1a and ORF1b, which make up two-thirds of the viral genome. To allow translation of ORF1b, a ribosomal frameshifting mediated by pseudoknot structural element occurs (Bredenbeek *et al.*, 1990). Translation of ORF1a and 1b yields two polyproteins, pp1a and pp1ab, which further guide the viral replication and transcription (see below), regulate cellular processes and potentially also fulfil other yet unknown functions (Neuman *et al.*, 2014). The remaining 3' proximal third of the genome contains 6 additional ORFs, encoding the structural proteins S, E, M, and N (ORF2, ORF4, ORF5, and ORF6, respectively), and the non-structural, accessory proteins 3a, 3b, 3c (ORF3), and 7a, 7b (ORF7). As for eukaryotic mRNA, the viral RNA genome also contains non-translated regions such as the 5' cap, the 5' untranslated region (UTR), the 3' UTR and the 3' poly-A tail. The 5' UTR contains the leader sequence and the ORF1 transcription regulatory sequence (TRS), the latter which is also found in front of each ORF (Sawicki *et al.*, 2007).

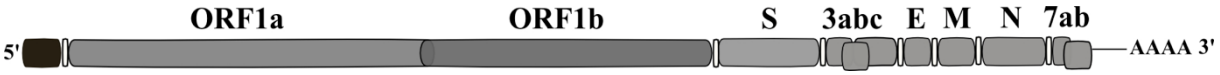


Figure 1.2. FCoV genome organization. The 5' end of the genome contains the leader sequence (black box), followed by the transcription regulatory sequence (TRS, white box) of the polymerase gene, which is comprised of the overlapping open reading frame (ORF)1a and ORF1b. The other ORFs, each of them preceded by a TRS, encode for the structural (S, E, M, N) and accessory proteins (3abc and 7ab).

1.1.2.2 Structural proteins

The trimeric, club-like **spike (S) proteins** project from the surface of the virions, giving the particle its corona-like appearance (Figure 1.1 and 1.3). S proteins are involved in entry by mediating attachment and virus-cell fusion processes and are essential determinants of host-range and pathogenicity of coronaviruses (Cowley & Weiss, 2010; Krempl *et al.*, 1997; Sanchez *et al.*, 1999). They are class I fusion proteins of about 170-222 kilodalton (kDa) in size, and are highly N-glycosylated with complex and mannose-rich oligosaccharides (Bosch *et al.*, 2003; Siddell *et al.*, 1983). S proteins are integral transmembrane proteins consisting of an N-terminal ectodomain, a transmembrane helix and a short C-terminal cytoplasmic tail. They have common structural features with the fusion proteins of other enveloped viruses such as orthomyxo-, paramyxo-, retro-, filo-, and arenaviruses (Bosch *et al.*, 2003; White *et al.*, 2008). Typically, these fusion proteins contain a region rich in hydrophobic residues, called the fusion peptide. In addition, they are characterized by 2 heptad repeat (HR) regions, consisting of a series heptapeptides in which the first and the fourth amino acids are typically hydrophobic. These give the HR regions their homotrimeric coiled-coil structure. Viral fusion proteins are synthesized as precursor proteins that undergo endoproteolytical cleavage by host proteases, generating a metastable complex of the receptor binding and the fusion subunit, which remain covalently or non-covalently linked, dependent on the virus (Eckert & Kim, 2001). This brings the protein in a fusion competent state, allowing the rapid dissociation when encountering the fusion trigger (see paragraph 1.2). In contrast to other viruses, coronaviruses differ with regard to the cleavage of their S proteins in between the receptor binding subunit (S1) and the fusion subunit (S2). It seems that cleavage does not occur for most alphacoronaviruses, whereas cleavage of beta-and gammacoronavirus' spikes depends on the virus strain and cell type. This implies that many coronavirus exit infected cells with uncleaved spikes. However, these viruses seem to use cellular proteases encountered during viral entry for activation of their fusion proteins (see paragraph 1.2). As exception in the genus *Alphacoronavirus*, serotype I FCoVs can carry a furin cleavage site, and potentially carry pre-cleaved spikes (de Haan *et al.*, 2008). Mutations in this furin cleavage site have recently been linked to the pathotype switch (Licitra *et al.*, 2013).

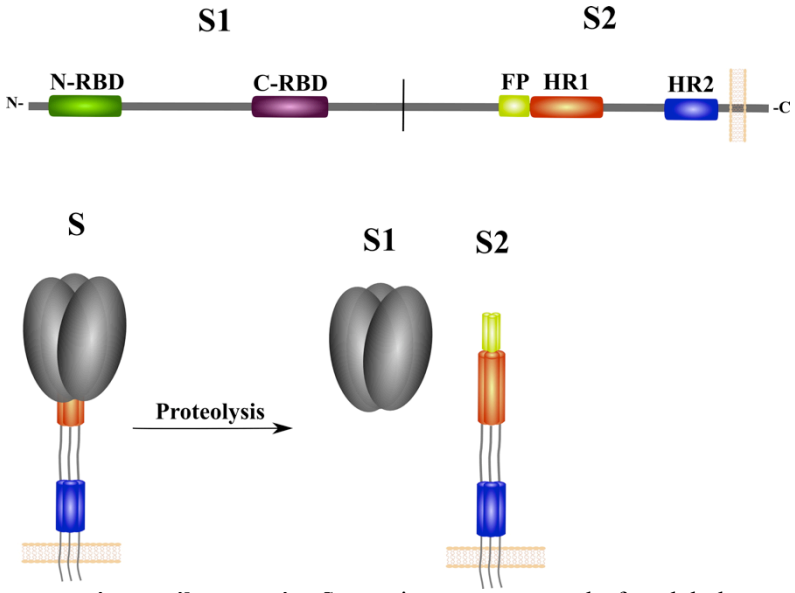


Figure 1.3. The coronavirus spike protein. S proteins are composed of a globular receptor binding subunit (S1) and a stalk-like transmembrane fusion (S2) subunit. S proteins protrude from the viral envelope as homotrimer complexes. The globular head represents the S1 subunit and mediates the attachment, which can be performed by the N-terminal and/or C-terminal receptor binding domain (RBD). The stalk-like S2 subunit contains a fusion peptide (FP) and 2 heptad repeat regions (HR 1 and 2) and is responsible for the membrane fusion after proteolytical dissociation from the S1 subunit.

The 25-35 kDa triple spanning **membrane (M) protein** is the most abundant envelope protein. It is a type III integral membrane protein with a short N-terminal glycosylated ectodomain, 3 transmembrane domains and a long C-terminal endodomain (Rottier, 1995). During virus assembly, M proteins interact with each other and with N and S proteins (de Haan *et al.*, 2000; Narayanan *et al.*, 2000; Opstelten *et al.*, 1995). These M-S interactions are needed to retain the spike proteins at the budding site, allowing their integration in the virion (Opstelten *et al.*, 1995). M proteins have key roles in virus assembly/budding, and mediate induction of neutralizing antibodies and immune-evasion processes (Dewerchin *et al.*, 2006; Rottier, 1995).

The **envelope (E) protein** is a small, non-glycosylated, hydrophobic protein of 9-12 kDa in size. It is only in restricted numbers present in the virus envelope and is an integral membrane protein spanning the envelope twice with both ends oriented to, and the C-terminal end even extending in, the viral lumen (Maeda *et al.*, 2001). Together with the M protein, the E protein plays crucial roles in virus assembly and budding (Lim & Liu, 2001). This multifunctional protein also displays ion channel activity, contributing to virus virulence and pathogenesis of at least severe acute respiratory syndrome coronavirus (SARS-CoV) (Nieto-Torres *et al.*, 2014).

Nucleocapsid (N) proteins are highly phosphorylated structural proteins (50-60 kDa) involved in packaging the viral genomic RNA to form the helical nucleocapsid (Spaan *et al.*, 1988). Incorporation of the nucleocapsid into the virion is mediated by N-M interactions. In addition to the RNA packaging and protecting role, N proteins fulfil many other functions, such as facilitating viral RNA synthesis (Baric *et al.*, 1988; Sawicki *et al.*, 2007) and perturbation of several cellular processes such as IFN-induced responses (Kopecky-Bromberg *et al.*, 2007; Ye *et al.*, 2007). In addition to S proteins, N proteins are important inducers of cell-mediated immunity (Takano *et al.*, 2014a; Zhao *et al.*, 2005).

1.1.2.3 Accessory proteins

Apart from the structural genes, the 3' one-third of the genome also contains several non-structural genes. Homologous genes are found in viruses from the same genus (formerly called group, and hence often referred to as group-specific genes), but have no or very few similarity with genes of coronaviruses from different genera. Although their evident role in virulence *in vivo*, they are often described as 'accessory' genes, since it has been shown that the proteins encoded by these genes are largely dispensable for *in vitro* growth (de Haan *et al.*, 2002; Haijema *et al.*, 2004; Ortego *et al.*, 2003). Two gene clusters, ORF3abc and ORF7ab, code for the 5 non-structural proteins of FCoVs. Although shown to be of key importance for efficient viral-host interactions *in vivo* (Haijema *et al.*, 2004), the exact function of most of these proteins is still largely unknown and a matter of speculation (Table 1.3).

Table 1.3. Properties and potential function of the FCoV accessory proteins.

Protein	Properties	Role during FCoV infections
3abc	Combines the effect of 3a, 3b and 3c proteins	• Determinant of virulence *in vivo* (Haijema *et al.*, 2004) • Deletion enhances (FIPV DF-2) (Balint *et al.*, 2012) or impairs (FIPV 79-1146) (Dedeurwaerder *et al.*, 2013) replication in monocytes, but has no effect in bone marrow-derived macrophages (BMDM) *in vitro* (Rottier *et al.*, 2005) • Restoration of deletion converts FIPV DF-2 into an enteric biotype *in vivo* (Balint *et al.*, 2014a) • Necessary for ORF7-dependent protection against interferon (IFN)-α *in vitro* (Dedeurwaerder *et al.*, 2014) • Not involved in antibody-mediated internalisation of viral glycoproteins or in escape from antibody-dependent complement mediated lysis (Cornelissen *et al.*, 2009)
3a	• Soluble protein (70-71 amino acids) • Well conserved among FCoVs of the same serotype	• Not necessary for replication in BMDM *in vitro* (Rottier *et al.*, 2005)
3b	• Soluble protein (72-73 amino acids) • Well conserved among FCoVs of same serotype	
3c	• Class III triple spanning membrane protein, similar to SARS 3a protein • Intact and well conserved in all faecal strains, deleteriously mutated in 60-71.4 % of FIPV strains (Chang *et al.*, 2010; Pedersen *et al.*, 2012)	• Potential determinant of intestinal replication, and hence efficient oro-faecal transmission in between cats (Chang *et al.*, 2010; Pedersen *et al.*, 2009; Pedersen *et al.*, 2012)
7ab	Combines the effect of 7a and 7b proteins	• Determinant of virulence *in vivo* (Haijema *et al.*, 2004) • Deletion negatively affects sustainability of FIPV replication in monocytes (Dedeurwaerder *et al.*, 2013), but has no effect in BMDM *in vitro* (Rottier *et al.*, 2005) • Not involved in antibody-mediated internalisation of viral glycoproteins or in escape from antibody-dependent complement mediated lysis (Cornelissen *et al.*, 2009)
7a	• 10 kDa membrane protein • 72% homologous to TGEV protein 7 • Relatively well conserved among FCoVs	• IFN-α antagonist (Dedeurwaerder *et al.*, 2014)
7b	• 24 kDa soluble glycoprotein, secreted from infected cells • Least well conserved among FCoVs • Specific for FCoVs, CCoVs, and ferret CoVs • Deleted/truncated in cell culture adapted strains	• Potential competitive inhibitor of host cytokines and/or inducer of T-cell apoptosis (Haagmans *et al.*, 1996; Herrewegh *et al.*, 1995; Rottier, 1999)

1.1.3 Replication cycle

As obligate intracellular parasites, coronaviruses depend on host cell machinery for their replication, a highly organised multistep process that takes about 9-12 hours to complete (Figure 1.4).

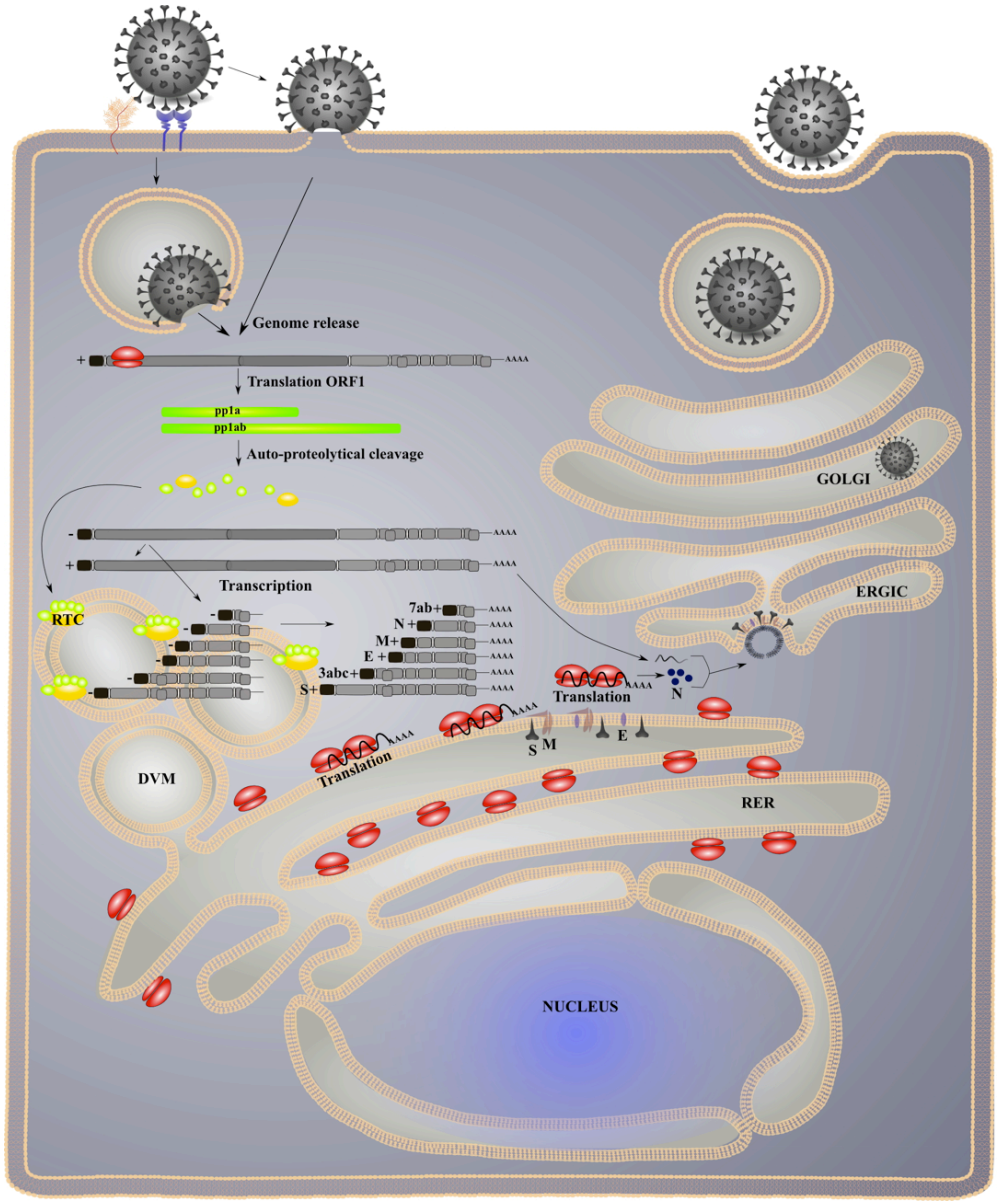


Figure 1.4. FCoV replication cycle. The positive sense genomic RNA, released into the cytosol upon receptor binding and subsequent fusion processes, directly serves as mRNA for the translation of ORF1a and ORF1b, yielding 2 polyproteins, pp1a and pp1ab. Autoproteolytical cleavage of pp1a and pp1ab yields at least 16 proteins, many of them forming the replication transcription complex (RTC) at ER-derived double membrane vesicles (DMV). The RNA-dependent RNA polymerase makes a negative stranded copy of the genome, which subsequently serves as a template for the generation of new genomic RNA, as well as the formation of minus-strand, subgenomic mRNAs via discontinuous transcription. These minus-strand intermediates then serve as template for the generation of the actual subgenomic mRNAs, from which all non-polymerase viral proteins are translated. Structural proteins accumulate at the ER-to-Golgi intermediate compartment (ERGIC), from where budding of new virions occurs. These new particles are subsequently transported through the secretory pathway and are released in the extracellular environment by exocytosis.

1.1.3.1 Entry

In order to gain access to the host cell transcription and translation tools, coronaviruses have to release their genome in the cytosol of the target cell. This entry process requires cell attachment and subsequent fusion between the viral envelope and the host plasma- or endosomal membrane. Both steps of the entry process are carried out by the viral S protein and are discussed in detail in paragraph 1.2.

1.1.3.2 Replication and transcription

Once the nucleocapsid is released into the cellular cytoplasm, the ribonucleoprotein complex disassembles and the 2 precursor proteins pp1a and pp1ab are directly synthesized from the genomic RNA. Subsequent autoproteolytical cleavage of these polyproteins yield 16 mature non-structural proteins (nsp). Together with the N proteins and some cellular proteins, these nsp assemble in the replication-transcription complex (RTC) where both genome replication and production of subgenome-sized mRNA occur. These RTCs are associated with double membrane vesicles at the perinuclear region of infected cells. Since only ORFs at the beginning of a mRNA can be read by eukaryotic ribosomes, coronaviruses, as for all nidoviruses, typically generate a nested-set of subgenomic mRNAs during their replication (Siddell *et al.*, 1983). These subgenomic mRNAs are generated by a process of discontinuous transcription, which via minus-strand intermediates result in 6 subgenomic RNAs with a common 5' leader and the ORF-specific TRS, followed by a variable length sequence containing 1 or more ORFs and a 3' poly(A) stretch (Figure 1.4) (Sawicki *et al.*, 2007). Although most of the subgenomic mRNAs are structurally polycistronic, only the first ORF of each mRNA is generally translated. However, translation of coronavirus accessory proteins results from functional polycistronic subgenomic mRNAs, generating 3 (ORF3) or 2 (ORF7) proteins from 1 ORF through a leaky scanning mechanism (Schaecher *et al.*, 2007).

1.1.3.3 Assembly and release

To produce progeny viruses, new nucleocapsids have to be assembled and subsequently need to be enveloped by a lipid membrane in which all structural proteins are embedded. Assembly of ribonucleoproteins occurs in the cytoplasm, after which they bud through the endoplasmic reticulum-to-Golgi intermediate compartment (ERGIC) within which the S, M, and E proteins are membrane-embedded. M and E proteins play a central role in this assembly/budding process (Vennema *et al.*, 1996). M proteins interact with both genomic RNA and N proteins to exclude the incorporation of non-genomic RNA (as N proteins can bind non-selectively to all RNA present in the cell), they associate with S proteins to ensure their incorporation, and

they form homotypic interactions necessary for the morphology of the envelop (de Haan *et al.*, 2000; Narayanan *et al.*, 2000; Opstelten *et al.*, 1995). E proteins contribute to the envelope formation and determine the site of budding (Fischer *et al.*, 1998; Lim & Liu, 2001). Assembled virions are transported out of the infected cell by the secretory pathway, during which glycosylation of S and M proteins occurs, and S proteins may become proteolytically cleaved, the latter depending on the virus and the strain (see paragraph 1.2.2). To allow S-M interactions and hence incorporation of S proteins in virions, posttranslational palmitoylation of cysteine residues in the cytoplasmic tail of S proteins is required (Thorp *et al.*, 2006). This palmitoylation contributes to the sorting process of S proteins, as only abundantly palmitoylated spike are incoported in the virions, whereas other S proteins are sorted to the cell surface where they can mediate cell-cell fusion and enhance the viral cell-to-cell spread (Shulla & Gallagher, 2009).

1.1.4 Epizootiology and pathogenesis

The exact viral and host key players in the onset of FIP are still largely unknown, and after many years of research it has become increasingly clear that the pathogenesis of FCoVs is much more complex than initially thought. Although described as an enteric virus, FECV is not confined to the intestinal tract. Eighty to ninety percent of all healthy coronavirus-infected cats show a monocyte-associated viraemia which can last for at least 12 months (Gunn-Moore *et al.*, 1998). In addition, FCoV RNA can be detected in all parenchymal organs of healthy cats, showing that systemic dissemination and widespread tissue distribution is not a hallmark of FIPVs (Meli *et al.*, 2004). However, the viral load detected in parenchymal tissues of FIP cats is substantially higher than in healthy coronavirus-infected animals. Hence, not the ability to spread systemically, but the rate of viral replication in monocytic cells and/or the ability to clear these infected cells have been linked to the development of FIP (see below) (Kipar *et al.*, 2006a). The pathogenesis of FCoVs is depicted in Figure 1.5 and will be further divided into 3 phases for discussion.

1.1.4.1 Intestinal replication and faecal-oral transmission of FCoVs

It is widely accepted that the majority of all FIP cases are the consequence of mutations arising in the viral genome during a common FECV infection (Chang *et al.*, 2010; Pedersen *et al.*, 2009; Pedersen *et al.*, 2012; Poland *et al.*, 1996). Although this internal mutation theory can be questioned during infrequently observed epizootics of FIP, i.e. when FIP deaths greatly exceed the normally encountered 5-12% of all seropositive cats (Addie & Jarrett,

1992; Kipar & Meli, 2014; Pedersen, 2009; Wang *et al.*, 2013), there is so far no firm proof for horizontal transmission of FIPVs (Barker *et al.*, 2013).

FECVs are found in virtually all multi-cat environments worldwide (Addie & Jarrett, 1992; Pedersen *et al.*, 1981), except for the Falkland Islands, which have remained FCoV seronegative so far by extensive testing of incoming cats (Addie *et al.*, 2012). Faeces from shedders are highly contagious, resulting in a very fast and efficient faecal-oral transmission of the virus to non-infected susceptible cats, which in turn start to shed a high amount of FECV in their faeces within one week after uptake (Pedersen *et al.*, 2008; Pedersen *et al.*, 2004; Pedersen *et al.*, 1981; Vogel *et al.*, 2010). Kittens in endemic environments are usually protected by maternal immunity and often do not shed virus before the age of 5-10 weeks (Addie *et al.*, 2009; Foley *et al.*, 1997; Harpold *et al.*, 1999; Pedersen *et al.*, 2008). However, shedding by kittens from 2 weeks of age has been described, explaining why the success of early weaning in preventing FIP is variable (see below) (Lutz *et al.*, 2002).

After ingestion, FECV proceeds to the intestinal tract where it finds its target cell, the enterocyte, notably lining the jejunum, ileum, caecum and colon, from which subsequent shedding of progeny virus occurs (Herrewegh *et al.*, 1997; Kipar *et al.*, 2010; Meli *et al.*, 2004; Pedersen *et al.*, 1981). This replication causes a transient enteritis, occasionally accompanied by loss of appetite and/or diarrhoea, but which is most often too mild to be noticed (Hickman *et al.*, 1995; Pedersen *et al.*, 1981; Vogel *et al.*, 2010). Faecal shedding is much higher in young (<2 years), immunocompromised, and, to a lesser extent, old (> 8 years) cats compared to adult cats. This increased rate of replication potentially favours the onset of pathotype switching mutations, which can, apart from the impaired capacity of these animals to clear mutated viruses, additionally explains the higher incidence of FIP in these groups of animals (Pedersen *et al.*, 2008; Poland *et al.*, 1996). However, it remains elusive so far whether these mutations indeed arise during intestinal replication or whether their introduction results from selective pressure when taken up by monocytes/macrophages, allowing this mutated virus to eventually adapt to and efficiently replicate in these new target cells, with all known consequences (Pedersen *et al.*, 2012).

In contrast to many other enteric viruses, FECVs, and notably serotype I strains, are known to establish long-lasting infections, as duration of shedding usually takes 2-18 months to eventually wane (Addie & Jarrett, 2001; Addie *et al.*, 2003; Pedersen, 2009; Pedersen *et al.*, 2008). In addition, around 13% of all FECV infected cats will become life-long shedders, comprising a continuous threat for susceptible animals (Addie & Jarrett, 2001). Endemic

coronavirus infections are the result of the readily declining local immunity. This lack of memory immune response allows continuous reinfections of cats, resulting from the high population number (and hence great chance that one cat is still shedding why the other one has become negative), but notably resulting from the persistently shedding cats in multi-cat households (Addie & Jarrett, 2001; Foley *et al.*, 1997; Pedersen, 2009). Although cats can become reinfected several times with the same or a different strain, reinfections do not increase the onset of FIP (Addie *et al.*, 2003). The long-lasting presence of FECV in the cat's body implies that, as known for FIPV (see below), FECV potentially uses some immune evasion strategies, but these have yet to be characterized.

1.1.4.2 Systemic dissemination of FCoVs by monocyte-associated viraemia

As mentioned before, viruses are not confined to the intestinal tract in the majority of all FECV infections, but additionally undergo a notably cell-associated viraemia with viral distribution throughout the cat's body (Gunn-Moore *et al.*, 1998; Meli *et al.*, 2004). It remains unclear whether this cell-associated viraemia results from uptake of FCoVs by monocytic cells underneath the epithelium in the intestine, or even tonsils as previously proposed for FIPV (Stoddart *et al.*, 1988b), or whether cell-free virus enters the draining lymph vessels and subsequently finds monocytic cells in or on its way to the blood. Nevertheless, systemic spread occurs in virtually every healthy cat and this has not only complicated the diagnosis of FIP, but also the search for FIP-inducing mutations, as viral RNA found in cats' tissues does not always reflect a real FIP-inducing strain. Indeed, whereas one report found a typical 'FIPV mutation' (M1058L or S1060A) in the spike protein by comparing faecal strains of healthy cats with tissue strains found in FIP cats (Chang *et al.*, 2012), a subsequent study revealed these mutations to be a hallmark of all systemic FCoVs found in tissues of both non-FIP and FIP cats (Porter *et al.*, 2014).

A peculiar observation is that intraperitoneal inoculation of FECV can occasionally result in faecal shedding which is very similar in onset and shedding levels to the shedding seen in orally inoculated cats (Foley *et al.*, 1997; Pedersen *et al.*, 2012). This implies that most probably FECV can also be carried back by monocytic cells from the periphery to the intestine. Whether this way of enterocyte infection is also the source of the persistent shedding remains to be investigated.

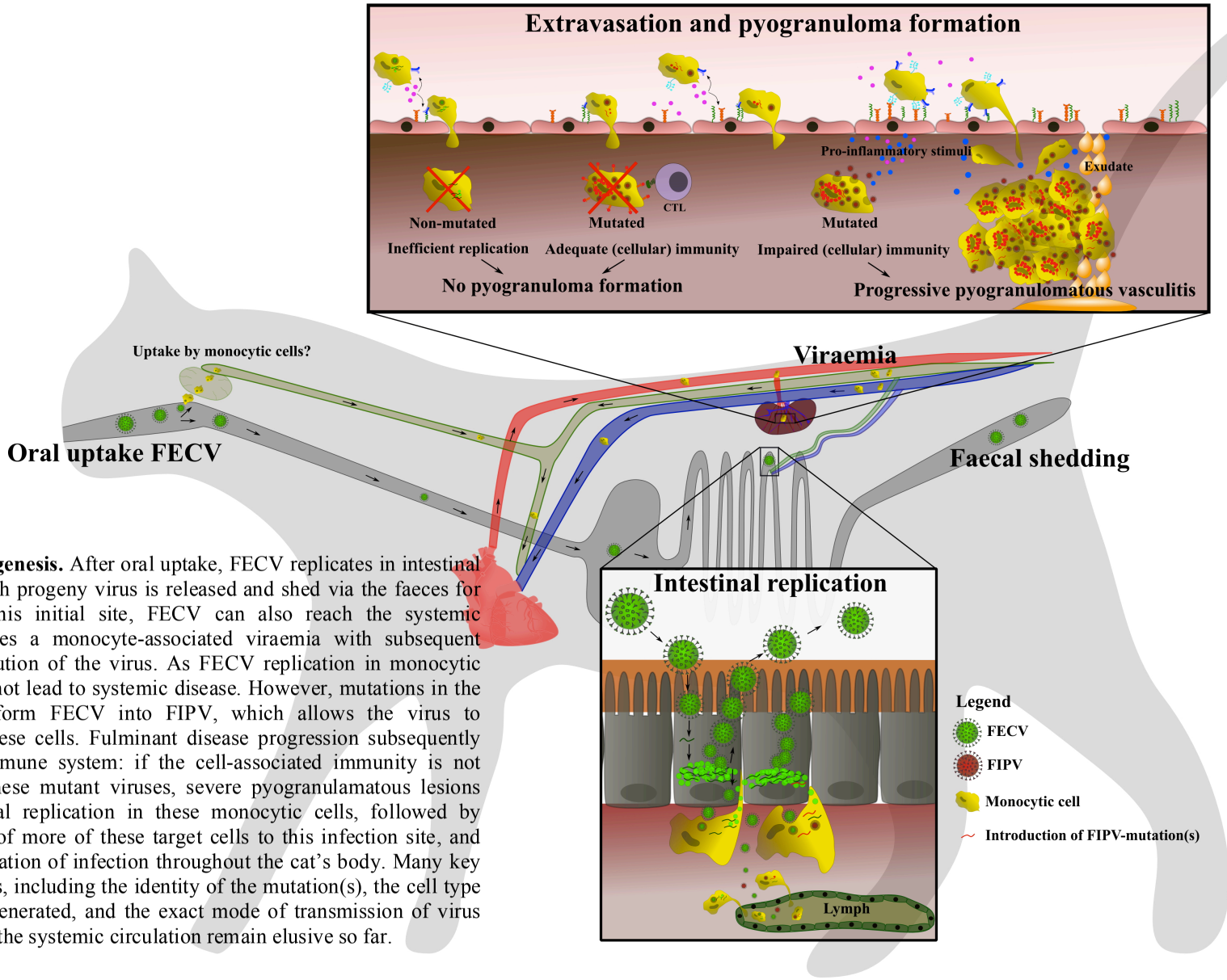


Figure 1.5. FCoV pathogenesis. After oral uptake, FECV replicates in intestinal epithelial cells from which progeny virus is released and shed via the faeces for several months. From this initial site, FECV can also reach the systemic circulation, and undergoes a monocyte-associated viraemia with subsequent widespread tissue distribution of the virus. As FECV replication in monocytic cells is limited, this will not lead to systemic disease. However, mutations in the viral genome can transform FECV into FIPV, which allows the virus to efficiently replicate in these cells. Fulminant disease progression subsequently depends on the cat's immune system: if the cell-associated immunity is not capable of combatting these mutant viruses, severe pyogranulamatous lesions will result from the viral replication in these monocytic cells, followed by activation and attraction of more of these target cells to this infection site, and further systemic dissemination of infection throughout the cat's body. Many key events in the pathogenesis, including the identity of the mutation(s), the cell type in which mutations are generated, and the exact mode of transmission of virus from mucosal surfaces to the systemic circulation remain elusive so far.

1.1.4.3 Virus distribution and replication in parenchymal organs

Although systemic spread occurs in both healthy and FIP cats, FCoV RNA is found in a far greater proportion of tissue samples and in much higher copy numbers in FIP cats compared to healthy cats (Kipar *et al.*, 2006a; Porter *et al.*, 2014). Moreover, except for some antigen positive sinus macrophages in mesenteric lymph nodes and pulmonary intravascular macrophages in persistently infected healthy cats, viral antigen detection has only been successful in tissues of FIP cats so far (Kipar *et al.*, 2010; Porter *et al.*, 2014). These findings strongly indicate that the pathotype difference is related to the level of viral replication in parenchymal organs. Indeed, *in vitro* experiments have shown that key determinants of the FIPV pathotype are the ability of the virus to induce efficient and sustainable replication in and subsequent activation of monocytes (Dewerchin *et al.*, 2005; Regan *et al.*, 2009; Rottier *et al.*, 2005; Stoddart & Scott, 1989). This activation contributes to the typical (pyo)granulomatous vasculitis, as it does not only cause enhanced extravasation, but potentially also renders these cells more susceptible to FIPV infection (Kipar & Meli, 2014; Kipar *et al.*, 2005). Infected cells release several inflammatory mediators that further contribute to the typical progressive granuloma formation by 1) continuous chemotactic attraction of neutrophils and new infectable monocytes, the latter which can be continuously supplied by indirect virus-induced monocyte/macrophage proliferation in haemolyphatic tissues, and by 2) mediating tissue damage (Goitsuka *et al.*, 1990; Hasegawa & Hasegawa, 1991; Kipar *et al.*, 2001; Kipar *et al.*, 2005; Weiss *et al.*, 1988). In addition, at least one of those substances released from infected cells, vascular endothelial growth factor (VEGF), additionally increases the vascular permeability and is a determining factor in effusion formation (Takano *et al.*, 2011). The exaggerated extravasation of leukocytes at the site of granulomatous vascular lesions has been shown to be the consequence of a general systemic upregulation of leukocyte-associated adhesion molecules combined with the restricted upregulation of endothelial counter ligands (P-selectin, E-selectin, intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1)) at the site of infection. The latter is the result of specific mediators released by infected monocytes and explains why, despite the systemic activation of leukocytes, extravasation is confined to these specific sites (Olyslaegers, 2014; Olyslaegers *et al.*, 2013).

Although the consequences of the pathotype switch are clear, the exact mutations causing the virus to change its cell tropism remain elusive. Mutations in several viral proteins (notably S and 3c) have been proposed to be involved (Chang *et al.*, 2010; Chang *et al.*, 2012; Licitra *et*

al., 2013; Pedersen *et al.*, 2009; Pedersen *et al.*, 2012; Rottier *et al.*, 2005), but the lack of a clinically relevant serotype I FECV/FIPV infectious clone and notably a susceptible cell line to grow and study these viruses have seriously hampered this search so far.

Regardless of the viral genetics, the onset of FIP is additionally determined by the cat's genetics. Indeed, cats in the same environment are exposed to the same strains, but only a restricted number of them will eventually develop FIP, and some breeds or notably lines within breeds have a higher incidence of FIP (Pedersen, 2009). It is now well established that not the humoral but only a strong cellular immunity is of key importance in the survival from FIPV infection. Cats experimentally infected with highly virulent FIPV typically show waves of viral replication and viraemia, provoked by acute indirect virus-induced T-cell lymphopenia, which coincide with fever and weight loss. These waves of disease are interspersed with a period of apparent recovery, during which (partial) reconstitution of antiviral T-cell immunity seems to temporally confine the infection in all cats. Subsequent disease progression and final outcome of this infection is cat-dependent: whereas some cats develop a fulminant T cell lymphopenia in blood and lymphoid tissues, consequently resulting in rapid disease progression and death, others gain control over the infection, resulting in complete recovery and long-term survival (de Groot-Mijnes *et al.*, 2005). This prolonged survival of FIPV infection does not necessarily mean that the virus is cleared from the body, as inducing immune suppression in surviving cats still evoked the onset of FIP (Pedersen, 1987, 2009). This and the fact that in normal conditions FIPV arises during FECV infections, make it hard to determine the incubation period and the source of infection in naturally occurring FIP cases.

The substantial loss of immunological control during FIP development is not confined to the cellular immunity. FIPV-infected cats typically mount an excessive antibody response, but antibodies are not protective and can even enhance the course of the infection in FCoV seropositive cats, the latter at least upon experimental inoculation with certain FIPV strains (Pedersen, 2009; Pedersen & Boyle, 1980; Vennema *et al.*, 1990; Weiss & Scott, 1981) . This antibody-dependent enhancement of infectivity (ADEI) has been attributed to the promotion of Fc-receptor mediated uptake of FIPV by macrophages (Hohdatsu *et al.*, 1991b). However, as in natural conditions development of FIP often occurs on first exposure to FCoVs, and cats are not exposed to FIPV but to FECV, the role of ADEI in naturally occurring FIP is questioned and is believed to be a non-natural phenomenon occurring during experimental infections (Addie *et al.*, 1995; Pedersen, 2009). In addition, ADEI cannot explain why the

cat's immune system is not able to clear the infected monocytes/macrophages. An explanation for the latter was found *in vitro,* showing that half of the FIPV-infected monocytes do not express viral proteins on the cell surface (= retention), whereas the others rapidly internalize the membrane-expressed viral proteins upon anti-FCoV antibody addition. These 2 phenomena, retention and antibody-mediated internalization of viral proteins, protect infected cells from antibody-dependent cell-mediated cytotoxicity (ADCC) and antibody-dependent complement-mediated lysis (ADCML) and hence explains why FIPV cannot be cleared from the body despite the enormous amount of antibodies present in those cats (Cornelissen *et al.*, 2007; Dewerchin *et al.*, 2005, 2006).

Cytokine expression in healthy and FIP cats (including tumor necrosis factor alpha, interleukin (IL) 1, 2, 4, 6, 10, and 12, and IFN-γ) have been extensively studied in order to find out how the immune system is involved in the onset of FIP (Berg *et al.*, 2005; Dean *et al.*, 2003; Goitsuka *et al.*, 1987; Goitsuka *et al.*, 1990; Hasegawa & Hasegawa, 1991; Kipar *et al.*, 2006b; Kiss *et al.*, 2004; Takano *et al.*, 2007). However, great variability is observed between individual animals, and even between different tissues within 1 animal, often resulting in contradictory conclusions on the expression level of a specific cytokine in FIP cats. But although the triggers for the onset of FIP remain largely elusive, the consequences are unambiguously clear: FIP is associated with a proliferation and activation of the FIPV target cells (monocytes/and macrophages) and with an evident suppression of protective innate (NK-cells) and adaptive (CD4+ and CD8+ T cells, and regulatory T cells) immunity to attack the virus and to deal with excessive damaging inflammatory processes (de Groot-Mijnes *et al.*, 2005; Kipar & Meli, 2014; Kipar *et al.*, 2005; Vermeulen *et al.*, 2013).

1.1.5 Lesions and symptoms

1.1.5.1 FECV infections

Although affecting nearly all cats in multi-cat environments, FECV infections pass unnoticed in most of these cases, as they are generally associated with no or rather mild and non-specific (transient anorexia and/or diarrhoea) symptoms. Consequently, most cattery owners are not aware of the presence of FECV until one or more of their cats suddenly succumb(s) to FIP.

1.1.5.2 FIP

FIP was first described as a disease typically characterised by a diffuse granulomatous peritonitis in presence of ascites (Holzworth, 1963). However, it became rapidly clear that peritonitis is only one amongst several other pathological changes seen in FIP, affecting many

organs and tissues besides the peritoneum. FIP manifests in different clinical forms, depending on the presence (effusive or wet FIP) or absence (non-effusive or dry FIP) of effusions, and depending on the affected organ(s). Effusions (abdominal, thoracic and/or pericardial) are seen in 60-80% of all cases, but care should be taken when using this as the only factor to consider FIP as diagnosis, as only 51% of all cats with effusion do actually have FIP (Hartmann *et al.*, 2003). Moreover, the highly variable and non-specific clinical signs do not further help clinicians in finding the right aetiology for the cats' disease (see below). Mostly, FIP cats are presented with lethargy, anorexia, and weight loss, but it is not uncommon that seemingly normally conditioned cats, initially presented with other symptoms as dyspnoea, polyuria/polydipsia (due to kidney disease or pancreas damage-induced diabetes), neurological signs and/or ocular lesions, are finally diagnosed with FIP. Upon physical examination, many FIP cats show jaundice (which can have a hepatic and pre-hepatic origin) and/or mild fever, which upon treatment, shows to be unresponsive to antibiotics. Palpation/medical imaging will confirm the presence of abdominal fluid (which is present in 65% of all effusive forms), and can reveal enlargement or deformation of kidneys, liver, intestines and/or mesenteric lymph nodes in the dry form of the disease (Addie *et al.*, 2009).

Distinction between the effusive and non-effusive form was initially thought to be a reflection of the lesions, serosal and parenchymatous granulomatous lesions, respectively. However, profound pathological examinations have shown that this is merely a clinical distinction, as a mixture of both serosal and parenchymatous (pyo)granulomas are found in nearly all cats (Kipar & Meli, 2014).

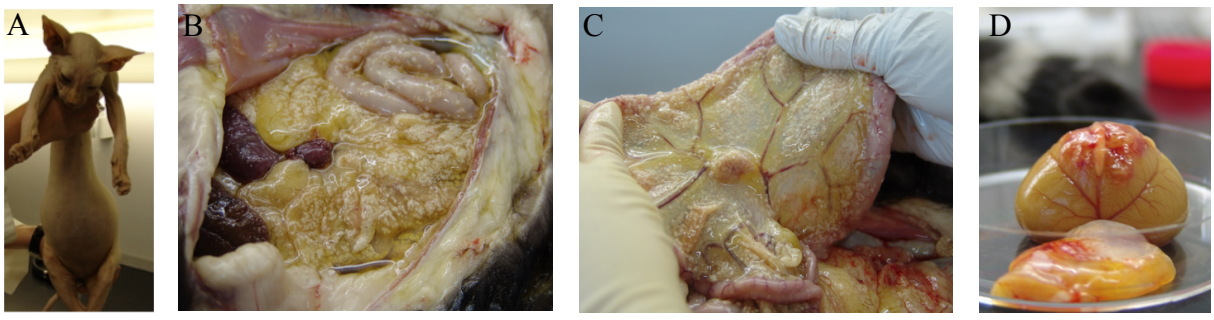


Figure 1.6. FIP pathology. (**A**) Typically big-bellied FIP cat due to abdominal effusion. (**B**) Macroscopic lesions consisting of diffuse granulomatous serositis affecting the omentum, spleen, liver, intestines, and peritoneum. (**C**) Diffuse, small granulomas on mesentery with enlargement of mesenteric lymph node. (**D**) Vasculitis and large granulomas on the kidney.

1.1.6 Diagnosis

Ante-mortem diagnosis of FIP is often non-conclusive for the following reasons: 1) the symptoms are too vague to discriminate from other diseases and vary in between FIP cats depending on the affected organs, 2) serology is hampered by the detection of anti-FECV antibodies, and 3) changes in haematological parameters and protein electrophoresis can be suggestive but are certainly not pathognomonic. FIP can hitherto only undoubtedly be demonstrated by detection of FCoV antigens in macrophages in effusions (by means of immunofluorescence) or affected tissue samples (by means of immunohistochemistry). Detection of positive macrophages in effusion is 100% predictive of FIP (Hartmann *et al.*, 2003), but is currently only done in a few laboratories. Tissue samples for the more widely available immunohistochemical detection of antigens can be obtained by laparotomy or percutaneously, but both methods have their advantages and disadvantages. Whereas percutaneous biopsy is less risky and hence preferred to perform on these seriously ill cats, laparotomy allows a better-controlled sample taking of affected sites and hence creates less false negative results (Giordano *et al.*, 2005). In many cases, FIP cannot be confirmed and the diagnosis remains a probability diagnosis, whereby history, clinical signs, and analysis of effusion, blood and in some cases cerebrospinal fluid should be combined to obtain a high FIP probability (Figure 1.7) (Addie *et al.*, 2009; Kipar & Meli, 2014; Pedersen, 2009, 2014). Diagnosing FIP with the highest probability is important, as misdiagnosis will often unnecessarily lead to the cat's death, since the main 'treatment' for FIP remains euthanasia. In addition, as in some cases prednisolone is used to try to prolong the cat's survival, other infectious FIP differential diagnoses (Table 1.4) should be excluded, as for most of them prednisolone is highly contra-indicated and can also result in unnecessary cat's death.

Recently, a new FECV-FIPV discriminatory test (IDEXX FIP Virus RealPCR™ Test) was launched based on 2 mutations (M1058L or S1060A) in the S2 domain of the spike protein, which are found in 96 % of all FIP tissues, but not in faeces of healthy cats (Chang *et al.*, 2012). It has been recommended to use this test on effusions or biopsies taken from FIP suspected cats to make a definite FIP diagnosis. However, another study reports that the mutations can be found in tissues from both non-FIP and FIP cats (Porter *et al.*, 2014), and more details on the sensitivity and notably specificity of the test on biopsies/effusions of both non-FIP and FIP cats should therefore reveal the value of this test. If a specificity of nearly 100% can be shown, this test will add an alternative/additional test method (apart from antigen detection in macrophages) to ascertain that a cat has FIP.

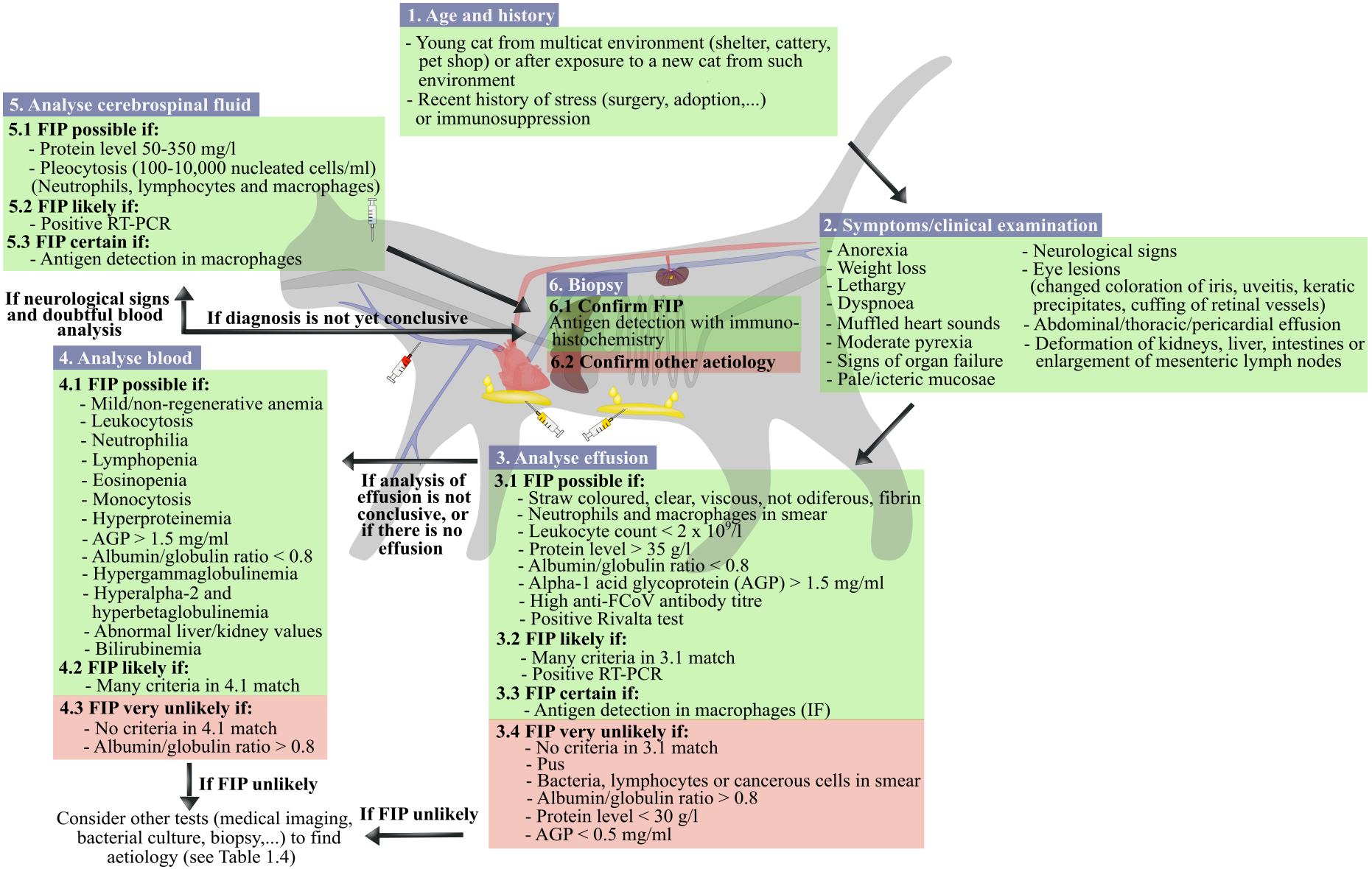


Figure 1.7. Ante-mortem FIP diagnosis.

Table 1.4. Important FIP differential diagnoses to take into consideration if testing is not conclusive or unlikely for FIP (Davies & Forrester, 1996; Jones, 1975; Pedersen, 2009, 2014; Poindessault Santa-Croce, 2006).

Effusive FIP	Non-effusive FIP
1. Abdominal effusion	**1. Systemic infections**
• Hypoproteinemia (liver or kidney disease, protein losing enteropathy)	• Toxoplasmosis
• Congestive heart failure	• Mycosis
• Infections (bacterial, parasitic)	• Feline leukemia virus (FeLV)
• Traumatic hemo- or uro-peritoneum	• Feline immunodeficiency virus (FIV)
• Cholangitis	• Tuberculosis
• Pancreatitis	• Actinomycosis
• Tumours	**2. Tumours**
2. Thoracic effusion/dyspnoea	**3. Other disorders**
• Heart failure	• Degenerative disease of CNS
• Infections (bacterial, parasitic)	• Meningitis
• Chylothorax	• Trauma
• Hemothorax	• Storage diseases
• Tumour	• Idiopathic uveitis
• Intoxication	• Hepatic/renal amyloidosis
• Hernia diaphragmatica	

1.1.7 Prevention

1.1.7.1 Vaccination

Vaccination is a very effective approach for the eradication of viral infections, but despite numerous attempts, development of a safe and effective vaccine against FIPV has been largely unsuccessful so far. Since cell-mediated immune responses are needed for protection against FIP, many studies focused on the use of live or modified live viruses. Vaccination with avirulent FIPV strains (Pedersen & Black, 1983), recombinant viruses carrying the FIPV S, N, or M proteins (Glansbeek *et al.*, 2002; Hebben *et al.*, 2004; Klepfer *et al.*, 1995; Vennema *et al.*, 1990; Wasmoen *et al.*, 1995), and closely related coronaviruses (CCoV, TGEV and human coronavirus (HCoV) 229E) did not protect cats against challenge with FIPV (Barlough *et al.*, 1984; Barlough *et al.*, 1985; Stoddart *et al.*, 1988a; Woods & Pedersen, 1979). Moreover, antibodies induced by vaccination, enhanced development of FIP

after challenge in most of those studies. The use of a sublethal dose of a virulent FIPV showed to be effective, but this virus was not safe enough to be considered as a vaccine candidate (Pedersen & Black, 1983). In the late eighties, a modified-live temperature sensitive FIPV was derived from the virulent DF2 FIPV strain and used as an intranasal vaccine (Christianson *et al.*, 1989; Gerber *et al.*, 1990a; Gerber *et al.*, 1990b). This vaccine was the first and to date only commercially marketed vaccine (Primucell® FIP) and has been found to be safe for vaccination of healthy cats, immunosuppressed cats (dexamethasone treated or FeLV viraemic) and cats with pre-existing coronavirus antibodies. However, Primucell® FIP has not been approved for pregnant queens and kittens before the age of 16 weeks. Moreover, the efficacy of the Primucell® vaccine is highly dependent on the successful prevention of exposure to FCoVs prior to vaccination (Fehr *et al.*, 1997). Since most kittens are infected at 5-10 weeks of age and vaccination is only safe and efficacious in seronegative animals from the age of 16 weeks, vaccination will never be successful if the infection pressure is not correctly managed (see below). In addition, it remains questionable if this serotype II-based vaccine is efficacious against the predominant serotype I strains (Olsen, 1993; Pedersen, 2009). Promising results were obtained using FIPV 79-1146 deletion mutants, truncated in their ORF3 or ORF7 genes, as vaccine candidates. These ORF3 and ORF7 deleted viruses conferred 100% and 80% protection to homologous challenge with the highly virulent FIPV 79-1146, respectively (Haijema *et al.*, 2004). However, reports on follow-up research are still lacking. A similar approach was recently described by Balint *et al.* (2014). Two infectious FIPV DF2 clones were generated, one with a truncated ORF3 and the other with an intact ORF3, displaying a low virulent and completely avirulent phenotype, respectively. Both vaccine candidates conferred 100% protection against homologous challenge with virulent FIPV DF2 in specific pathogen free (SPF) cats. However, application of the same vaccination strategy in purebred British Shorthair cats resulted in 100% deaths after challenge, again highlighting the role of the cat's genetics and immunological responses in the determination of outcome of a FIPV infection (Balint *et al.*, 2014b).

Recently, a new vaccination approach has been considered. As Th1-responses, and consequently IFN-γ production, can confer protection against FIP, this strategy is based on the induction of Th1-responses by using Th1-stimulating peptides derived from viral structural proteins. These peptide-based vaccines were administered with feline CpG-oligodeoxynucleotides as vaccine adjuvant, and peptide-vaccines with Th1-epitopes from the N protein of serotype I FIPV KU-2 conferred slightly better protection against FIPV 79-1146.

However, immune tolerance was also reported and hence more studies are required to optimize the concentration of peptides and fCpG-ODNs, along with dose, frequency and route of administration (Takano *et al.*, 2014a). In another report, these researchers showed that peripheral blood mononuclear cells (PBMCs) of cats vaccinated with an M- or S-derived peptide had significantly higher IFN-γ production compared to controls (Takano *et al.*, 2014b). Whether these peptide-vaccines will also be able to protect cats from FIP development should be further investigated by challenge studies.

1.1.7.2 Management

As FIP is the consequence of a common FECV infection, prevention of FIP can be directed at controlling transmission of this parent virus (Addie *et al.*, 2004). FECV is shed via the faeces and can survive for 7 weeks in a dry environment. Consequently, measures should be taken to prevent faecal contamination of the environment (floors, water, food) by regular (and preferably daily) cleaning en disinfection of floors, litter trays, water- and food bowls (Addie *et al.*, 2009). Infected cats shed the virus for many weeks, months, or in case of persistent shedders, years and these cats are a continuous source of (re)infection for negative cats (Addie & Jarrett, 2001; Addie *et al.*, 2003; Pedersen, 2009; Pedersen *et al.*, 2008). Therefore, if one wants to prevent FECV infection, the most important measure to be taken is to prevent any contact between shedders and naive animals. In the past, this separation policy has been successfully applied to either completely eradicate FCoVs (Hickman *et al.*, 1995), or to control transmission to the most susceptible population in breeding catteries, namely the several weeks old kittens (Addie & Jarrett, 1992). Hickman *et al.* reported the introduction of FECV in a closed SPF facility of the University of California, Davis, which had been unnoticed until several cats started to die from FIP. As these cats were of high value, the researchers decided to completely eradicate FCoVs from the colony by serological testing and grouping based on antibody titres, since no diagnostic PCR tests were available at that time. Only seronegative animals were kept to create offspring, and cats that remained seropositive were removed from the colony. By regular testing and strict quarantine measurements, these researchers were able to recreate a FCoV-negative SPF population (Hickman *et al.*, 1995). In practice, however, this method has many drawbacks and is hardly feasible. Indeed, by using PCR to measure faecal shedding, many researchers reported inconsistent conclusions on the correlation between antibody titre and shedding, indicating that it is very difficult to reliably isolate shedders from non-shedders based on their serum antibody titre (Addie *et al.*, 2003; Foley *et al.*, 1997; Harpold *et al.*, 1999; Pedersen *et al.*, 2008). In addition, even if this would

be successful, it is quite challenging in practice to remain FCoV-negative, as FCoV infections are present in virtually all multi-cat environments from where new cats are frequently imported. FIP is typically seen in kittens in the post-weaning period and most kittens are protected by maternally derived antibodies until 5-10 weeks of age. Therefore, it has been recommended to direct the control of FIP towards the control of FECV transmission from shedders to the kittens, more specifically by isolating the queens 2-3 weeks prior to partus, taking the kittens away from their mother at 4-6 weeks of age (= early weaning) and raise them in complete isolation (Addie *et al.*, 2004). Although clearly demonstrated that early weaning can strongly decrease the incidence of FIP (Addie & Jarrett, 1992), the success rate is variable and depends on the isolation procedure and the shedding state of the queen (Addie *et al.*, 2004). When kittens are faced with a high infection pressure, viruses break through the maternal immunity and kittens can become infected as soon as 2 weeks of age (Lutz *et al.*, 2002). In addition, early weaning has been questioned by its negative impact on the socialisation of kittens, and is therefore not regularly applied in practice.

FECV remains enzootic by continuous faecal-oral transmission of the virus from shedders to non-shedders (Addie *et al.*, 2003; Foley *et al.*, 1997). Consequently, grouping of cats based on their shedding state has been opposed to avoid (re)infections, but no reports on successful isolation of shedders from non-shedders have yet been published. However, this strategy would allow protection of FECV exposure to kittens without the need for early weaning and complete isolation, as this would allow selection of negative animals for breeding and socialisation of kittens. Although cattery owners are aware of the fact that adapting management is currently the only way to deal with FIP, the time- and money-consuming measures to be taken (due to the long-lasting and highly contagious character of FECV) remain a drawback for many of them.

1.1.8 Treatment

To date, there is no treatment with proven efficacy available to cure cats from FIP. Some FIP-affected cats will undergo a spontaneous remission without ever showing clear symptoms, but once the clinical signs become apparent, mortality is nearly 100%. Several attempts have been made to treat FIP, including the use of immune-suppressive drugs, viral replication inhibitors, and non-specific immunostimulant drugs (reviewed by (Pedersen, 2014)), but, despite some claims, properly controlled clinical trials to evaluate the real efficacy is still lacking for many of those products. Although the lack of evidence on efficacy for any of those products, some of them including prednisolone, interferon omega, polyprenyl immunostimulant, and

pentoxyfillin, have been frequently used in practice. Although it will not cure the cat, prednisolone is probably the most rational one to use, as it makes the cat feel better and stimulates appetite, which will certainly enhance the quality of the cat's life. In addition, prednisolone is the treatment of choice to cure lymphocytic cholangitis, one of the most difficult differential diagnoses to make with FIP. Hence, if one would have misdiagnosed a cat with FIP, prednisolone can make this cat survive. Recently, promising results were obtained *in vitro* with synthetic peptides targeting either the viral spike (Liu *et al.*, 2013) or the viral 3C-like protease (Kim *et al.*, 2013), but results of *in vivo* safety and efficacy testing are still to be reported.

1.2 ENTRY OF CORONAVIRUSES

Coronaviruses infect a wide range of mammalian species and birds, causing medically and economically important diseases in humans, birds, lifestock and pets. The host and the tissue(s) infected by a certain strain are largely determined by the expression of the viral receptor(s) and fusion triggers, which vary greatly amongst the different members of the *Coronaviridae* family. Both coronavirus' receptors and fusion processes are reviewed below.

1.2.1 Receptors and attachment factors involved in coronavirus infections

Every virus infection is initiated by attachment of the virus particle to one or more cell surface molecules. Whereas some of these 'receptors' only mediate absorption of the virus to the host cells and hence should merely be considered as 'attachment factors', others are absolutely necessary to guide the infectious entry of the virus in its target cell by generating fusion-competent spikes or allowing the endocytosis of the virus. A wide variety of cell surface receptors/attachment factors have been described for coronaviruses, including both proteins and sugars, but for some coronaviruses, including serotype I FCoVs, receptors remain elusive to date (Table 1.5). In general, coronavirus attachment occurs by spike-carbohydrate, spike-protein, and/or viral mannose carbohydrate-host lectin interactions. All three classes of receptors are discussed below with respect to their role in coronavirus infections.

1.2.1.1 Carbohydrates: sialic acids and heparan sulfate

Two types of carbohydrate receptors have been described for coronaviruses: sialic acids and heparan sulfate. However, whereas sialic acids are clearly involved during *in vivo* infections, heparan sulphate is thought to be less, if not at all, relevant, as it is only reported as receptor for some cell-culture propagated strains (de Haan *et al.*, 2005; de Haan *et al.*, 2008; Madu *et al.*, 2007).

Sialic acids are acidic monosaccharides, typically found at the outermost end of N-glycans, O-glycans, and glycosphingolipids. They occur in many diverse forms (all of them derivatives of neuraminic acid), depending on the substitutions at the 4-, 5-, 7-, 8-, and 9-carbon group (Figure 1.8) (Schauer, 2004; Varki & Schauer, 2009). N-acetylneuraminic acid (Neu5Ac), N-glycolylneuraminic acid (Neu5Gc) and N-acetyl-9-O-acetylneuraminic acid (Neu5,9Ac_2) are the most predominant forms in mammalian cells (Schauer, 2004). From the 2-carbon, sialic acids are α-glycosidically linked to the underlying sugar chain. This can occur to the 3- or 6-carbon position of galactose, N-acetyl-D-glucosamine, or N-acetyl-D-galactosamine residues. Some sialic acids can even occupy internal positions, most commonly attached to another

sialic acid at the 8-carbon position. These 2,3-, 2,6-, and 2,8-α-linkages highly impact the glycan structure and further contribute the diversity within the sialic acids family (Varki & Schauer, 2009).

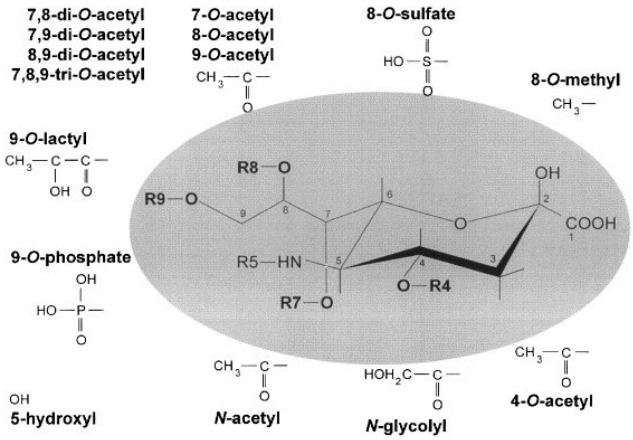


Figure 1.8. Overview of naturally occurring sialic acids. Adapted from (Schauer, 2004)

Many viruses, including influenzavirus, feline calicivirus, some rotaviruses, adenoviruses, and coronaviruses, have evolved to use a specific type of sialic acids for their entry (Haselhorst *et al.*, 2009; Kaludov *et al.*, 2001; Schwegmann-Wessels & Herrler, 2006; Skehel & Wiley, 2000; Stuart & Brown, 2007). Given the species-, tissue-, or even molecule-specific expression of sialic acid linkages and modifications (Varki & Schauer, 2009), sialic acids are often major determinants of virus tropism.

Within the coronaviruses, TGEV and the related PRCV are probably the most well known examples of how sialic acid binding activity can determine virus tropism. TGEV is an enteric pathogen, causing fatal diarrhoea in newborn piglets. PRCV emerged in 1984 from TGEV by mutations and shares an overall homology of 96% (Pensaert *et al.*, 1986; Rasschaert *et al.*, 1990). Although both viruses use the same receptor, porcine aminopeptidase N, PRCV has lost its enterotropism and replicates very efficiently in the respiratory tract (Cox *et al.*, 1990; Delmas *et al.*, 1993; Delmas *et al.*, 1992). A major difference between both viruses lies in the spike gene, as PRCV spike lacks 224 to 227 amino acids in its S1 subunit (Rasschaert *et al.*, 1990; Wesley *et al.*, 1991), thereby missing 2 antigenic sites (Sanchez *et al.*, 1990) and the sialic acid (preferentially N-glycolylneuraminic acid) binding capacity found in TGEV (Schultze *et al.*, 1996). Although dispensable for *in vitro* infections, the TGEV sialic acid binding activity is undoubtedly involved in the virus' enterotropism *in vivo*, as not only

PRCV, but also mutants lacking the sialic acid binding site were no longer capable of inducing enteropathy (Bernard & Laude, 1995; Krempl *et al.*, 1997). Indeed, TGEV binds to a second receptor in the intestinal brush border, a 200 kDa mucin-type glycoprotein, which seems indispensable to allow intestinal infections, most probably by attaching to and passing through the mucus layer covering the intestinal epithelial cells (Schwegmann-Wessels *et al.*, 2003).

In addition to TGEV, many other coronaviruses possess sialic acid binding potential, including porcine epidemic diarrhoea virus (PEDV), bovine coronavirus (BCoV), human coronavirus OC43, porcine haemagglutinating encephalomyelitis virus (HEV), and avian infectious bronchitis virus (IBV) (Table 1.5) (Kunkel & Herrler, 1993; Schultze & Herrler, 1992; Schultze *et al.*, 1990; Vlasak *et al.*, 1988; Winter *et al.*, 2006). The betacoronaviruses BCoV, HCoV-OC43, and HEV recognize 9-O-acetyl-5-N-acetylneuramininc acid (Neu 5,9 Ac2), which at least for BCoV is a receptor determinant, as treatment of cells with neuraminidase or acetylesterase renders cells resistant to infection (Schultze & Herrler, 1992). Those viruses share the characteristic feature to express an additional structural protein in their envelope, the haemagglutinin esterase (HE) glycoprotein. This HE protein serves as a receptor destroying enzyme and potentially has similar functions to the receptor-destroying enzyme of orthomyxoviruses, such as facilitating viral spread by enhancing virus release from infected cells and by preventing the formation of virus aggregates (Schwegmann-Wessels & Herrler, 2006). Some strains of murine hepatitis virus (MHV) also contain such HE protein, but they rather hydrolyse 4-O-acetyl-5-N-acetylneuraminic acid (Smits *et al.*, 2005). For MHV, the expression of HE and the ability to attach to O-acetylated sialic acids have been linked to neurovirulence (Kazi *et al.*, 2005; Yokomori *et al.*, 1995).

For FCoVs, no information is available on the role of sialic acids in virus infection. However, it has been shown that healthy FCoV-positive cats typically mounted an acute phase reaction consisting of hypersialylated serum alpha1-acid glycoprotein (AGP), whereas cats that went on to develop FIP had hyposialylated serum AGP (Ceciliani *et al.*, 2004). If and how this feature contributes to FIP pathogenesis remains elusive.

Table 1.5. Overview of coronavirus receptors.

Genus	Species	Subspecies	Receptor	Type of molecule	Cell type used to study receptor
Alpha-CoV	*Alphacoronavirus 1*	CCoV type I	Unknown	Unknown	
		CCoV type II	APN	N-terminal exopeptidase	BHK cells expressing chimeric human/canine APN (Benbacer *et al.*, 1997)
		FCoV type I			
		FIPV	DC-SIGN	C-type lectin	CRFK expressing DC-SIGN and monocyte-derived DCs (Regan *et al.*, 2010), monocytes (Van Hamme *et al.*, 2011)
			Unknown	Unknown	Monocytes (Van Hamme *et al.*, 2011)
		FECV	Unknown	Unknown	
		FCoV type II			
		FIPV	APN	N-terminal exopeptidase	BHK and 3T3 cells expressing fAPN (Tresnan *et al.*, 1996); fcwf cells (Hohdatsu *et al.*, 1998); monocytes (Van Hamme *et al.*, 2011)
			DC-SIGN	C-type lectin	3T3 and CrFK cells expressing hDC-SIGN; monocytes (Regan & Whittaker, 2008); CRFK expressing fDC-SIGN and monocyte-derived DCs (Regan *et al.*, 2010); monocytes (Van Hamme *et al.*, 2011)
			Unknown	Unknown	Monocytes (Van Hamme *et al.*, 2011)
		FECV	APN	N-terminal exopeptidase	Fcwf cells (Hohdatsu *et al.*, 1998)[a]
		PRCV	APN	N-terminal exopeptidase	BHK cells expressing pAPN (Delmas *et al.*, 1993)
		TGEV	APN	N-terminal exopeptidase	MDCK cells expressing pAPN; ST-cells (Delmas *et al.*, 1992)
			Sialo-glycoprotein	Carbohydrate	Brush border membranes (Schwegmann-Wessels *et al.*, 2003)
	Alphacoronavirus 2	Ferret enteric CoV	Unknown	Unknown	
		Ferret systemic CoV	Unknown	Unknown	
		Mink CoV	Unknown	Unknown	
	HCoV 229E		APN	N-terminal exopeptidase	Murine 3T3 cells expressing hAPN; WI38 cells (Yeager *et al.*, 1992)
			L-SIGN	C-type lectin	CHO cells expressing L-SIGN (Jeffers *et al.*, 2006)
	HCoV NL63		ACE2	C-terminal exopeptidase	Huh-7, LLC-MK2, and 293-T cells; 293-T cells overexpressing ACE2 (Hofmann *et al.*, 2005)
			DC-SIGN/ L-SIGN	C-type lectin	BHK cells co-expressing ACE2 and DC-SIGN/R (Hofmann *et al.*, 2006)
	PEDV		APN	N-terminal exopeptidase	Vero cells (Oh *et al.*, 2003)

Beta-CoV	*Betacoronavirus 1*	BCoV	Sialic acids (Neu 5,9 Ac2)	Carbohydrate	MDCK, LLC-PK1, and Caco-2 cells; red blood cells (haemagglutination) (Schultze & Herrler, 1992; Vlasak *et al.*, 1988)
		HCoV OC43	Sialic acids (Neu 5,9 Ac2)	Carbohydrate	Red blood cells (haemagglutination) (Kunkel & Herrler, 1993; Vlasak *et al.*, 1988)
		Equine CoV	Unknown	Unknown	
		Human enteric CoV	Unknown	Unknown	
		PHEV	Sialic acids (Neu 5,9 Ac2)	Carbohydrate	Red blood cells (haemagglutination) (Schultze *et al.*, 1990)
		Canine respiratory CoV	Unknown	Unknown	
	HCoV HKU1		Unknown	Unknown	
	Murine CoV	MHV	CEACAM1	Ig-like superfamily	BHK and RD cells expressing CEACAM1; L2 cells (Dveksler *et al.*, 1991)
			Heparan sulphate (MHV/BHK)	Carbohydrate	LR7 and HeLa cells (de Haan *et al.*, 2005)
		Puffinosis CoV	Unknown	Unknown	
		Rat CoV	Unknown	Unknown	
	Pipistrellus bat CoV HKU5		Unknown	Unknown	
	Rousettus bat CoV HKU9		Unknown	Unknown	
	SARS-related CoV	Human SARS-CoV	ACE2	C-terminal exopeptidase	293T cells expressing ACE2; Vero E6 cells (Li *et al.*, 2003)
			DC-SIGN/ L-SIGN	C-type lectin	HeLa-, CHO-, and 293T cells expressing DC-/L-SIGN (Han *et al.*, 2007; Jeffers *et al.*, 2004; Marzi *et al.*, 2004)
		Bat SL-CoV WIV1	ACE2	C-terminal exopeptidase	HeLa cells expressing ACE2; Vero cells (Ge *et al.*, 2013)
	Tylonycteris bat CoV HKU4		DPP4	N-terminal exopeptidase	HEK293T and Tb1-Lu cells expressing DPP4; Huh-7, Calu-3 and MRC-5 cells (Yang *et al.*, 2014)
	MERS-CoV		DPP4	N-terminal exopeptidase	Huh-7 and primary bronchiolar epithelial cells; COS-7 cells expressing DPP4 (Raj *et al.*, 2013)
Gamma-CoV	*Avian CoV*	IBV	Sialic acids (Neu 5,9 Ac2)	Carbohydrate	Vero cells (Winter *et al.*, 2006)

[a]Serotype II FCoV WSU 79-1683 is the only studied enteritis-inducing strain so far, but this strain is believed to rather be an avirulent FIPV than a real FECV (Pedersen, 2009).

1.2.1.2 Protein receptors

For most coronaviruses, attachment to specific transmembrane proteins, notably peptidases, is the key determinant for the initiation of infection. Although coronaviruses need enzymatic cleavage of their spikes to allow efficient fusion processes, the enzymatic activity of these receptor peptidase is not involved in this process for most, if not all, of them (Delmas *et al.*, 1994; Li *et al.*, 2003; Raj *et al.*, 2013).

Aminopeptidase N (APN) or **CD13** is a type II transmembrane Zn^{2+}-dependent protease (metalloprotease) with a wide tissue/cell type distribution (epithelial cells, endothelial cells, fibroblasts, and leukocytes). It is an ectoenzyme cleaving N-terminal neutral amino acids from peptides and proteins, thereby fulfilling many different functions such as regulation of peptides, tumour-cell invasion, differentiation, migration, proliferation, apoptosis, chemotaxis, and antigen presentation. In addition, APN is known to execute many other enzymatic-activity-independent processes by mediating endocytosis or initiating cell-signalling cascades upon ligand binding (for a comprehensive review see (Mina-Osorio, 2008)). One of the protease-independent processes includes its receptor function for many alphacoronaviruses, including serotype II FCoVs, PRCV, TGEV, CCoV, PEDV, and HCoV 229E (Benbacer *et al.*, 1997; Delmas *et al.*, 1993; Delmas *et al.*, 1992; Hohdatsu *et al.*, 1998; Oh *et al.*, 2003; Van Hamme *et al.*, 2011; Yeager *et al.*, 1992). Apart from their species specific APN, TGEV, CCoV, and HCoV 229E also bind to feline (f)APN, and it has consequently been proposed that cats can be potential mixing vessels for new emerging viruses (Tresnan *et al.*, 1996). fAPN is the sole receptor for serotype II FCoV in continuous cell cultures (Hohdatsu *et al.*, 1998; Tresnan *et al.*, 1996; Van Hamme *et al.*, 2011). In monocytes, however, at least serotype II FIPV is able to use an alternative yet unidentified receptor to induce infection (Van Hamme *et al.*, 2011). In contrast to the serotype II viruses, the entry factors involved in serotype I infections are still mainly unknown. There have been some conflicting evidences regarding the use of fAPN for the functional entry of serotype I FCoVs. Tresnan *et al.* reported replication of the serotype I FIPV UCD-1 after transfection of fAPN cDNA in otherwise unsusceptible hamster and mouse cells (Tresnan *et al.*, 1996). However, further reports evidenced against the role of fAPN in the serotype I infection (Dye *et al.*, 2007; Hohdatsu *et al.*, 1998; Van Hamme *et al.*, 2011), which can explain the difficulties to propagate serotype I FCoVs on all (fAPN-expressing) feline cell lines.

Angiotensin-converting enzyme (ACE2) is a type I integral membrane carboxy-metallopeptidase expressed by alveolar and intestinal epithelial cells, and arterial and venous

endothelial cells (Hamming *et al.*, 2004). ACE2 functions as a receptor for SARS-CoV, HCoV NL63, and bat SARS-like CoV WIV1 (Ge *et al.*, 2013; Hofmann *et al.*, 2005; Li *et al.*, 2003).

Dipeptidyl peptidase 4 (DPP4) was recognized in 2013 as an additional coronavirus receptor used by the recently emerged Middle East respiratory syndrome coronavirus (MERS-CoV) (Raj *et al.*, 2013) and the related bat CoV HKU4 (Yang *et al.*, 2014). It is a cell surface serine protease expressed on epithelial cells, endothelial cells, and leukocytes in various tissues, but it also occurs in a soluble form in plasma or other body fluids (Boonacker & Van Noorden, 2003; Lambeir *et al.*, 2003). DPP4 typically releases proline-containing dipeptides from polypeptide chains, thereby regulating bioactivity of many molecules, but this enzymatic activity is not involved in MERS-CoV entry (Raj *et al.*, 2013).

Carcinoembryonic antigen-cell adhesion molecule 1 (CEACAM1), a type I transmembrane protein belonging to the immunoglobulin superfamily, has been identified as the MHV receptor (Dveksler *et al.*, 1991). Both isoforms of CEACAM1, CEACAM1a and 1b, can function as MHV receptor, but CEACAM1a has much higher affinity (Ohtsuka *et al.*, 1996). In contrast to all other coronaviruses, the amino acids responsible for the spike-CEACAM1 receptor interaction have not been mapped to the C-RBD, but to the N-RBD of the spike (Figure 1.3), a region that is typically involved in sialic acid binding in other coronaviruses. Interestingly, the crystal structure of the MHV receptor-binding domain revealed a similar galectin-like structure of the N-RBD as found in sialic acid binding viruses as BCoV and HCoV OC43, but the lack of a peptide loop makes the MHV spike not operative as a lectin, but as a ligand for CEACAM1 receptor engagement (Peng *et al.*, 2011). One MHV strain, MHV/BHK, has lost its CEACAM1 tropism by acquiring 2 heparan sulfate binding sites (de Haan *et al.*, 2005).

1.2.1.3 C-type lectins

Many coronaviruses use dendritic cell-specific ICAM-3 grabbing nonintegrin (**DC-SIGN** or CD209), or DC-SIGN related (DC-SIGNR), also called **L-SIGN** (liver/lymph node specific) or CD209L as entry factor. DC-SIGN and L-SIGN are transmembrane Ca^{2+}-dependent carbohydrate binding proteins (C-type lectins). DC-SIGN is expressed on subsets of dendritic cells/macrophages, whereas L-SIGN is found in liver, lung, lymph node, and intestine, expressed on endothelial cells or alveolar cells (Khoo *et al.*, 2008). They specifically recognize high-mannose carbohydrates and serve as adhesion molecules and pattern recognition receptors, as these mannoses are expressed by many microbial proteins, including

the highly glycosylated spike protein of coronaviruses (Siddell *et al.*, 1983). The (mis)use of C-type lectins as receptor determinants for coronaviruses was first noticed for human coronaviruses. For SARS-CoV, DC-SIGN and L-SIGN can function either as alternative receptors (Han *et al.*, 2007; Jeffers *et al.*, 2004) or as enhancing factors during ACE2-mediated entry (Marzi *et al.*, 2004). L-SIGN can also act as an additional receptor for HCoV 229E (Jeffers *et al.*, 2006), whereas DC-SIGN is an enhancing factor in the ACE2-medated entry for HCoV NL63 (Hofmann *et al.*, 2006). For animal coronaviruses, the role of C-type lectins as entry factors has only been reported for FCoVs. Serotype II FCoV, including strains 79-1146, DF2 and 79-1683, use DC-SIGN as a co-entry factor in APN-expressing susceptible cells (Regan & Whittaker, 2008; Van Hamme *et al.*, 2011). In addition, DC-SIGN also acts as a co-receptor in serotype I FIPV infections of feline monocytes and DCs (Regan *et al.*, 2010; Van Hamme *et al.*, 2011), and both serotype I and serotype II FIPV infections can be reduced in cell cultures by addition of mannose-binding lectins (Keyaerts *et al.*, 2007; van der Meer *et al.*, 2007). However, the primary receptor for serotype I FCoV infections remains unidentified so far.

1.2.2 Fusion processes in coronavirus biology

Coronavirus spike proteins are class I fusion proteins, mediating fusion processes during two events in the replication cycle, namely very early to deliver the nucleocapsid from virions into the host cell (virus-cell fusion), and late in the infection cycle to spread the infection from the infected cell to the neighbouring uninfected cell without the need for cell-free viruses to be formed (cell-cell fusion). Viral class I fusion proteins are typically synthesized as inactive precursor proteins and require proteolytical cleavage to acquire their fusion competent state. This fusion competent protein, comprised of a metastable complex of receptor binding subunit and fusion subunit, undergoes subsequent conformational changes upon receptor binding, acidification, and/or additional proteolysis (= fusion triggers), resulting in the dissociation of both subunits, which finally allows the insertion of the hydrophobic fusion peptide into the host membrane (Figure 1.9, I-II). By refolding to its most stable conformation, i.e. the formation of a 6 helical bundle (6HB) by the association of the trihelical HR1 and HR2 domains, the fusion protein mediates close apposition and subsequent fusion of the viral envelope with the host membrane (Figure 1.9, III-V) (Bosch *et al.*, 2003; Eckert & Kim, 2001; White *et al.*, 2008).

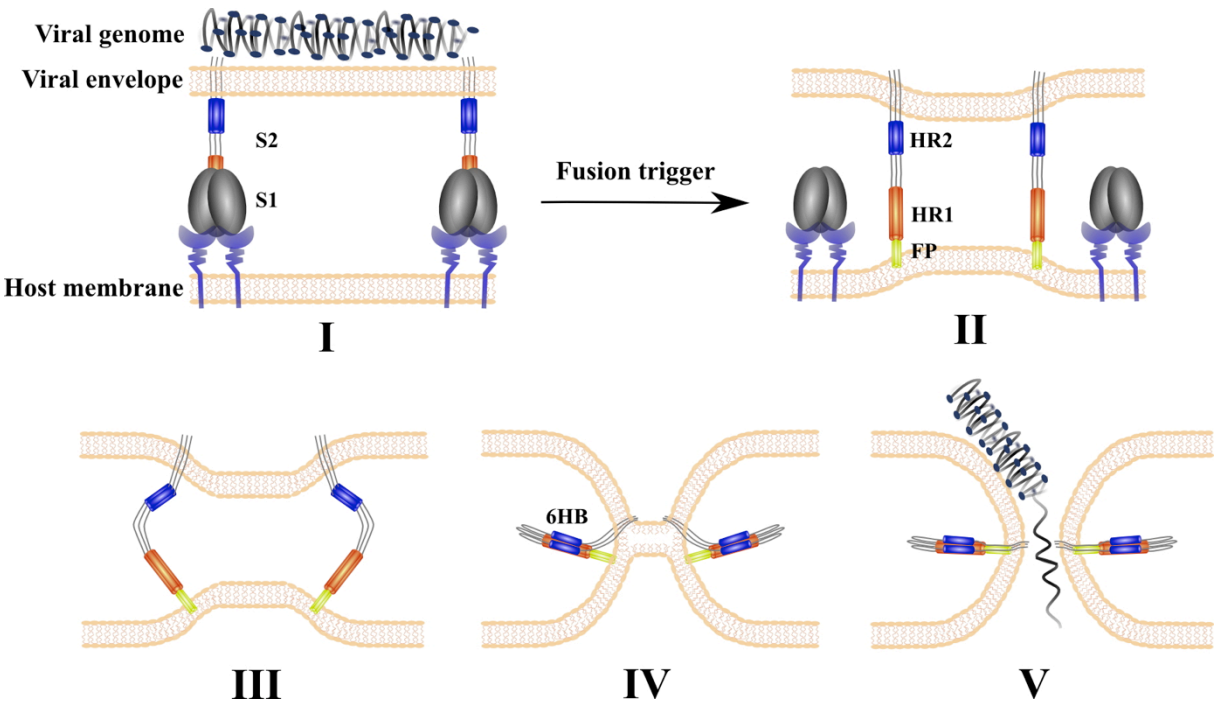
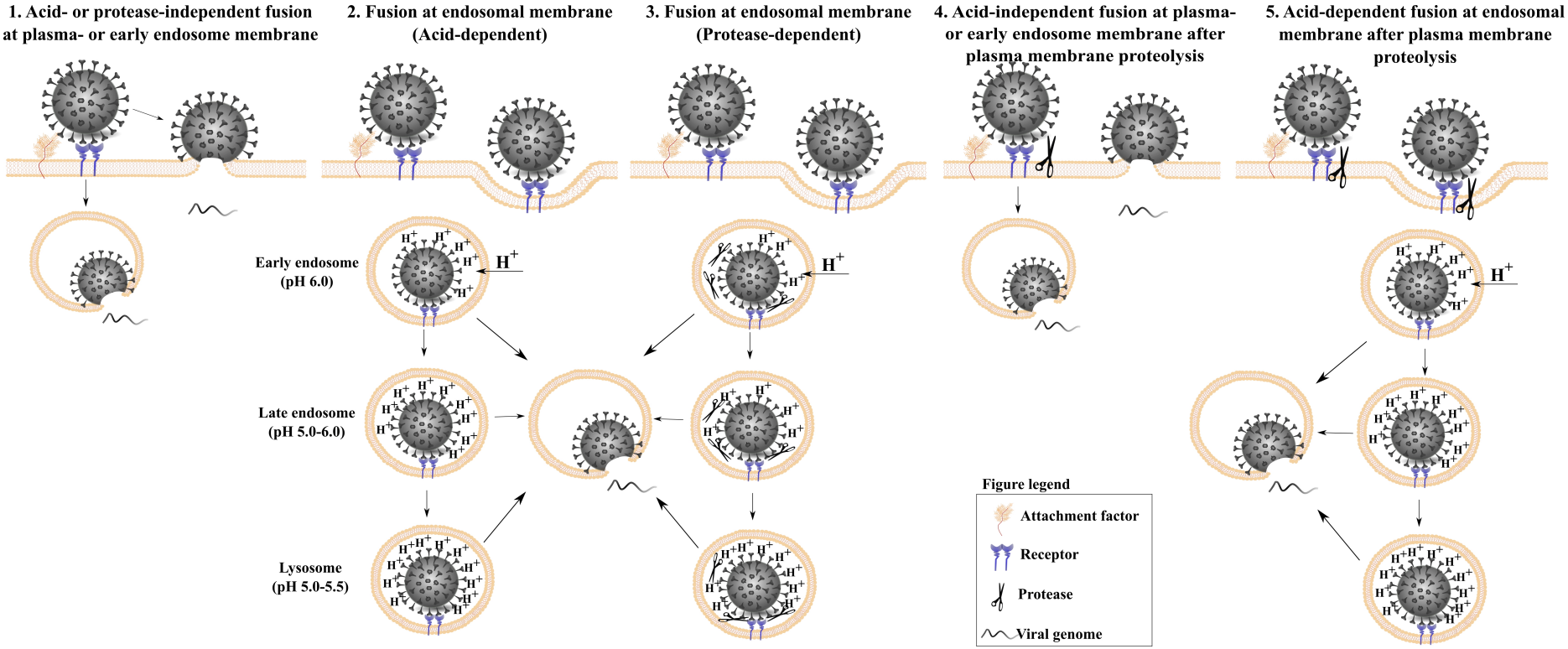


Figure 1.9. Schematic representation of membrane fusion processes mediated by coronavirus S proteins. Dissociation of the S1 receptor binding subunit and the S2 fusion subunit upon proteolysis, receptor binding, and/or pH reduction liberates the viral fusion peptide, which becomes inserted into the host membrane (I-II). Subsequent refolding of HR1 and HR2 into a 6 helix bundle (6HB) finally results in fusion of viral and host membrane and the release of the viral genome in the cytoplasm (III-V).

As for the receptor usage, coronaviruses also show great distinctions in entry pathways and fusion activating triggers. These differences largely influence virus tropism and pathogenicity, and can explain why 2 viruses or even strains using the same receptor can show such great distinction in cell tropism. In addition, it seems that some coronaviruses have evolved to use multiple cell entry routes, depending on the cell type the virus is faced with. Figure 1.10 reviews entry pathways and fusion triggers of different coronaviruses.



Pathway 1 examples
MHV-4 (JHM)
(Gallagher *et al.*, 1991)
MHV-2 mutants with S1-S2 furin cleavage site
(Qiu *et al.*, 2006)
SARS-CoV mutants with furin cleavage site
(Watanabe *et al.*, 2008)

Pathway 2 examples
MHV-4 OBLV60
(Gallagher *et al.*, 1991)
MHV-A59
(Eifart *et al.*, 2007)
IBV Beaudette, M41
(Chu *et al.*, 2006)

Pathway 3 examples
MHV-2: cathepsin B & L
(Qiu *et al.*, 2006)
SARS-CoV: cathepsin L
(Huang *et al.*, 2006; Simmons *et al.*, 2005)
FCoV 79-1683: cathepsin B & L
(Regan *et al.*, 2008)
FCoV 79-1146: cathepsin B
(Regan *et al.*, 2008)
HCoV 229E: cathepsin L
(Kawase *et al.*, 2009)
MERS-CoV: cathepsin B and/or L
(Gierer *et al.*, 2013)
IBV Beaudette: furin
(Yamada & Liu, 2009)

Pathway 4 examples
SARS-CoV: Trypsin, thermolysin, elastase, TMPRSS2
(Matsuyama *et al.*, 2005)

Pathway 5 examples
MERS-CoV
(Barlan *et al.*, 2014)

Figure 1.10. Entry pathways and fusion triggers used by coronaviruses.

Many viruses carrying class I fusion proteins require an early proteolytical activation of their fusion proteins in the virus-producing cells, usually mediated by furin-like cellular proteases encountered during exocytosis processes. For most of them, this proteolytical cleavage occurs directly N-proximal of the hydrophobic fusion peptide, allowing its immediate insertion into the target cell membrane after introducing conformational changes upon receptor binding and/or low pH exposure (Dimitrov, 2004). The exact location of the coronavirus fusion peptide is not known, but based on the sequence, the structure, the position within the S trimer, and the conservation among coronaviruses, the sequence SFIEDLLFNKVTLADAGF of SARS-CoV, and the related sequence in other coronaviruses, has been suggested as the putative coronavirus fusion peptide (Belouzard *et al.*, 2012; Madu *et al.*, 2009). Remarkably, furin cleavage of the coronavirus spike into the receptor binding S1 subunit and the S2 fusion subunit occurs not directly adjacent to this fusion peptide (Figure 1.11, CS1) (Bosch *et al.*, 2008; Bosch *et al.*, 2004). In addition, many coronaviruses do not posses the multibasic furin cleavage motif (R-X-R(K)-R) at the S1/S2 boundary, and hence carry uncleaved spikes (Belouzard *et al.*, 2012; de Haan *et al.*, 2008; Wesseling *et al.*, 1994; Yao *et al.*, 2004). For viruses that carry pre-cleaved spikes, such as IBV, MHV-A59, and MHV-4, receptor engagement and/or exposure to acid pH have generally been believed to be sufficient to allow genome release in the cell (Figure 1.10 pathway 1 and 2), although it has recently been questioned if these are the only triggers. Indeed, furin cleavage occurs not directly adjacent to the putative fusion peptide, and both MHV-A59 and IBV spikes seem to become additionally cleaved within their S2 subunit during entry (Wicht *et al.*, 2014a; Yamada & Liu, 2009). In contrast to IBV, MHV-4 and MHV-A59, most coronaviruses carry uncleaved spikes and indisputably rely on proteolytical activation with proteases encountered during virus entry to allow infections (Figure 1.10 pathway 3, 4, and 5). It has become clear that the presence or absence of furin cleavage has no impact on coronavirus infectivity (Hingley *et al.*, 2002), although it can impact the fusion trigger/entry pathway required to initiate infections. Indeed, SARS-CoV and MHV-2, which lacks the furin recognition site, normally depend on endosomal cathepsins, but this dependence can be counteracted by introducing a consensus furin cleavage site (Qiu *et al.*, 2006; Watanabe *et al.*, 2008). In addition, rendering cleaved spikes uncleavable by mutations in the multibasic motif or by furin inhibition did not affect MHV-A59 infectivity (de Haan *et al.*, 2004; Gombold *et al.*, 1993), but it made the virus more susceptible to inhibitors of endocytosis (de Haan *et al.*, 2004). In contrast to the virus-cell fusion, S1-S2 cleavage during virus infections increases the cell-cell fusion (de Haan *et al.*, 2004; Yamada *et al.*, 1997), explaining the advantage for those viruses to produce pre-

cleaved spikes, and highlighting the fact that virus-cell and cell-cell fusion processes are differently regulated.

Different, alternative entry pathways have been demonstrated for many coronaviruses, and the pathway that is used seems to be determined by the target cell/tissue. It is well established that infectivity of many coronaviruses, such as MHV, SARS-CoV, and PEDV, can be enhanced by exogenous treatment of trypsin, and that this treatment can bypass the endocytosis pathway (Matsuyama *et al.*, 2005; Qiu *et al.*, 2006; Sturman *et al.*, 1985; Wicht *et al.*, 2014b). In addition to trypsin, SARS-CoV fusion at the plasmamembrane can also be induced by treatment with elastase or thermolysin, or by expression of transmembrane serine proteases such as TMPRSS2 (Belouzard *et al.*, 2010; Bertram *et al.*, 2011; Glowacka *et al.*, 2011; Matsuyama *et al.*, 2005; Shulla *et al.*, 2011). At least for SARS-CoV, this mode of entry is much more efficient than the cathepsin-dependent entry (Matsuyama *et al.*, 2005). The monobasic cleavage sites recognized by these proteases are not only found at the S1/S2 boundary, but also directly upstream of the putative fusion peptide (Figure 1.11, CS2) (Belouzard *et al.*, 2009; Belouzard *et al.*, 2012; Bosch *et al.*, 2008; Matsuyama *et al.*, 2005; Simmons *et al.*, 2004). As cleavage at the S1/S2 boundary seems not to be a determinant of infectivity and cannot liberate the internal fusion peptide, cleavage at CS2 is considered as the key fusion-determining factor for many coronaviruses (Belouzard *et al.*, 2009). Indeed, for IBV Beaudette (and related strains), 2 furin cleavage sites are found, one at amino acid position 531-538 (CS1) and the other at amino acid position 684-692 (CS2) (Figure 1.11). Whereas the first cleavage site can promote cell-cell fusion, only the latter is the key determinant for induction of both virus-cell and cell-cell fusion. At that position, FCoV 79-1683, TGEV, CCoV, and bat HKU5-1 also have a furin cleavable motif (RKYR), whereas other coronaviruses have a highly conserved trypsin cleavable site (Yamada & Liu, 2009).

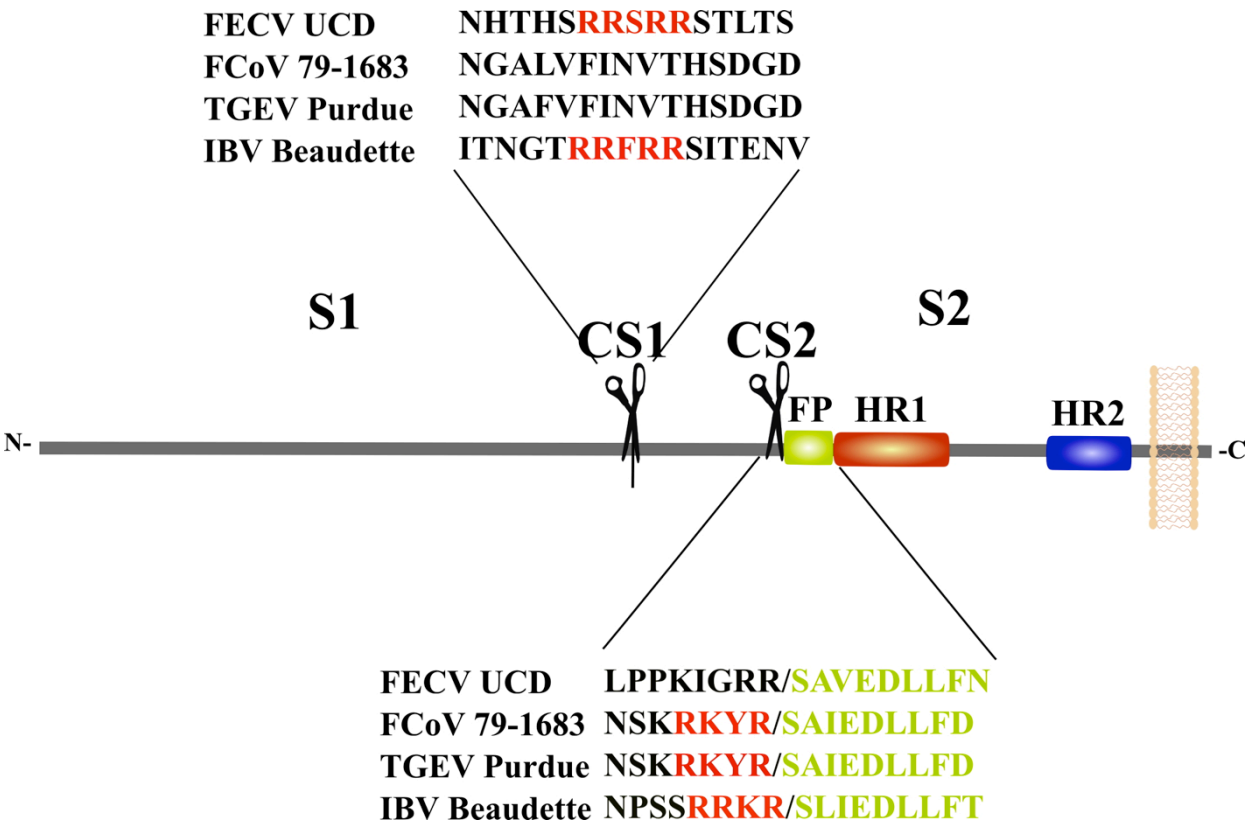


Figure 1.11. Coronavirus spike cleavage sites and sequence of different coronaviruses at these sites. Coronavirus S proteins are composed of the S1 receptor binding (S1) and the transmembrane fusion (S2) subunit. The S2 subunit contains a fusion peptide (FP) and 2 heptad repeat regions (HR 1 and 2) and mediates fusion of the viral envelope with the host membrane after proteolytical activation. Two proteolytic cleavage sites (CS) have so far been identified in coronaviruses: CS1 is located at the S1/S2 boundary, whereas CS2 is located just upstream of the putative fusion peptide. Furin recognition motifs are indicated in red.

Viruses carrying uncleaved spike can theoretically be proteolytically activated during either extracellular transit (e.g. by trypsin in the gastro-intestinal tract) or during entry of the virus by proteases at the plasma membrane or in endosomes. For most coronaviruses, however, only receptor-associated S proteins seem to be available for proteolysis, potentially due to fusion-promoting conformational changes upon receptor engagement (Kam *et al.*, 2009; Matsuyama *et al.*, 2005; Park *et al.*, 2011; Simmons *et al.*, 2005; Wicht *et al.*, 2014b).

With some exceptions, endocytosis is the main route for entry of most coronaviruses, potentially because this route advantages the virus to pass through the cortical actin network and allows genome release 'deep' into the cytoplasm near the nucleus were replication occurs (Heald-Sargent & Gallagher, 2012). Consequently, it is not surprising that most coronaviruses rely on endosomal proteases and/or acidification for fusion activation (Figure 1.10). Endocytosis has also been described as the main entry route for serotype II FCoVs (Van Hamme *et al.*, 2007). After APN engagement, FIPV 79-1146 undergoes clathrin- and

caveolae- independent, but dynamin-dependent endocystosis (Van Hamme *et al.*, 2008). Proteolytical cleavage is mediated by cathepsin B, and is only mildly dependent on low pH. In contrast, the avirulent 79-1683 strain depends on both cathepsin B and low pH-activated cathepsin L activity for infection, at least in cell culture (Regan *et al.*, 2008). If this strain also uses the same dynamin-dependent endocytosis pathway as its virulent counterpart is unknown. Based on the molecular weight of the cleavage products, it has been suggested that cleavage of serotype II spikes by cathepsins occurs at the CS2 site (Belouzard *et al.*, 2012; Regan *et al.*, 2008). As for attachment factors and receptors, no information is available on fusion triggers for serotype I FCoVs.

The determinants of the FCoV tropism have fascinated researchers for years, and the spike protein is considered of key importance in the FCoV pathotype switch (Chang *et al.*, 2012; Licitra *et al.*, 2013; Rottier *et al.*, 2005). So far, 2 regions in the spike protein have been found very often affected by mutations when comparing faecal with tissue strains, one which is located at the S1/S2 boundary comprising the furin cleavage site (Licitra *et al.*, 2013), and the other which is located in the S2 subunit (Chang *et al.*, 2012). As described above, it has recently been shown that the second mutation typically occurs in viruses (both FIP-inducing as avirulent ones) that can undergo systemic dissemination, but it remains elusive if and how these mutations contribute to the FIP pathogenesis (Porter *et al.*, 2014). In addition, the onset of FIP is most probably the result of various mutations in the genome. Indeed, although highly important, the spike is not the sole determinant of coronavirus tissue tropism and pathogenicity. This has already been shown for other coronaviruses, as introduction of MHV-A59 spike in MHV-JHM background did not confer hepatotropism to the virus (Navas & Weiss, 2003), and chimeric viruses carrying IBV M41 spikes in a Beaudette background was still attenuated *in vivo* (Hodgson et al., 2004). For FIPV, the 3c protein has been proposed as one of the proteins for which mutation can contribute to the pathotype switch, but if and how this protein is involved remains enigmatic (Chang *et al.*, 2010; Pedersen *et al.*, 2009; Pedersen *et al.*, 2012). Establishing a reverse genetic system and notably cell lines to grow and study both serotype I FECVs and FIPVs, would certainly be an enormous leap forward to unravel this intriguing mystery.

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Aims

Coronaviruses are associated with either harmless or highly fatal intestinal, respiratory, and systemic infections in many animal species and humans. These RNA viruses are prone to genetic changes and recombination events, which not only allow them to cross species barriers and cause new (zoonotic) emerging diseases, but also enable them to switch virulence within their host. Feline infectious peritonitis virus (FIPV) is probably the most well known coronavirus emerging from mutational changes occurring in the viral genome during replication of its parent virus, feline enteric coronavirus (FECV). Although FECV often passes unnoticed, it affects 90-100% of all cats in virtually all multi-cat environments worldwide, and up to 12% will finally develop and succumb to FIP. Despite decades of research and attempts to combat this highly feared and dreadful disease, effective vaccines and/or antivirals are still lacking, ante-mortem diagnosis of FIP is still challenging, and the complex pathogenesis remains an enigma.

FIP is the consequence of mutations arising in the viral genome during a common FECV infection, but almost no information is available on the interaction of this parent virus with its host. Therefore, this thesis aimed at contributing to the complex puzzle by focussing on these roots of the FIP pathogenesis. At the start of this project, no relevant FECV strains were available for *in vitro* research, as these faecal strains had been uncultivable in the available cell lines. In fact, the lack of susceptible cell lines is the most important factor why information on these viruses is missing, and why unravelling of the pathotype switching mutations has been hampered. To address this lack, the first study (**Chapter 3**) aimed at establishing intestinal epithelial cell lines to enable the propagation and further study of these viruses. These cultures and strains were then used to further unravel the early beginning of FECV infections, and hence to define some of the entry factors involved in enterocyte infections (**Chapter 4.1 and 4.2**).

A second part of the present thesis aimed to add upon the knowledge on *in vivo* FECV infections and to investigate the feasibility to control this virus in the fight against FIP. Indeed, since every FECV replication cycle holds the risk for mutated viruses to emerge, prevention of FIP can be directed towards controlling faecal-oral transmission of this parent virus. To broaden our knowledge on the FECV pathogenesis and to validate detection methods, three feline leukaemia virus-, feline immunodeficiency virus-, and feline coronavirus-negative cats were inoculated with the serotype I FECV strain UCD and immunological, virological and clinical parameters were followed during 3 months after inoculation (**Chapter 5.1**). As there is a great demand for effective measures to control FCoV infections in practice, it was investigated in 2 catteries whether successful prevention of transmission of FECV from shedders to naive

cats/kittens is feasible by adapting the management. Since FECV shedding is known to be long-lasting and immunity does not protect against reinfections, control of cat-to-cat transmission in both catteries was based on regular monitoring of faecal shedding and grouping of cats (**Chapter 5.2**).

Establishment of feline intestinal epithelial cell cultures for the propagation and study of feline enteric coronaviruses

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Adapted from: Veterinary Research 2013; 44: 71

Summary

Feline infectious peritonitis (FIP) is the most feared infectious cause of death in cats, induced by feline infectious peritonitis virus (FIPV). This coronavirus is a virulent mutant of the harmless, ubiquitous feline enteric coronavirus (FECV). To date, feline coronavirus (FCoV) research has been hampered by the lack of susceptible cell lines for the propagation of serotype I FCoVs. In this study, long-term feline intestinal epithelial cell cultures were established from primary ileocytes and colonocytes by simian virus 40 (SV40) T-antigen- and human Telomerase Reverse Transcriptase (hTERT)-induced immortalization. Subsequently, these cultures were evaluated for their usability in FCoV research. Firstly, the replication capacity of the serotype II strains WSU 79-1683 and WSU 79-1146 was studied in the continuous cultures as was done for the primary cultures. In accordance with the results obtained in primary cultures, FCoV WSU 79-1683 replicated significantly more efficient compared to FCoV WSU 79-1146 in both continuous cultures. In addition, the cultures were inoculated with faecal suspensions from healthy cats and with faecal or tissue suspensions from FIP cats. The cultures were susceptible to infection with different serotype I enteric strains and two of these strains were further propagated. No infection was seen in cultures inoculated with FIPV tissue homogenates. In conclusion, a new reliable model for FCoV investigation and growth of enteric field strains was established. In contrast to FIPV strains, FECVs showed a clear tropism for intestinal epithelial cells, giving an explanation for the observation that FECV is the main pathotype circulating among cats.

3.1 Introduction

Feline coronaviruses (FCoVs) are associated with both enteric and systemic diseases in domestic and wild *Felidae*. The feline enteric coronavirus (FECV) is an ubiquitous enteropathogenic virus, replicating in epithelial cells of both small and large intestine after oral uptake (Hayashi *et al.*, 1982; Herrewegh *et al.*, 1997; Kipar *et al.*, 2010; Meli *et al.*, 2004; Pedersen *et al.*, 1981). The mild enteritis caused by this replication is usually unapparent or is manifested by a transient diarrhoea in young kittens (Pedersen *et al.*, 1981). Around 13% of all infected cats are not able to clear the virus (Addie & Jarrett, 2001). In these cats, the virus persists for several months or even years in the epithelium of the large intestine (Hayashi *et al.*, 1982; Herrewegh *et al.*, 1997; Kipar *et al.*, 2010; Pedersen *et al.*, 1981; Stoddart *et al.*, 1988). Since FECVs are easily transmitted from cat to cat by faecal-oral route, they are enzootic among most cat populations (Addie & Jarrett, 1992; Pedersen *et al.*, 1981). Although FECV infections manifest subclinically, they may be the start of a lethal outcome. During replication, mutations can occur in the viral genome, providing the virus with tools to productively replicate in monocytes/macrophages (Dewerchin *et al.*, 2005; Rottier *et al.*, 2005; Stoddart & Scott, 1989; Vennema *et al.*, 1998). This mutational variant, designated feline infectious peritonitis virus (FIPV), causes a chronic and highly fatal systemic disease, FIP, characterized by a diffuse pyogranulomatous (peri)phlebitis and serositis in presence (wet form) or absence (dry form) of fibrinous exudate in the affected body cavities (Horzinek & Osterhaus, 1979; Kipar *et al.*, 1998; Montali & Strandberg, 1972). In contrast to FECV, which is highly infectious but seldom causes disease, FIPV shows a low infectivity but high mortality (95-100%) (Addie *et al.*, 1995). Losses from FIP are typically unpredictable and occur in only a restricted fraction (<12%) of all seropositive cats (Addie & Jarrett, 2001; Addie *et al.*, 1995; Pedersen, 2009; Pedersen *et al.*, 2012). However, the lack of tools to successfully prevent and control the disease has an enormous financial, emotional and ethical impact, and makes FIP the most feared infectious cause of death in cats (Wolf, 1995). To date, it remains unknown why FECV and FIPV show such a clinically (mild enteritis versus FIP) and epidemiologically (easy versus restricted transmission) different behaviour.

Besides the two pathotypes, FCoVs also occur as two serotypes (Fiscus & Teramoto, 1987). Worldwide, the majority of all strains (both FECVs and FIPVs) are serotype I viruses (Addie *et al.*, 2003; Benetka *et al.*, 2004; Hohdatsu *et al.*, 1992; Kummrow *et al.*, 2005; Lin *et al.*, 2009; Vennema, 1999). In contrast to the type I viruses that are 100% feline, type II viruses possess spike and adjacent genes of canine origin, since they have arisen by double

recombination events between type I FCoVs and canine coronavirus (CCoV) (Herrewegh *et al.*, 1998; Lin *et al.,* 2013). Despite their lower prevalence, most comparative *in vitro* studies have been performed with the easily cell culture growing serotype II strains WSU 79-1683 and WSU 79-1146 (Dewerchin *et al.*, 2005; McKeirnan *et al.*, 1987; Rottier *et al.*, 2005; Stoddart & Scott, 1989). FCoV WSU 79-1146 has been shown to be a highly virulent, readily FIP-inducing virus due to its efficient infection of monocytes/macrophages. FCoV WSU 79-1683, on the other hand, is an avirulent virus, inducing at most a mild enteritis in kittens. The poor systemic dissemination of this virus has been attributed to a restricted, inefficient infection of monocytes/macrophages (Dewerchin *et al.*, 2005; Pedersen *et al.*, 1984; Rottier *et al.*, 2005; Stoddart & Scott, 1989). To date, cell culture propagation of the abundantly present serotype I FECVs has never been achieved and only few serotype I FIPV strains have been adapted to grow in felis catus whole fetus (fcwf) cells. However, most of these strains have lost their pathogenicity through cell culture adaptation (Pedersen, 2009; Tekes *et al.*, 2012). Hence, comparative studies between non-culture adapted FECVs and FIPV have only been possible by comparing genomes of both naturally occurring strains (Chang *et al.*, 2011; Chang *et al.*, 2010; Chang *et al.*, 2012; Pedersen *et al.*, 2009; Pedersen *et al.*, 2012). To date, it remains unclear which genetic determinants make up a certain pathotype.

In the present study, cultures of intestinal epithelial cells from the ileum (ileocytes) and colon (colonocytes) were established by inducing a combined expression of SV40 T-antigen and hTERT in primary ileocytes and colonocytes. The reliability of these cultures for their use in FCoV-research was first investigated by comparing replication capacities of the, at high titre available, avirulent FCoV WSU 79-1683 and the highly virulent FCoV WSU 79-1146 with results obtained for the primary cultures. Since those serotype II strains have been heavily cell culture adapted, the usability of the intestinal epithelial cell cultures in FCoV research was further evaluated by investigating their susceptibility for different field strains, present in faeces and tissues of coronavirus-infected cats.

3.2 Materials and methods

3.2.1 Cats

Since cats are euthanized every day in practice, tissues of these animals can be used in research in order to reduce the number of laboratory cats. Using tissues of euthanized animals is in agreement with the statements of the Local Ethical Committee. Therefore, the intestines of euthanized conventional cats were used in this study and were a kind contribution to research by the owners. Faecal extracts from SPF cats (Harlan laboratories, Indianapolis, IN,

USA) experimentally infected with FECV UCD were used as a source of this enteric field strain. These infection experiments were approved by the Local Ethical and Welfare Committee of the Faculty of Veterinary Medicine of Ghent University (EC2010/043).

3.2.2 Isolation and cultivation of primary ileocytes and colonocytes

Cats were sedated by intramuscular injection of a mixture of Ketamin (0.05 ml/kg; Anesketin®, Eurovet, Heusden-Zolder, Belgium) and Midazolam (0.05 ml/kg; Dormicum®, Roche, Brussels, Belgium). Subsequently, the cats were euthanized by intracardial injection of 20% Sodium Pentobarbital (1 ml/1.5 kg; Kela Laboratories, Hoogstraten, Belgium). The protocol used for the isolation of primary ileocytes and colonocytes was based on the one described by Rusu and co-workers, with minor adaptations (Rusu *et al.*, 2005). Directly after euthanasia, the colon was aseptically removed and transported in ice-cold Dulbecco's Modified Eagle Medium (DMEM; Gibco BRL, Merelbeke, Belgium) supplemented with 100 U/ml penicillin (Continental Pharma Inc., Puurs, Belgium), 0.1 mg/ml streptomycin (Certa, Braine l'Alleud, Belgium), 0.1 mg/ml gentamycine (Gibco BRL) and 10% foetal bovine serum (FBS; Gibco BRL). Subsequently, the pieces of intestine were inverted, i.e. mucosal side facing outwards, and the intestinal content was removed by three vigorous washings in ice-cold DMEM supplemented with antibiotics. The intestinal mucosa was digested in DMEM containing collagenase I (0.4 mg/ml, Invitrogen, Paisley, UK) and dispase (1.2 mg/ml, Sigma, St. Louis, MO, USA) for 15 minutes (ileum) or 20 minutes (colon) at 37°C. Then, the digestion medium was refreshed and the pieces were incubated for another 45 minutes (ileum) or 60 minutes (colon) at 37°C. Subsequently, the pieces were longitudinally opened and the digested mucosa was scraped with a sterile scalpel blade. The scrapings were incubated in warm DMEM supplemented with antibiotics and dispase (1.2 mg/ml) for 10 minutes whilst pipetting. After centrifugation (140 g, 3 min) the pellet was resuspended in DMEM containing 2% D-Sorbitol (Sigma) and 10% FBS, and centrifuged (50 g, 3 min) in order to separate as much single cells (most probably contaminating stromal cells) as possible from the epithelial cell clusters. This sorbitol centrifugation was repeated 5 times. The resulting pellet was subsequently resuspended 1:3 (vol:vol) in culture medium consisting of DMEM/F-12 supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.1 mg/ml gentamycin, 10% FBS (Gibco BRL), 10 ng/ml epidermal growth factor (Sigma), 1% insulin-transferrin-selenuim-X (Invitrogen), 100 nM hydrocortisone (Sigma), 1% non-essential amino acids 100x (Gibco BRL), and 1 µg/ml 3,3',5-Triiodo-L-thyronine sodium salt (Sigma). The cells were seeded on glass coverslips coated with collagen type I (Roche Diagnostics,

Vilvoorde, Belgium). The cells were cultivated in a 37°C / 5% CO_2 atmosphere. After 24 hours, the culture medium was replaced by medium containing 2% FBS to restrict the outgrowth of non-epithelial cells. Medium was changed every other day. Morphological features of the primary cultures were evaluated every day by light microscopy (Olympus).

3.2.3 Characterization of the primary cultures

To assess the origin of the primary cells, double-immunostainings were performed against pancytokeratin and vimentin. Therefore, the cells were fixed with 4% paraformaldehyde in PBS for 10 minutes at room temperature (RT) followed by permeabilization with 0.1% Triton X-100 for 2 minutes at RT. The cells were incubated with monoclonal anti-cytokeratin antibodies (Dako Denmark A/S) containing 10% normal goat serum for 1 h at 37°C, followed by goat anti-mouse-Texas Red labelled antibodies for 1 h at 37°C (Molecular Probes, Eugene, Oregon, USA). Afterwards, the cells were incubated for 45 minutes at 37°C with monoclonal anti-vimentin antibodies (Lab Vision Corporation, Fremont, CA, USA) labelled with Zenon® Alexa Fluor 488 (Invitrogen) according to the manufacturer's protocol. Nuclei were stained with Hoechst 33342 (Molecular Probes) for 10 minutes at RT. The slides were mounted using glycerine-PBS solution (0.9:0.1, vol:vol) with 2.5% 1,4-diazabicyclo[2.2.2]octane (Janssen Chimica, Beerse, Belgium) and analysed by fluorescence microscopy (DM B fluorescence microscope, Leica Microsystems GmbH, Heidelberg, Germany).

3.2.4 Immortalization of primary feline ileocytes and colonocytes

At 4 days post isolation, primary cultures of ileocytes and colonocytes from the same cat were transduced with both recombinant lentiviruses expressing either the SV40 large T antigen or the hTERT protein (Applied Biological Materials Inc., Canada) in addition of polybrene (8 μg/ml, Applied Biological Materials Inc.). After 30 minutes, medium was added and the cells were further incubated with the virus (1:1 vol:vol in medium) overnight. The following day, the viral supernatant was removed and cells were further incubated in medium. After 5 days, the cells were detached by trypsinization with 0.25% trypsin - 0.02% EDTA, subcultured in collagen-coated wells (split ratio 1:2) and evaluated daily for clonal expansion by light microscopy (Olympus). Clusters of cells with epithelial (cobblestone-like) morphology were marked and other cells in the well were removed by scraping. Subsequently, the epithelial clusters were detached by trypsinization and further expanded in collagen-coated flasks to generate a long-term culture of both small and large intestinal epithelial cells.

3.2.5 Characterization of the ileocyte and colonocyte cell lines

To confirm the epithelial character of both cell lines, double-immunostainings were performed against cytokeratin and vimentin as described above. The success of transduction was assessed by performing immunocytochemical stainings against the SV 40 large T antigen and hTERT. Therefore, cells seeded on collagen-coated glass coverslips were fixed with 4% paraformaldehyde, followed by permeabilization with 0.1% Triton X-100. The cells were incubated with polyclonal rabbit antibodies against hTERT (Applied Biological Materials Inc.) containing 10% normal goat serum for 1 h at 37°C, followed by goat ant-rabbit-FITC labelled antibodies (Molecular Probes) for 1 h at 37°C. Subsequently, the cells were incubated with monoclonal antibodies against the SV40 large T antigen (Applied Biological Materials Inc.) containing 10% normal goat serum, followed by goat anti-mouse-AF594 labelled antibodies (Molecular Probes), each for 1 h at 37°C. Nuclei were stained and slides were mounted as described above. The cells were analysed by fluorescence microscopy (DM B fluorescence microscope, Leica Microsystems GmbH). In addition, immunocytochemical stainings against the intestinal brush border hydrolase aminopeptidase N were performed. Therefore, cells were fixed with 1% paraformaldehyde and incubated with the monoclonal antibody R-G-4 (kindly provided by Dr. Hohdatsu, Department of Veterinary Infectious Diseases, Towada, Japan) containing 10% normal goat serum followed by goat anti-mouse-FITC labelled antibodies (Molecular Probes), each for 1 h at 37°C. Images were obtained using a Leica TCS SPE laser scanning spectral confocal system linked to a DM B fluorescence microscope (Leica Microsystems). Argon and He/Ne lasers were used for exciting FITC and Texas Red fluorochromes, respectively. Leica confocal software was used for image acquisition.

3.2.6 Expression kinetics of viral antigens in FCoV WSU 79-1683 and FCoV 79-1146 infected cells

A third passage of the FCoV strains 79-1683 and 79-1146 grown in Crandell feline kidney (CrFK) cells were used. FCoV WSU 79-1683 was obtained from the American Type Culture Collection (ATCC) and FCoV WSU 79-1146 was kindly provided by Dr. Egberink (Department of Infectious Diseases and Immunology, Utrecht University, the Netherlands). At 4 days post isolation, primary cells of three cats were inoculated at a multiplicity of infection (m.o.i.) of 1. After 1 h incubation (37°C, 5% CO_2) the cells were washed 3 times with warm DMEM and further incubated in medium. Monolayers of continuous ileocyte and colonocyte cultures were inoculated in the same way. At different time points (0, 3, 6, 9, 12 and 24 h) post inoculation, cells were fixed with 4% paraformaldehyde for 10 minutes and

permeabilized with 0.1% Triton X-100 for 2 minutes at RT. For the primary cultures, double-immunostainings against both FCoV-antigens and cytokeratin were performed to visualize the infected epithelial cells. For the continuous cultures, only viral antigens were stained. Viral antigens were visualized with polyclonal FITC-labelled anti-FCoV antibodies (VMRD, Pullman, USA). Cytokeratin-positive cells were visualized as described above. Nuclei were stained with Hoechst, the slides were mounted and analysed by fluorescence microscopy (Leica Microsystems GmbH). All experiments were performed 3 times. The area under the curve was determined for each experiment. Triplicate assays were compared using a Mann-Whitney U test. Statistical analysis was performed using GraphPad Prism version 5.0c (GraphPad software, San Diego, CA, USA). P values ≤ 0.05 were considered significantly different.

Using primary cells of conventional cats holds the risk that cultured cells are already infected with FCoVs. Therefore, mock-infected cells were accurately screened to exclude the presence of inherent infected cells. All cells were negative for inherent coronavirus.

3.2.7 One-step real time RT-PCR for the detection of the viral load in field strain suspensions

RNA was extracted from the faecal suspensions using the QIAamp Viral RNA Mini Kit (Qiagen, Benelux BV, Belgium) and from tissue suspensions with the RNeasy Mini Kit (Qiagen). To avoid detection of subgenomic mRNA's, primers were designed using the Primer 3 plus software within a conserved region of ORF1b based on FCoV sequences available in GenBank. A 20 µl PCR mixture was used per reaction and contained 10 µl Precision OneStep™ qRT-PCR Mastermix with SYBR Green and ROX (PrimerDesign, Southampton, UK), 0.2 µM forward primer ORF1bFW (5'-TGGACCATGAGCAAGT CTGTT-3'), 0.4 µM reverse primer ORF1bRV (5'-CAGATCCATCATTGTGTACTT TGTAAGA-3') and 3 µl RNA or diluted standard RNA (see below). A reverse transcription step of 10 min at 55°C and an enzyme activation step at 95°C for 8 min were followed by 40 cycles, each 10 s at 95°C and 60 s at 58°C. A first-derivative melting curve analysis was performed by heating the mixture to 95°C for 15 s, then cooling to 60°C for 1 min, and heating back to 95°C at 0.3°C increments. Reverse transcription, amplification, monitoring, and melting curve analysis were carried out in a Step One Plus™ real-time PCR system (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA).

3.2.8 Synthetic RNA standards for absolute quantitation

RNA was extracted from faecal suspensions containing FECV UCD using the QIAamp Viral RNA Mini Kit (Qiagen). The RNA was reverse-transcribed into cDNA using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen). Briefly, 250 ng RNA was incubated for 5 min at 65°C with 2 µM reverse primer ORF1bRV and 10 mM dNTP mix. Afterwards, an equal volume of cDNA synthesis mix, containing 10x RT buffer, 25 mM $MgCl_2$, 0.1 M DTT, 40 U/µl RNase OUT and 200 U/µl Superscript III RT was added and incubated for 50 min at 50°C. The reaction was terminated at 85°C for 5 min. RNA was removed by incubation with RNase H for 20 min at 37°C. The 50 µl PCR mixture for the amplification of the cDNA contained 10 µl 5x Herculase II reaction buffer, 0.8 µl dNTP mix, 2 µl DNA template, 0.25 µM forward primer ORF1bFW modified with a T7 promoter sequence at its 5' end (5'-TAATACGACTCACTATAGGGTGGACCATGAGCAAGTCT GTT-3'), 0.25 µM reverse primer ORF1bRV, and 1 µl Herculase II fusion DNA polymerase (Agilent Technologies Inc., Santa Clara, CA, USA). After a denaturation step for 1 min at 95°C, 30 cycles of amplification, each 20 s at 95°C, 20 s at 50°C, and 60 s at 68°C, were followed by a terminal elongation of 4 min at 68°C. Fragment length was controlled by agarose gel electrophoresis and fragments with the correct length were excised and purified from the gel using the Nucleospin® Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany). cRNA standards were transcribed by incubation for 1 h at 37°C with 10x transcription buffer, 500 µM rNTPs and 20 U T7 RNA polymerase-Plus Enzyme Mix (Applied Biosystems). Transcription reactions were DNase I treated and the amount of RNA was determined using the Nanodrop 2000 system. Ten-fold serial dilutions of the RNA were made over a range of 6 log units (10^7-10^2) for the generation of the standard curve (Efficiency: 93.96 ± 0.76%; R^2: 0.999).

3.2.9 Assessment of the infectious coronavirus titre in faecal and tissue suspensions

Faecal samples were collected from healthy cats housed in 3 different catteries / multi-cat environments that have dealt with FIP in the past. Faecal extracts of experimentally infected cats containing an unknown titre of FECV strain UCD (originally isolated at UC Davis, (Pedersen *et al.*, 1981)) were a kind gift of Dr. Rottier (Department of Infectious Diseases and Immunology, Utrecht University, the Netherlands). This suspension was clarified by centrifugation at 4000 g for 10 min and SPF cats were infected with the supernatant. Faecal extracts from one cat were used as a source of this enteric field strain. From all faecal samples, 20% suspensions were made in DMEM supplemented with 2% FBS (Gibco, BRL),

100 U/ml penicillin (Continental Pharma Inc.), 0.1 mg/ml streptomycin (Certa), and 0.1 mg/ml gentamycin (Gibco BRL). From 4 cats with FIP (immunohistochemically confirmed), faeces and affected tissues were collected. From tissue homogenates, 20% suspensions were made in DMEM supplemented with 100 U/ml penicillin (Continental Pharma Inc.), 0.1 mg/ml streptomycin (Certa), and 0.1 mg/ml gentamycin (Gibco BRL). Suspensions were centrifuged (1200 g, 4°C, 20 min), and the supernatant was aliquoted and stored at -70°C until use. All samples were initially screened by immunofluorescence in both cell lines by inoculating monolayers, seeded on collagen coated coverslips, with 250 µl of the suspensions for 1 h at 37°C. Thereafter, cells were washed and further incubated in medium for 24 h. After fixation and permeabilization, infected cells were visualized as described above. In addition, the amount of infectious virus was quantified in all samples, including the initially negative ones. Therefore, monolayers of colonocytes, seeded in collagen I coated 96-well plates, were inoculated with 50 µl of serially diluted (1/10) faecal or tissue suspensions (ranging from 10^0 to 10^{-7}). After 1 h (37°C, 5% CO_2), medium was added and the cells were further incubated for 72 h. To reduce cell loss due to toxicity, undiluted suspensions were removed from the wells 1 h p.i. and the cells were washed 2 times before they were further incubated in medium. Then, plates were washed with PBS, air-dried (1 h, 37°C) and frozen (-20°C). The 50% tissue culture infectious dose ($TCID_{50}$) was determined by means of immunoperoxidase monolayer assay (IPMA). Therefore, cells were fixed and permeabilized by incubation with PF 4% (10 minutes, RT), followed by incubation with methanol containing 1% H_2O_2 (5 minutes, RT). Then, cells were incubated with PBS containing 10% normal goat serum and 0.1% Tween 80 for 30 minutes at 37°C. Subsequently, cells were incubated with monoclonal antibodies against the N-protein (produced and characterized in the laboratory of the authors), followed by incubation with goat anti-mouse HRP-labelled antibodies. Infected cells were visualized by adding sodium-acetate buffer containing amino-ethylcarbazole (AEC) and H_2O_2 for 10 minutes at RT. The fifty percent end-point was calculated according to the method of Reed and Muench. The serotype of all samples was determined by means of RT-PCR described by Addie et al. (Addie *et al.*, 2003).

3.2.10 Determination of infectious virus in FIPV-suspensions by inoculation of monocyte-derived macrophages

Feline monocytes were isolated and seeded on glass coverslips as previously described (Dewerchin *et al.*, 2005). At 7 days post seeding, cells were inoculated with 250 µl of the suspensions. After 1 h at 37°C, cells were washed and further incubated in medium for 24 h.

After fixation and permeabilization, infected cells were visualized by immunofluorescence staining as described above.

3.2.11 Propagation and titration of FECV UCD and UG-FH8

Two different faecal strains, UCD and UG-FH8, were passaged 3 times in continuous colonocyte cultures, starting from the faecal suspensions. After 3 passages, the $TCID_{50}$ was determined as described above. In addition, sequencing of ORF3 and ORF7 was performed to check for their integrity. Therefore, primers were designed using published sequences of FCoV ORF3 and ORF7 in GenBank. Viral RNA was extracted from the faecal suspensions with the QIAamp Viral RNA Mini Kit (Qiagen) and cDNA was generated using SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen). Amplification was carried out in a 50 µl reaction using Herculase II fusion DNA polymerase (Agilent Technologies Inc., Santa Clara, CA, USA). The Nucleospin® Gel and PCR Clean-up kit (Macherey-Nagel) was used for purification of the PCR products. Sequencing was performed by the GATC Biotech Company (Konstanz, Germany). Additionally, it was investigated if both third passage strains still showed a specific enterotropism by inoculating other feline cell lines (CrFK and fcwf cells). Twenty-four hours p.i., infected cells were visualized by immunofluorescence staining as described above.

3.3 Results

3.3.1 Morphological features and characterization of the primary cultures

By using a combination of dispase and collagenase, epithelial cells were isolated from the underlying basement membrane in clusters (Figure 3.1A). Four hours post seeding, the majority of the cells had attached and foci of polygonal cells became visible within 24 h post seeding (Figure 3.1B/D). Primary ileum cultures were always ‘contaminated’ with a lot of elongated or stellate-like cells, present in between the epithelial foci, while the colon cultures were more pure. For the ileum, the epithelial cells did not further grow beyond 24 h post seeding, whereas mesenchymal cells started to expand in between the epithelial cell clusters. In the colonic cultures, the epithelial cells showed a confined proliferation within 3-4 days post isolation, resulting in the formation of (sub)confluent cobblestone-like layers (Figure 3.1E). Then, these cells had reached a state of replicative senescence, which became typically characterized by morphological changes such as increase in cell size and development of multiple nuclei at 6-7 days post isolation. The growth arrest seemed not to be the result of the confluent state since, despite many attempts, it was not possible to subculture the cells. A part of the cells started to degenerate from 7 days post seeding. However, most of the cells could

be kept for another week. To prevent cell loss due to inherent degeneration and to prevent overgrowth by mesenchymal cells, both ileum and colon cultures were always infected at 4 days post isolation for studying the viral replication.

Immunofluorescence stainings against cytokeratin (intermediate filaments typically found in the cytoskeleton of epithelial cells) and vimentin (intermediate filaments expressed by mesenchymal cells) confirmed the epithelial nature of the polygonal, cobblestone-like cells (Figure 3.1C/F). At 4 days post isolation, the majority of the cells (> 90%) in the colon cultures was still of epithelial origin. For the ileum cultures, the vimentin positive mesenchymal cells had expanded in between the epithelial clusters, occupying around 50% of the wells. Remarkably, some of the ileum epithelial cells did also express vimentin, resembling dedifferentiated epithelial cells typically found after injury or in tumours (Figure 3.1C).

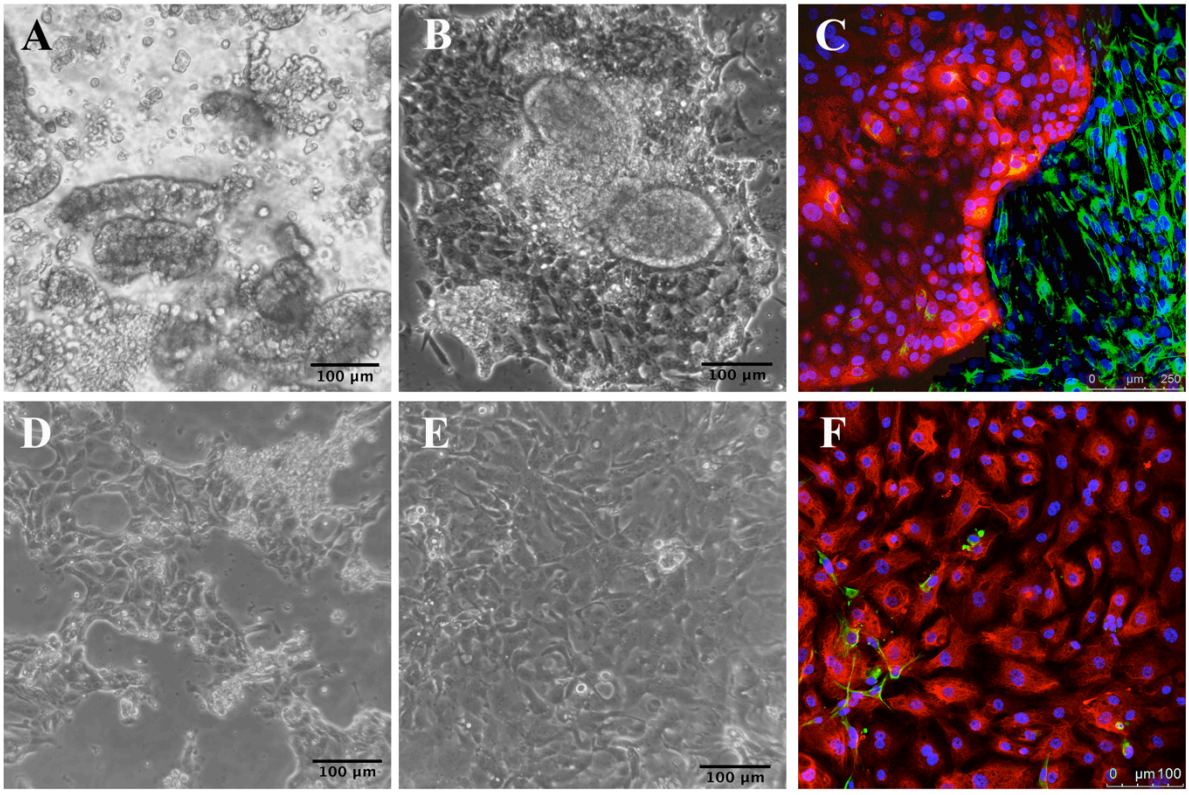

Figure 3.1. Morphological features and immunocytochemical characterization of the primary ileum (A-C) and colon (D-F) cultures. (**A**) Epithelial cells were isolated in cell clusters. (**B, D**) Polygonal cells started to spread from these clusters giving rise to several foci of cells. (**E**) (Sub)confluent layers were reached 3-4 days after seeding due to a restricted proliferation of the cells. (**C, F**) Double-immunostainings against cytokeratin (red) and vimentin (green) filaments 4 days after isolation, confirming the epithelial nature of the polygonal cells.

3.3.2 Expression kinetics of viral antigens in FCoV WSU 79-1683 and WSU 79-1146-infected primary ileocytes and colonocytes

Primary ileocytes and colonocytes were susceptible to infection with both serotype II FCoV strains. However, the antigen expression kinetics differed greatly between the avirulent FCoV WSU 79-1683 and the virulent FCoV WSU 79-1146 (Figure 3.2). For both strains, the first antigen-positive cells appeared at 6 h p.i. and increased further over time. However, the avirulent enterotropic WSU 79-1683 strain infected the cells significantly more efficient (P= 0.05 for both ileum and colon) compared to WSU 79-1146.

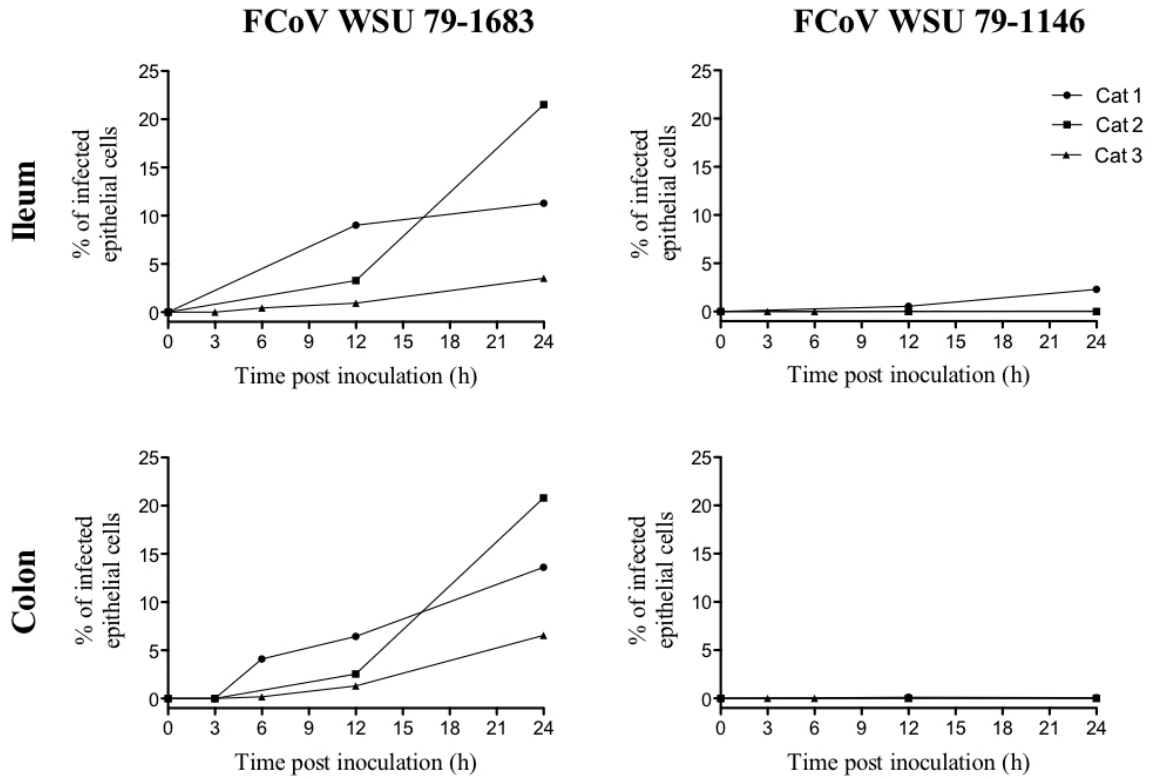

Figure 3.2. Kinetics of FCoV replication in primary ileum and colon cultures from 3 conventional cats. Cells were inoculated with FCoV WSU 79-1683 or FCoV WSU 79-1146 at a m.o.i. = 1. At different time points post inoculation, cytoplasmically expressed viral proteins were visualized and the percentage of infected epithelial cells was determined.

3.3.3 Morphological features and characterization of the established continuous ileocyte and colonocyte cultures

By introducing a combinational expression of SV40 large T-antigen and hTERT, a successfully transformed cell line was generated for both ileocytes and colonocytes (Figure 3.3 and 3.4). Indeed, a various number of the transduced cells started to proliferate from 1 week after transduction onwards, forming layers of cobblestone-like cells with a cell diameter of 20-25µm and 30-35µm for ileocytes and colonocytes, respectively. Both SV40 large T-antigen as hTERT expression was detected in these cultures, confirming the success of transduction. These cell lines could be further expanded and passaged for over 30 passages now, which is in sharp contrast to the primary cultures. Besides its typical cobblestone-like appearance, the epithelial character was confirmed by the expression of cytokeratin and dome formation in the cultures. The latter is indicative for the polarization of cells in monolayers. Remarkably, most of the cells in both cultures co-expressed both cytokeratin and vimentin in the freshly formed monolayers, suggesting a more dedifferentiated state of the cells. For further characterization, APN expression in the cultures was investigated, since APN is an intestinal brush border associated hydrolase, and moreover an important receptor for serotype II FCoVs. All cells expressed APN at their surface. However, the expression levels varied greatly from cell to cell in both cultures, most probably due to different differentiation levels of the cells in culture.

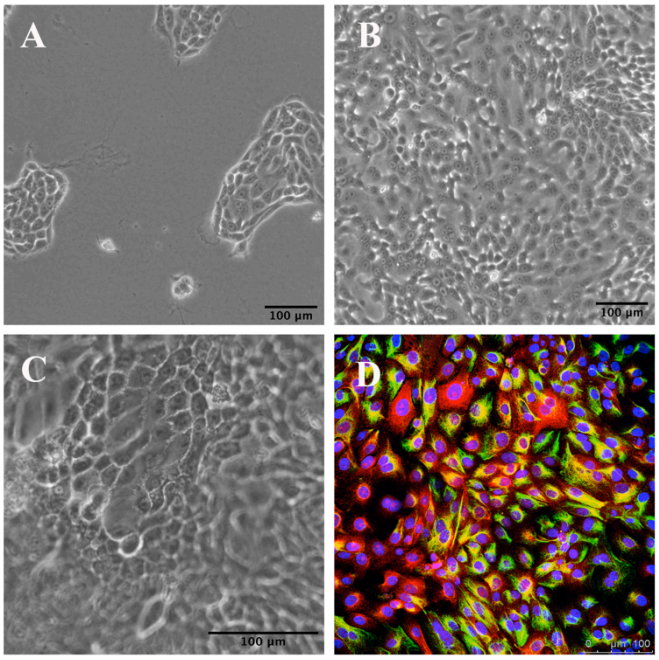

Figure 3.3. Morphological and immunocytochemical characterization of the continuous ileocyte cultures. (**A**) Proliferating isles. (**B**) Cobblestone morphology of the monolayer. (**C**) Dome formation. (**D**) Double-immunostaining against cytokeratin (red) and vimentin filaments.

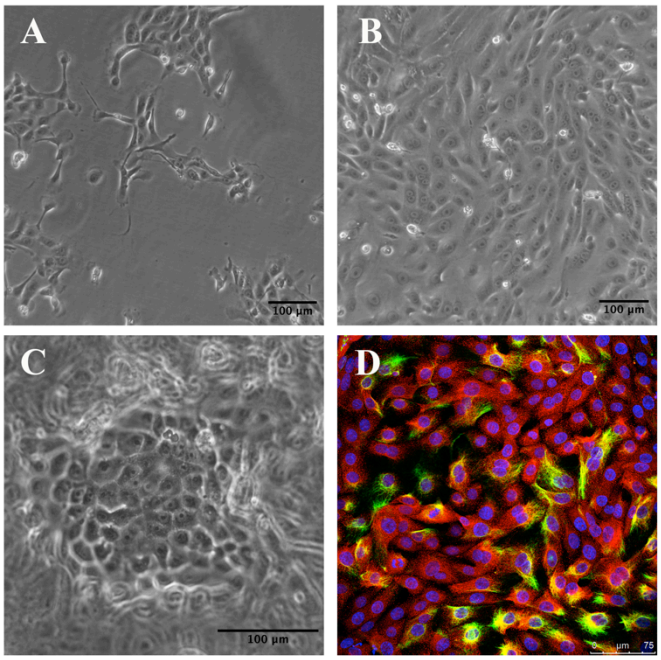

Figure 3.4. Morphological and immunocytochemical characterization of the continuous colonocyte cultures. (**A**) Proliferating isles. (**B**) Cobblestone morphology of the monolayer. (**C**) Dome formation. (**D**) Double-immunostaining against cytokeratin (red) and vimentin filaments.

3.3.4 Antigen expression kinetics of FCoV WSU 79-1683 and WSU 79-1146 in continuous ileocyte and colonocyte cultures

Since the continuous cultures seemed to be less differentiated compared to the primary cultures, the reliability of the established cell lines as model for intestinal epithelial cells was further investigated. Therefore, antigen expression kinetics were assessed in both continuous ileocyte and colonocyte cultures as was done for the primary cells (Figure 3.5). In accordance with the results obtained for the primary cultures, FCoV WSU 79-1683 significantly infected both ileocytes as colonocytes more efficiently than WSU 79-1146. At 24 h p.i., FCoV WSU 79-1683 had infected 19.46 ± 4.37 % and 18.47 ± 4.61% of the colonocytes and ileocytes, respectively, whereas only 0.03 ± 0.02% of the colonocytes and 0.22 ± 0.18% of the ileocytes were infected by FCoV WSU 79-1146 at that time point.

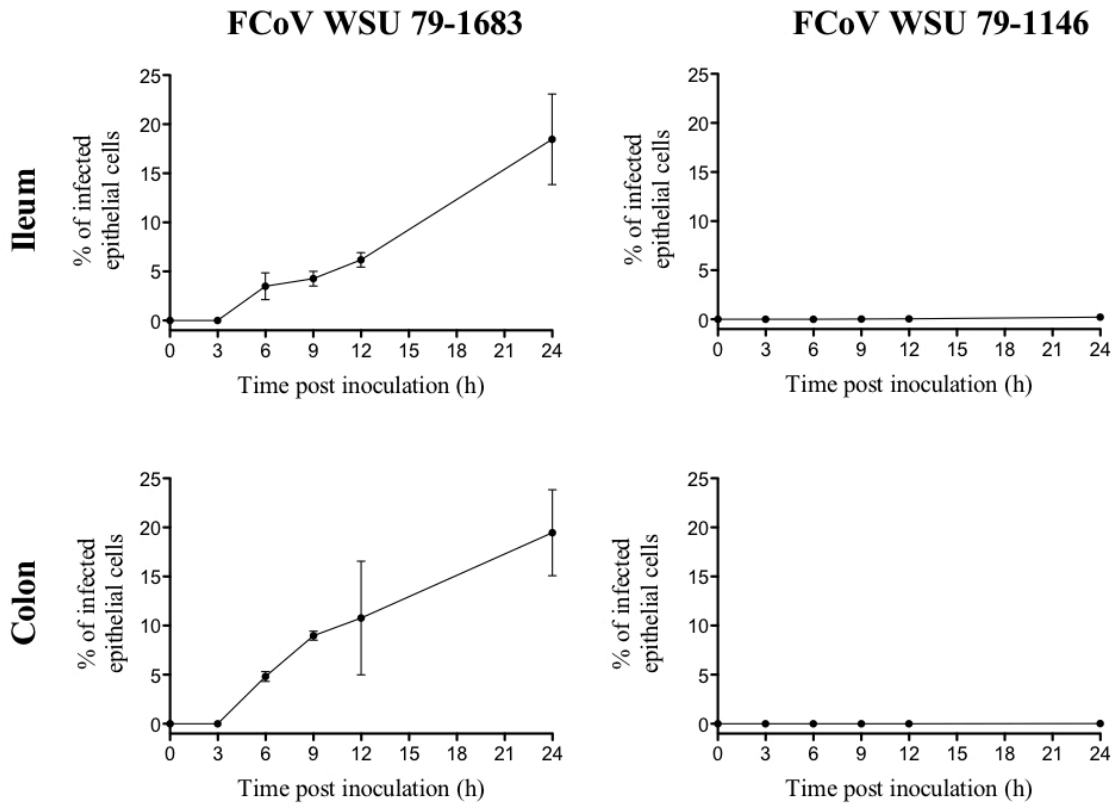

Figure 3.5. Kinetics of FCoV replication in continuous ileocyte and colonocyte cultures. Cells were inoculated with FCoV WSU 79-1683 or FCoV WSU 79-1146 at a m.o.i. = 1. At different time points post inoculation, the percentage of infected cells was determined. Data are expressed as the means ± standard deviation of the results of 3 separate experiments.

3.3.5 Titration of field strains in faecal and tissue suspensions

A major restriction in FCoV research is the lack of cell lines supporting the growth of serotype I enteric strains. Therefore, the newly established cell lines were further validated by investigating their susceptibility for different field strains. All those strains were serotype I viruses as confirmed by PCR. Table 3.1 gives the results obtained by titration of different faecal and tissue suspensions on colonocyte cultures. Comparable results were obtained by titration on ileocyte cultures with FECV UCD. Hence, titration of other field strains was not repeated on this cell line. All but two of the samples collected from healthy cats were positive for coronavirus, with qPCR titres ranging from $10^{4.18}$ to $10^{9.06}$ viral copies / g faeces. Infectious virus was detected by IPMA in 50% of all positive samples (8/16), with 57% of positivity in samples with qPCR titres above 10^5. This number increased to 64% (7/11) and 80% (4/5) when the cutoff was made at qPCR titres above 10^6 and 10^7 viral copies / g faeces, respectively. In the one sample (UG-FH9) with a qPCR above 10^7 that was negative on IPMA, enterotropic virus was detected by immunofluorescence staining. All but one of the

samples collected from FIP cats were positive for coronavirus on qPCR, with the number of viral copies / g ranging from $10^{3.98}$ to $10^{9.16}$. As determined by both IPMA and immunofluorescence staining, none of those samples, except for one, contained enterotropic virus. However, 3 tissue samples (UG-TF5, UG-TF9 and UG-TF17) did contain infectious virus as determined on monocyte-derived macrophages. Despite its high viral load, no infectious virus (neither on enterocytes nor on monocytes/macrophages) was found in faecal suspensions of FIP cat 1 (UG-FF1). Faeces of FIP cat 2 (UG-FF2) did contain enterotropic virus that was not infectious for macrophages.

3.3.6 Propagation and titration of FECV UCD and UG-FH8

To date, no serotype I enteric field strains have been propagated *in vitro* and availability of such FECV strains would be valuable in feline coronavirus research. Therefore, two faecal strains, FECV UCD and UG-FH8, were further propagated in colonocyte cultures (Table 3.2). After 3 passages, both strains were raised in titre with around 3 $\log_{10}$ $TCID_{50}$ / ml. In addition, ORF3 and ORF7 from each of the third passage strains were sequenced to check for signs of cell culture adaption. Both strains still carried intact accessory genes that were 100% identical to the original strain. Typically, a lot more CPE was noticed in UG-FH8 infected wells compared to FECV UCD (Figure 3.6). After 3 passages, both strains still showed a specific enterotropism, since no infection was seen after inoculation of other feline cell lines (fcwf and CrFK cells).

Table 3.1. QPCR- and infectious titre of different faecal and tissue suspensions from healthy and FIP cats.

Sample	Source	QPCR titre (Log_{10} copies / g)	Infectious titre (Log_{10} $TCID_{50}$ / g)
UG-FH1	Faeces healthy cats	6.03	-
UG-FH2	Faeces healthy cats	6.64	2.67
UG-FH3	Faeces healthy cats	5.51	-
UG-FH4	Faeces healthy cats	5.41	2.36
UG-FH5	Faeces healthy cats	7.22	2.50
UG-FH6	Faeces healthy cat	6.88	-
UG-FH7	Faeces healthy cat	-	-
UG-FH8	Faeces healthy cat	6.30	3.33
UG-FH9	Faeces healthy cats	7.69	-
UG-FH10	Faeces healthy cat	7.89	2.50
UG-FH11	Faeces healthy cats	8.44	2.67
UG-FH12	Faeces healthy cats	4.66	-
UG-FH13	Faeces healthy cats	-	-
UG-FH14	Faeces healthy cats	6.27	-
UG-FH15	Faeces healthy cat	6.62	2.50
UG-FH16	Faeces healthy cats	4.18	-
FECV UCD	Faeces healthy cat 6d p.i.	9.06	5.00
UG-FF1	Faeces FIP cat 1	7.57	-
UG-FF2	Faeces FIP cat 2	9.16	3.50
UG-FF3	Faeces FIP cat 3	-	-
UG-FF4	Faeces FIP cat 4	3.98	-
UG-TF2	Kidney FIP cat 1	6.79	-
UG-TF5	Omentum FIP cat 2	6.87	-
UG-TF9	Spleen FIP cat 3	5.83	-
UG-TF17	Omentum FIP cat 4	8.00	-

Table 3.2. Infectious titre and status of ORF3 and ORF7 in cell culture propagated viruses.

Strain	Infectious titre (Log_{10} $TCID_{50}$ / ml)		Status ORF3 at P_3	Status ORF7 at P_3
	P_0	P_3		
FECV UCD	3.97	6.30	Intact	Intact
UG-FH8	2.63	5.97	Intact	Intact

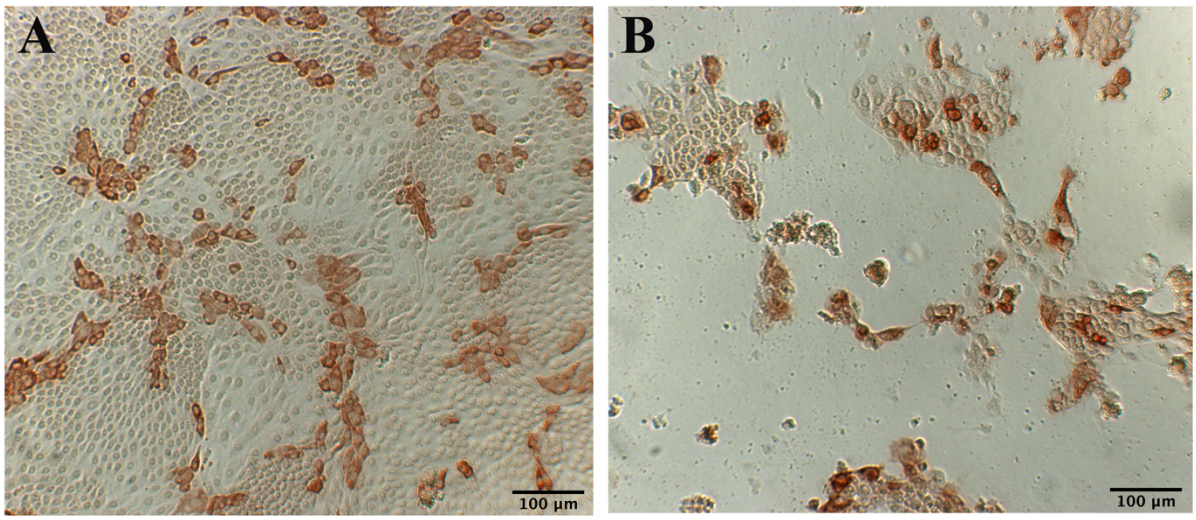

Figure 3.6. Immunoperoxidase staining of infected colonocytes. Infected colonocytes 3 days p.i. with (**A**) $10^{2.99}$ $TCID_{50}$ FECV UCD and (**B**) $10^{2.67}$ $TCID_{50}$ UG-FH8.

3.4 Discussion

In this study, immortalized cultures of both small (ileum) and large (colon) intestinal epithelial cells were established and validated for their use in feline coronavirus research. Intestinal epithelial cells are important target cells in FCoV pathogenesis, but to date such cell lines are not available. The establishment of primary intestinal epithelial cell cultures has been proven to be difficult because of the induction of programmed cell death after disruption from the extracellular matrix, the uncontrolled contamination with stromal cells, and the still unknown homeostatic components needed for the maintenance of these cultures (Kaeffer, 2002). To avoid induction of apoptotic signals by disrupting cell-matrix adhesions, a combination of collagenase and dispase was used in this study to digest the mucosa, allowing the isolation of epithelial cell clusters. These were subsequently separated as much as possible

from the contaminating single stromal cells by D-sorbitol density centrifugation. The primary colon cultures showed a relative high purity of epithelial cells, whereas primary ileum cultures were much more contaminated with stromal cells. The contamination with mesenchymal cells is intrinsic to the isolation method used and therefore inevitable. Yet, the epithelial cells could be cultured for a week without overgrowth by these cells, making both primary cultures ideal models for studying interactions with enterotropic infectious agents. Remarkably, some primary cells co-expressed cytokeratin and vimentin filaments, which is often found in injured epithelial cells, tumours and in primary cultures due to the detachment of the cells from their natural environment during isolation. In these cells, the epithelial differentiation is turned back to a more embryonic state, amongst others characterized by de novo expression of vimentin filaments (Baer & Bereiter-Hahn, 2012). Only a minority of the cells did express vimentin, suggesting that most cells were able to restore their differentiation with the used culture conditions.

Although the doubtful origin and clear signs of cell culture adaptation (Pedersen, 2009; Pedersen *et al.*, 2008), FCoV WSU 79-1683 and FCoV WSU 79-1146 were the only available strains representing an avirulent and related virulent strain at the time of the study. Hence, those strains were initially used for investigating the susceptibility of primary enterocytes to both virulent and avirulent FCoVs. Replication of both strains have been studied in CrFK cells, fcwf cells, peritoneal macrophages, bone marrow-derived macrophages and peripheral blood monocytes (Dewerchin *et al.*, 2005; McKeirnan *et al.*, 1987; Rottier *et al.*, 2005; Stoddart & Scott, 1989). In contrast to the available continuous cultures (CrFK and fcwf cells), the difference in virulence between both strains was reflected *in vitro* when using primary FIPV target cells (monocytes/macrophages). The highly efficient and mostly sustained infection of FIPV in macrophages and monocytes from susceptible cats, in contrast to an inefficient and not sustained infection of the avirulent WSU 79-1683 in those cells, may explain why FIPV behaves as a harmful invasive virus causing this progressive systemic disease (Dewerchin *et al.*, 2005; Rottier *et al.*, 2005; Stoddart & Scott, 1989). As was previously shown for monocytes/macrophages, the present study confirms that both strains exhibit clear differences in cell tropism. In contrast to FCoV WSU 79-1146, the avirulent WSU 79-1683 efficiently infected and replicated in intestinal epithelial cells, resulting in exactly opposing kinetics as were found for macrophages (Rottier *et al.*, 2005).

Primary cultures are ideal tools to reliably investigate virus-host interactions. Nevertheless, isolation of primary epithelial cells is labour-intensive, the cultures are often contaminated

with a various amount of mesenchymal cells and the yield is variable and rather low. To allow research with those cells, long-term cultures were derived from both primary ileocytes and colonocytes by SV40 T-antigen- and hTERT-induced immortalization, resulting in the generation of two feline intestinal epithelial cell cultures. The epithelial nature of both cell lines was confirmed by their cobblestone morphology, dome formation and cytokeratin expression. These newly established cell lines could be valuable tools for virus research. However, immortalized cell lines are often phenotypically transformed, making reliable research with these cells questionable. In the present study it was shown that, in contrast to the primary cultures, the majority of the cells co-expressed cytokeratin and vimentin filaments, suggesting that the cultures were less differentiated compared to their primary counterparts. Therefore, the reliability of the established cell lines for their use in feline coronavirus research was further investigated and confirmed. Antigen expression kinetics of FCoV WSU 79-1683 and FCoV WSU 79-1146 were comparable with the results obtained with the primary cultures, showing a significant difference in cell tropism between both strains. As mentioned before, comparative studies in the available continuous feline cell lines (CrFK and Fcwf cells) showed no replicative differences between both serotype II strains (Dewerchin *et al.*, 2005; McKeirnan *et al.*, 1987; Rottier *et al.*, 2005). However, both cultures are hardly sensitive to serotype I FCoVs. To date, cultivation of serotype I FECVs has never been achieved and only few serotype I FIPV strains could be adapted to grow in continuous cell cultures. In addition, most of these strains seem to have lost their pathogenicity through cell culture adaptation (Pedersen, 2009; Tekes *et al.*, 2012). In the present study, the newly established intestinal epithelial cell cultures were further evaluated for their susceptibility to serotype I field strains. Infectious, enterotropic virus was found in 57% (8/14) of all FCoV-positive faecal samples originating from healthy cats in 3 geographically distinct multi-cat environments. One of those samples was detected only by immunofluorescence staining. This higher sensitivity can be explained by the use of more inoculum in that test. In the majority of the positive samples, infectious titres were always between $10^{3.05}$ to $10^{5.77}$ times lower compared to the total virus titre. This difference can be attributed to the presence of defective particles, but infectious titres in such faecal samples can possibly be underestimated due to faecal toxicity to the cells and the presence of neutralizing IgA antibodies as well. In infection experiments with FECV UCD, the amount of infectious particles was typically 3-4 $\log_{10}$ times lower compared to the total amount of particles in the first week p.i., but this further increased thereafter most probably due to the generation of neutralizing antibodies (see chapter 5.1). It is impossible to estimate when cats in multi-cat environments became infected and the

presence of neutralizing antibodies can explain why infectious virus in some of the faecal samples with a quite high viral load was not detectable. Coronavirus was detected in 3/4 of the tested faecal samples from FIP cats. Previously, it has been shown that faecal viruses from FIP cats did not cause enteric infections or FIP upon inoculation of laboratory cats (Pedersen *et al.*, 2012). This can explain why, despite its high viral load, no infectious virus (neither in enterocytes nor in monocytes/macrophages) was found in the faeces of FIP cat 1 (UG-FF1). However, enterotropic virus was found in the faeces from another FIP cat (UG-FF2) that was housed in a Belgian shelter. To search for explanations for this discrepancy, accessory proteins of the virus in faecal and tissue suspensions of that cat were sequenced (data not shown). As in all faecally shed FCoVs sequenced so far (Chang *et al.*, 2010; Pedersen *et al.*, 2009; Pedersen *et al.*, 2012), the faecal strain carried an intact 3c gene. In addition, this strain showed only 96% and 89% homology with the tissue strain based on 7a and 7b protein respectively. So it seems that this cat was co-infected with another, most probably enteric strain circulating in that shelter, explaining the shedding of enterotropic infectious virus in that cat. In 3/4 of the tissue samples from FIP cats (UG-TF5, UG-TF9 and UG-TF17), infectious virus was found by inoculation of monocyte-derived macrophages. However, these viruses seemed to have lost their tropism for intestinal epithelial cells since no infection was detected after inoculation of the intestinal epithelial cell cultures. The fact that FECV is the only pathotype that is well adapted for growth in intestinal epithelial cells shows that FECVs have the advantage over FIPVs to spread amongst cats. These findings are in agreement with previous observations on FCoV epidemiology, explaining the restricted transmission of FIPVs and hence low incidence of cats with FIP (Addie & Jarrett, 2001; Addie *et al.*, 1995; Pedersen, 2009; Pedersen *et al.*, 2012).

Since no cell culture-propagated serotype I enteric strains are available, two of those strains, FECV UCD and UG-FH8, were further propagated in the established cultures. After 3 passages, both virus strains were raised in titre with 3 $\log_{10}$ $TCID_{50}$ / ml, making them usable for further *in vitro* experiments. It has been described that the 7b glycoprotein is not necessary for replication in cell cultures, and hence this gene is readily lost by *in vitro* propagation. Therefore, alterations in the 7b protein can be a sign for cell culture adaptation as seen in many of the cell culture propagated serotype I FIPVs (Herrewegh *et al.*, 1995). In present study, no such signs of cell culture adaptation were detected for both 3th passage strains, which still carried intact ORF7 genes identical to the original faecal strains. All field enteric strains sequenced so far carried intact 3c genes (Chang *et al.*, 2010; Pedersen *et al.*, 2009;

Pedersen *et al.*, 2012). To date, the only available avirulent, enteritis-inducing strain, WSU 79-1683, has a mutated 3c gene and for that reason doubt has been cast on the use of this strain as a typical enteric pathotype (Pedersen, 2009; Pedersen *et al.*, 2008). Both FECV UCD and UG-FH8 propagated in this study still carried an intact (and identical to the original strain) ORF3. In addition, the cell culture propagation of both strains did not extend their tropism to other non-enterocytic feline cells, making them useful as representatives of the enteric pathotype.

In conclusion, we established cultures of both feline small and large intestinal epithelial cells, providing new and reliable *in vitro* models for studying enteric pathogenesis processes of FCoVs. These enterocyte cultures were susceptible to different enteric serotype I field strains, while FIPVs were clearly restricted in their replication in intestinal epithelial cells. Two of the enteric strains were further propagated, providing relevant enteric strains for future FCoV research.

Acknowledgements

We are grateful to Dr. Egberink and Dr. Rottier for supplying the virus strains. We also acknowledge Ytse Noppe for her excellent technical support. We gratefully thank the vets, and particularly Laurent Pauwels, for the interaction with the owners of the cats and of course the owners as well for their contribution to this work. This research was funded by The Research Foundation-Flanders (FWO-Vlaanderen; G.0284.09N). LMBD was supported by a doctoral fellowship of The Research Foundation-Flanders. DAJO, AD, BLV and IDMR were supported by the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT Vlaanderen).

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Entry factors involved in feline enteric coronavirus infections

4.1 Role of sialic acids in feline enteric coronavirus infections

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Adapted from: Journal of General Virology 2014, 95, 1911-1918

4.2 Effect of lysosomotropic agents and protease inhibitors on feline enteric coronavirus infections in enterocytes

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Manuscript in preparation

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Role of sialic acids in feline enteric coronavirus infections

Summary

To initiate infections, many coronaviruses use sialic acids, either as receptor determinants or as attachment factors helping the virus find its receptor underneath the heavily glycosylated mucus layer. In the present study, the role of sialic acids in serotype I feline enteric coronavirus (FECV) infections was studied in feline intestinal epithelial cell cultures. Treatment of cells with neuraminidase (NA) enhanced infection efficiency, showing that terminal sialic acid residues on the cell surface are not receptor determinants and even hamper efficient virus-receptor engagement. Knowing that NA treatment of coronaviruses can unmask viral sialic acid binding activity, replication of untreated and NA-treated viruses was compared, showing that NA treatment of the virus enhanced infectivity in untreated cells but was detrimental in NA-treated cells. By using sialylated compounds as competitive inhibitors, it was demonstrated that sialyllactose (2,6-α-linked over 2,3-α-linked) notably reduced infectivity of NA-treated viruses, whereas bovine submaxillary mucin inhibited both treated and untreated viruses. In desialylated cells, however, viruses were less prone to competitive inhibition with sialylated compounds. In conclusion, this study demonstrated that FECV has a sialic acid binding capacity, which is partially masked by virus-associated sialic acids, and that attachment to sialylated compounds can facilitate enterocyte infections. However, sialic acid binding was not a prerequisite for the initiation of infection, and virus-receptor engagement was even more efficient after desialylation of cells, indicating that FECV requires sialidases for efficient enterocyte infections.

4.1.1 Introduction

Feline enteric coronavirus (FECV) is an enzootic enteropathogen in cats. The enteritis caused by its replication in intestinal epithelial cells is mild and mostly unnoticed (Addie & Jarrett, 1992; Pedersen *et al.*, 1981). However, mutations in the viral genome can allow the virus to replicate efficiently in monocytes/macrophages, resulting in the fatal feline infectious peritonitis (FIP) (Dewerchin *et al.*, 2005; Pedersen, 2009; Rottier *et al.*, 2005; Stoddart & Scott, 1989; Vennema *et al.*, 1998). Despite many attempts, treatment of FIP has remained palliative to date. In multi-cat environments, cat owners lose up to 12 % of their cats, and recurrent FIP deaths are still a major reason to stop breeding programs. Since FIP is the consequence of mutations arising in the viral genome during a common FECV infection, FECV is an attractive target in the fight against FIP. Despite the valuable information available from different *in vivo* studies (Meli *et al.*, 2004; Pedersen *et al.*, 2008; Pedersen *et al.*, 1981; Poland *et al.*, 1996; Vogel *et al.*, 2010), very little is known about the FECV-enterocyte interactions as these viruses had been uncultivable for many years. The propagation of these viruses in recently established feline intestinal epithelial cell cultures allows further unravelling of these FECV-enterocyte interactions *in vitro* (Desmarets *et al.*, 2013).

Coronaviruses mediate their entry into host cells by their spike (S) proteins. Coronavirus S proteins have been shown to possess at least 2 receptor-binding domains (RBDs), the S1 N-terminal RBD and the S1 C-terminal RBD. Whereas the C-terminal RBD of most coronaviruses is involved in protein binding, the N-terminal RBD can act as a lectin, recognizing various sialic acids (Peng *et al.*, 2011). Sialic acid binding has been described for members of the alpha-, beta-, and gammacoronaviruses. However, whereas some of these viruses rely on sialic acid binding for the initiation of host cell infections, others use sialic acids as attachment factors, but rely solely on another protein receptor to initiate their infections (Schwegmann-Wessels & Herrler, 2006).

Among alphacoronaviruses, a sialic acid binding capacity has been described for transmissible gastroenteritis virus (TGEV) and porcine epidemic diarrhoea virus (PEDV). For both viruses, this sialic acid binding activity becomes more pronounced when virions are pretreated with neuraminidase (NA), demonstrating that the sialic acid binding site is masked by virion-associated sialic acids (Park *et al.*, 2010; Schultze *et al.*, 1996). The role of this sialic acid binding during TGEV infections has been extensively studied. Sialic acid binding by TGEV Purdue is not essential in the initiation of *in vitro* infections as desialylation of cells

hardly affects its replication, and mutants lacking the sialic acid binding site replicate to the same extent in cell cultures (Krempl *et al.*, 1997; Schultze *et al.*, 1996). However, when the absorption time is reduced, sialic acid binding contributes to efficient infection, showing that the sialic acid binding activity helps TGEV infections under unfavourable conditions, as encountered during its passage through the intestinal tract (Schwegmann-Wessels *et al.*, 2011). Indeed, mutants lacking the sialic acid binding site were no longer capable of inducing enteropathy, showing that sialic acid binding is required to induce efficient intestinal infections *in vivo* (Bernard & Laude, 1995; Krempl *et al.*, 1997), possibly by allowing the virus to interact with and pass through the mucus layer covering the epithelial cells (Schwegmann-Wessels *et al.*, 2002). In contrast to TGEV Purdue, NA treatment renders cells more resistant to infection with TGEV Miller, bovine coronavirus (BCoV) (a betacoronavirus) and avian infectious bronchitis virus (IBV) (a gammacoronavirus*)*, showing that these viruses use sialic acids as receptor determinants to initiate infection into host cells (Schultze & Herrler, 1992; Schwegmann-Wessels *et al.*, 2011; Winter *et al.*, 2006).

The role of sialic acids in feline coronavirus (FCoV) infections is unknown. However, it has been shown that FIP cats have hyposialylated serum alpha1-acid glycoprotein (AGP), whereas healthy cats in the same environment tend to have hypersialylated AGP, suggesting that sialylated compounds can determine the outcome of a feline coronavirus infection, potentially by acting as a decoy for virus infections (Ceciliani *et al.*, 2004; Paltrinieri *et al.*, 2008). In the present study, the sialic acid binding capacity of FECV and the role of sialic acids in FECV infections was investigated in 2 different intestinal epithelial cell cultures with 2 different serotype I FECV strains.

4.1.2 Materials and methods

4.1.2.1 Viruses and cells

Two serotype I FECV strains, UCD and UG-FH8, were propagated in feline colonocyte cultures in foetal bovine serum (FBS)-depleted medium, and third passage strains were used for all infection experiments. All experiments were performed in both feline ileocyte and colonocyte cultures. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 Nutrient Mixture (1/1) supplemented with 100 U penicillin ml^{-1}, 0.1 mg streptomycin ml^{-1}, 0.1 mg gentamycin ml^{-1}, 5 % FBS (Gibco BRL) and 1 % non-essential amino acids 100x (Gibco BRL). The origin of the viruses and cells has previously been described (Desmarets *et al.*, 2013).

4.1.2.2 Neuraminidase treatment of cells

To remove sialic acids from the enterocytes, monolayers of continuous ileocytes and colonocytes were washed 2 times with warm Ca^{2+}- and Mg^{2+}-enriched phosphate buffered saline (PBS). Then, cells were incubated with 50 mU ml^{-1} NA from *Vibrio Cholerae* (Roche Diagnostics) in Ca^{2+}- and Mg^{2+}-enriched PBS. Cells that were not treated with NA were incubated in Ca^{2+}- and Mg^{2+}-enriched PBS and underwent the same manipulations as the NA-treated cells. After 1 h at 37 °C, cells were washed three times with medium to remove the neuraminidase. Viability of the cells was assessed by ethidium monoazide bromide (EMA) staining, ensuring > 99 % viability with the used NA concentration.

4.1.2.3 Neuraminidase treatment of viruses

To remove sialic acids from the virus, virus suspensions were incubated on a shaker for 1 h at 37 °C with 50 mU ml^{-1} Glycocleave® neuraminidase (*Vibrio Cholerae*) enzyme beads (GALAB Technologies GmbH) in Ca^{2+}- and Mg^{2+}-enriched PBS. Beads were washed 2 times with Ca^{2+}- and Mg^{2+}-enriched PBS before incubation with the virus to remove buffers. Before inoculation, NA beads were separated from the virus by centrifugation (200 g, 10 min, 4 °C). Untreated virions were incubated in Ca^{2+}- and Mg^{2+}-enriched PBS without beads and underwent the same manipulations as the NA-treated virus.

4.1.2.4 Infection experiments

Cells were inoculated with either NA-treated or untreated virus ($10^{5.8}$ $TCID_{50}$ ml^{-1} and $10^{5.97}$ $TCID_{50}$ ml^{-1} for FECV UCD and UG-FH8, respectively). After 5 or 60 minutes at 37 °C, the unbound virus particles were removed by three washing steps with medium, and the cells were further incubated in medium for 12 h (37 °C, 5 % CO_2). Then, cells were fixed with 4 % paraformaldehyde for 10 min at room temperature (RT).

4.1.2.5 Immunofluorescence staining of infected enterocytes

Cells were permeabilized with 0.1% Triton X-100 for 2 min at RT. Then, cells were incubated for 1 h at 37 °C with the monoclonal anti-nucleocapsid antibody 10A12 (produced and characterized in the laboratory of the authors) containing 10% normal goat serum, followed by incubation with goat anti-mouse FITC labelled antibodies (Molecular Probes) for 1 h at 37 °C. Nuclei were stained with Hoechst 33342 (Molecular Probes) for 10 min at RT. Slides were mounted using glycerine-PBS solution (0.9:0.1, vol:vol) with 2.5 % 1,4-diazabicyclo[2.2.2]octane (Janssen Chimica) and analysed by fluorescence microscopy (DM B fluorescence microscope, Leica Microsystems GmbH).

4.1.2.6 Infection inhibition assays

Porcine gastric mucin, bovine submaxillary mucin, fetuin, lactoferrin, lactose, D-galactose, N-acetyl-D-glucosamine, and N-acetyl-D-galactosamine were purchased from Sigma-Aldrich, α2,3-sialyllactose and α2,6-sialyllactose from Carbosynth Limited. NA-treated and untreated viruses were pre-incubated with different concentrations of each compound for 30 min at 37 °C. These virus-compound mixtures were used to inoculate feline colonocyte cultures. After 1 h at 37 °C, unbound virus particles were removed by 3 washing steps with medium and the cells were further incubated in medium for 12 h. Then, cells were fixed with 4 % paraformaldehyde and stained as described above. Viability of the cells was assessed by EMA staining, ensuring > 99 % viability with the used concentrations.

4.1.2.7 Statistical analysis

Experiments were independently repeated 4 times, and results were compared with the Mann-Whitney U test. Statistical analysis was performed using GraphPad Prism version 5.0c (GraphPad software, San Diego, CA, USA). P values ≤ 0.05 were considered significantly different.

4.1.3 Results

4.1.3.1 Effect of NA treatment of cells on FECV infection

To assess the role of sialic acids as receptor determinants, cells were pretreated with 50 mU ml^{-1} NA prior to inoculation with FECV strains UCD or UG-FH8. Surprisingly, removal of sialic acids greatly enhanced infectivity of both strains, even after 5 minutes absorption time (Figure 4.1.1). By determination of the percentage of infected cells 12 h p.i., it was shown that NA pretreatment of cells significantly ($p = 0.05$) enhanced infection efficiency from 0.05 ± 0.05 % to 3.63 ± 1.21 % for UCD and from 0.59 ± 0.14 % to 19.07 ± 18.86 % for UG-FH8 in ileocytes. For the colonocytes, NA treatment of the cells increased the percentage of infected cells from 0.29 ± 0.29 % to 3.36 ± 2.11 % for UCD, and from 0.80 ± 0.19 to 33.45 ± 20.57 % for UG-FH8 (Figure 4.1.2, white bars). Although both strains were inoculated at comparable multiplicity of infection (m.o.i) (m.o.i. 0.25 and 0.35 as determined on NA-treated cells for UCD and UG-FH8, respectively), consistently more cells (ranging from 2.8 to 11.8 times more) were infected 12 h p.i. by UG-FH8 compared to UCD in both untreated and treated cells. These results imply that FECV does not depend on terminal sialic acid residues on the enterocyte surface for the initiation of its infection and that FECV requires sialidases to allow efficient virus-receptor engagement.

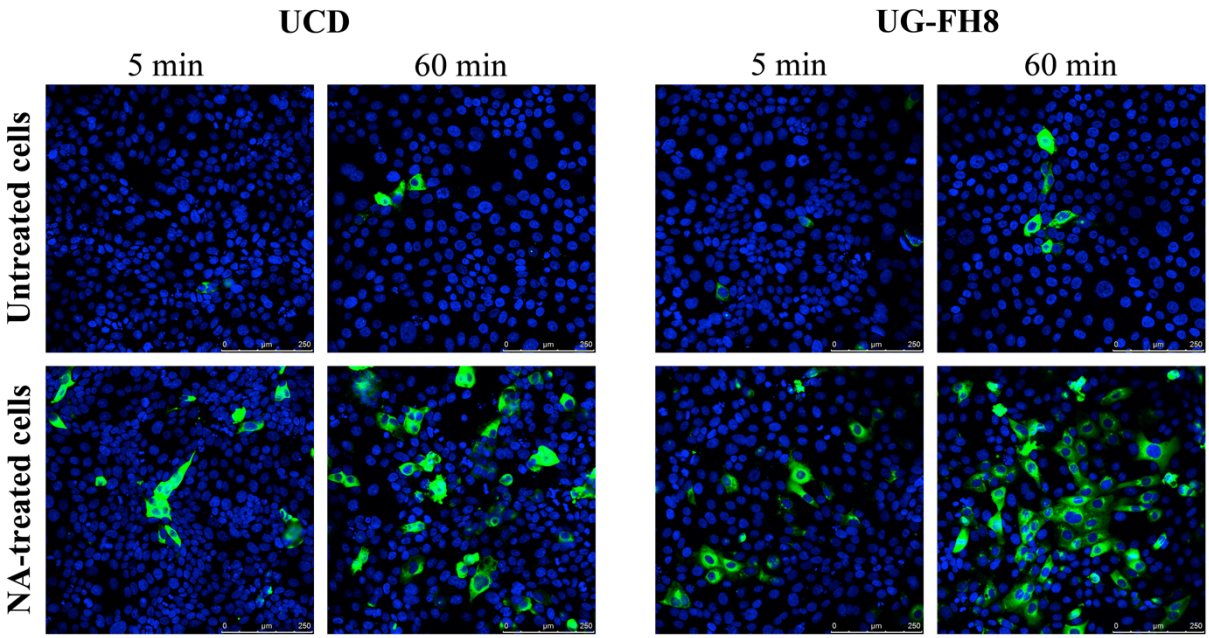

Figure 4.1.1. NA treatment of intestinal epithelial cells enhances FECV infection. Cells were pretreated with PBS or NA in PBS (50 mU ml^{-1}) and inoculated with FECV UCD (m.o.i. 0.25) or UG-FH8 (m.o.i. 0.35) for 5 minutes or 60 minutes at 37 °C. After 3 washings, cells were incubated in medium and infected cells were visualised 12 h p.i. by immunofluorescence staining.

When sialic acids are removed by NA treatment of cells, subterminal sugar residues are exposed. To investigate if these sugars were involved in the enhanced infection efficiency of FECV in desialylated cells, subterminal sugar residues including D-galactose, N-acetyl-D-glucosamine, and N-acetyl-D-galactosamine were used as competitive inhibitors. None of these sugars (in concentrations up to 50 mM) reduced FECV infection in NA-treated cells, showing that attachment to one of these exposed sugars is not the reason for the enhanced infectivity of FECV (data not shown).

4.1.3.2 Effect of NA treatment of FECV on the replication in NA-treated and untreated cells

Knowing that NA treatment of coronaviruses can unmask sialic acid binding activity, the replication of untreated and NA-pretreated viruses was compared in both untreated and NA-treated cells. The effect of these treatments was analysed 12 h post inoculation (p.i.) in ileocyte and colonocyte cultures for both FECV strains (Fig. 4.1.2). Removal of sialic acids from the virus enhanced infectivity for both strains in both untreated cell cultures, though not significantly for UCD in colonocytes. For ileocytes, treatment of the virus increased the number of infected cells on average 7 times for UCD ($p = 0.03$) and 3.3 times for UG-FH8 ($p = 0.03$), whereas for colonocytes percentage of infected cells was 1.6 times ($p = 0.34$) and 1.9 ($p = 0.03$) times higher for UCD and UG-FH8, respectively.

Desialylation of the cells also enhanced infection efficiency of NA-treated viruses (black bars) ($p = 0.03$ for UCD and UG-FH8 in ileocytes, and $p = 0.05$ for UCD in colonocytes), although not significant for UG-FH8 in colonocytes ($p = 0.2$). In contrast to untreated cells, NA-treatment of the virus seemed to have a detrimental effect in NA-treated cells, although this reduction was only significant for UG-FH8 in colonocytes ($p = 0.05$). Considering all treatments, inoculation of NA-treated cells with untreated virus resulted in the most efficient infection.

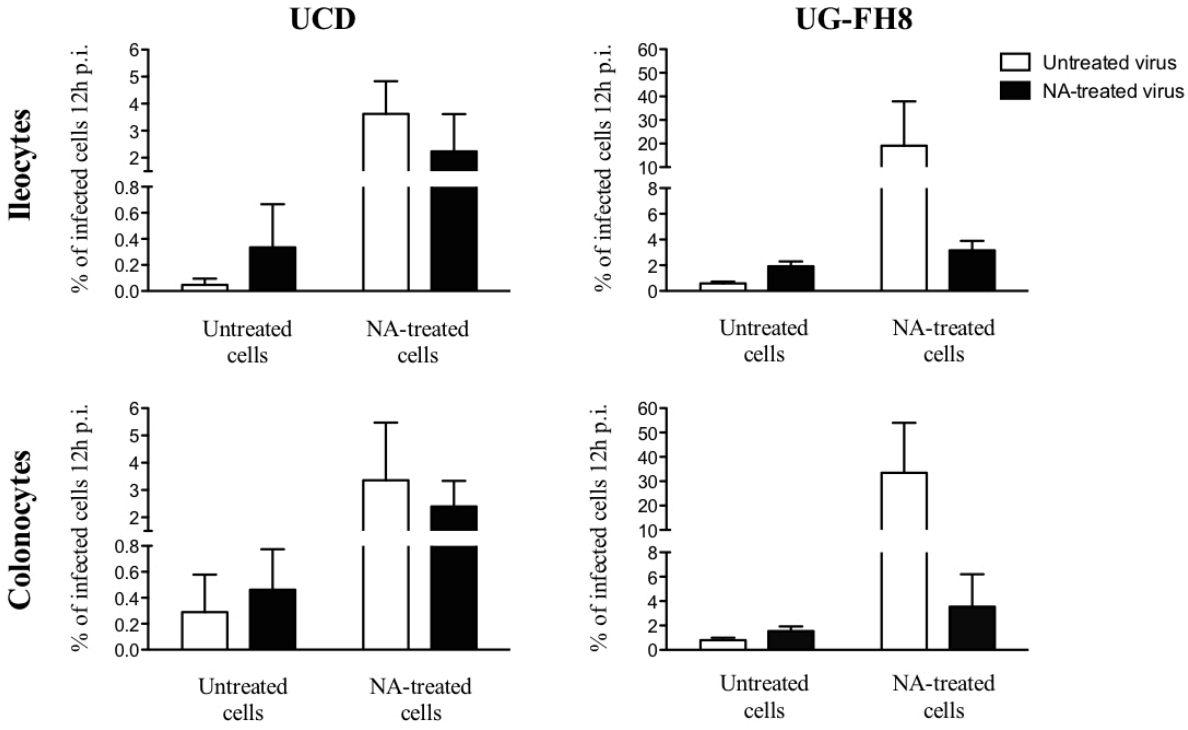

Figure 4.1.2. Effect of NA treatment of FECV on the replication in untreated and NA-treated cells. For both untreated and NA-treated FECV, percentage of infection was evaluated 12 h p.i. in both untreated and desialylated cells. Data are expressed as the means ± standard deviation of the results of 4 separate experiments.

4.1.3.3 Effect of sialylated compounds on the replication of FECV in enterocytes

To further analyse the FECV-sialic acid binding, and to investigate if the differences seen between untreated and NA-treated virions were due to sialic acid binding, the potential of different sialylated compounds (α2,3- and α2,6-sialyllactose, fetuin, porcine gastric mucin (PGM), bovine submaxillary mucin (BSM), and lactoferrin) to act as competitive inhibitor for FECV infection was studied. Therefore, both untreated and NA-treated virions were pre-incubated for 30 minutes with different concentrations of each compound before inoculation

of colonocytes. Of all tested compounds, only sialyllactoses and BSM acted as inhibitors for FECV infection, whereas fetuin, lactoferrin, and PGM had no effect on FECV infectivity in concentrations up to 200 μg ml^{-1}, 1 mg ml^{-1}, and 50 mg ml^{-1}, respectively.

Figure 4.1.3 shows the relative percentage of infected cells 12 h p.i. after pre-incubation of both untreated and NA-treated viruses with different concentrations of α2,3-sialyllactose, α2,6-sialyllactose or lactose. In contrast to lactose, sialyllactoses significantly reduced infection of NA-treated viruses. For both strains, 2,6-α-linked sialic acids had a slightly more pronounced inhibitory activity compared to 2,3-α-linked sialic acids and reduced the absolute percentage of infected cells to a similar level as the untreated viruses. In contrast to the NA-treated viruses, untreated viruses were hardly affected by the sialyllactoses. Only for the UG-FH8 strain, α2,6-sialyllactose at the highest concentration (1000 μM) significantly reduced infection efficiency of the untreated virus. In desialylated cells, sialyllactoses did not have any effect on the replication of FECV (data not shown).

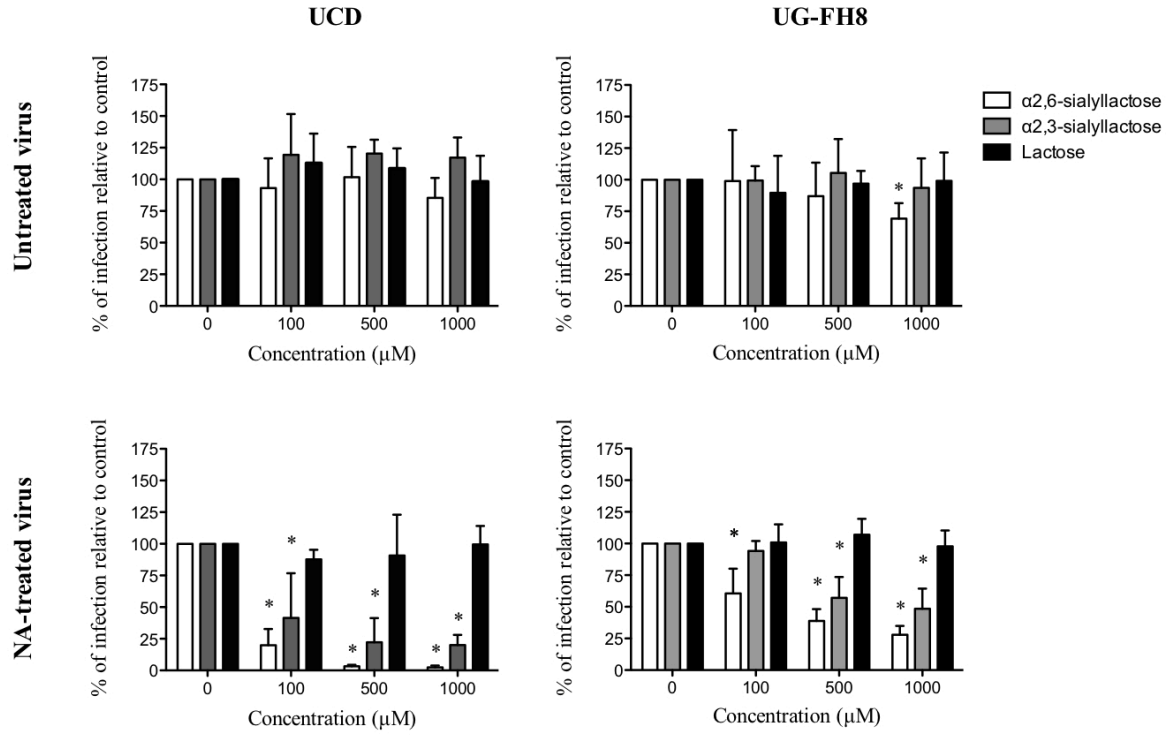

Figure 4.1.3. Effect of sialyllactoses on the infectivity of untreated and NA-treated viruses in untreated colonocytes. Both untreated and NA-treated virions were pre-incubated for 30 minutes at 37 °C with α2,3-sialyllactose, α2,6-sialyllactose or lactose before inoculation. One hour p.i., the inoculum was removed by 3 washings, and the relative percentage of infected cells was assessed 12 h p.i. Data are expressed as the means ± standard deviation of the results of 4 different experiments. Significant differences ($P \leq 0.05$) are indicated with an asterix.

Whereas sialyllactoses especially inhibited infection of NA-treated virions, BSM was a very potent inhibitor of both NA-treated and untreated viruses, with almost complete inhibition of infection in untreated cells at a concentration of 1 mg ml^{-1} (Figure 4.1.4). Pre-incubation of only the cells with 2 mg ml^{-1} BSM had no effect on the viral replication, showing that BSM specifically interacted with the virus (data not shown). In addition, BSM also had an inhibiting effect on desialylated cells, though to a lesser extent than in untreated cells, indicating that it is more difficult for sialylated compounds to compete with the viral attachment in NA-pretreated cultures. The concentration needed to completely inhibit FECV infections in desialylated cells could not be determined, since toxicity was seen with BSM from 5 mg ml^{-1} onwards.

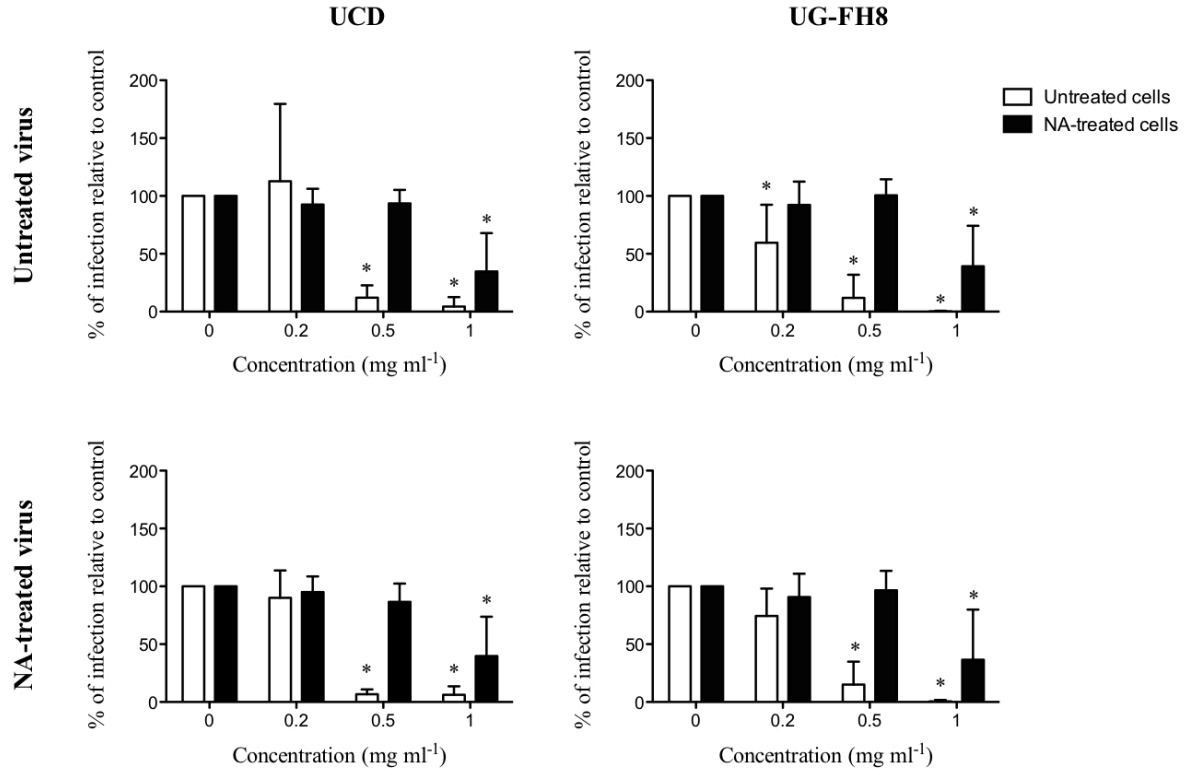

Figure 4.1.4. Effect of BSM on the replication of untreated and NA-treated viruses in both untreated and desialylated cells. Both untreated and NA-treated virions were pre-incubated with different concentrations of BSM for 30 minutes at 37 °C before inoculation. One hour p.i., the inoculum was removed by 3 washings, and the relative percentage of infected cells was assessed 12 h p.i. Data are expressed as the means ± standard deviation of the results of 4 different experiments. Significant differences ($P \leq 0.05$) are indicated with an asterix.

Taken together, these results show that FECV has a sialic acid binding capacity that is partially masked by virus-associated sialic acids and preferably recognizes 2,6-α-linked sialic acids over 2,3-α-linked sialic acids. In addition, attachment to sialylated compounds can facilitate FECV infection, especially in the untreated enterocyte cultures, which can explain the increased infectivity of NA-treated viruses. However, it seems that the receptor can be more efficiently reached when sialic acid binding is reduced/avoided by desialylation of the cells, indicating that attachment to terminal sialic acid residues is not a prerequisite for the initiation of infection *in vitro*.

4.1.4 Discussion

Coronaviruses are able to attach to host cells in 3 different ways: viral lectin-host carbohydrate (e.g. spike-sialic acids), protein-protein (e.g. spike-aminopeptidase N (APN)), and viral carbohydrate-host lectin (e.g. mannose-DC-SIGN) interactions. In the present study, the sialic acid binding capacity of FECV and the role of sialic acids in FECV infections of enterocytes was investigated. Sialic acid binding by the coronavirus S protein has been described for members of the alpha-, beta-, and gammacoronaviruses, including TGEV, PEDV, BCoV, human coronavirus (HCoV) OC43, and IBV (Kunkel & Herrler, 1993; Park *et al.*, 2010; Schultze *et al.*, 1992; Schultze *et al.*, 1991; Schultze *et al.*, 1996). In contrast to the alphacoronavirus TGEV, sialic acid binding is essential for initiating infection of host cells for members of both genera *Beta-* (BCoV and HCoV OC43) and *Gammacoronavirus* (IBV) (Schultze & Herrler, 1992; Schultze *et al.*, 1996; Winter *et al.*, 2006). For TGEV Purdue, it has been shown that NA treatment of the cells enhances APN-binding, but this seems to have no enhancing effect on the viral infectivity *in vitro* (Schwegmann-Wessels *et al.*, 2002; Shahwan *et al.*, 2013). In addition, when absorption time is reduced, desialylation of cells even reduces infectivity, clearly showing the role of sialic acid binding under unfavourable conditions as encountered in the intestinal tract (Schwegmann-Wessels *et al.*, 2011). In the present study, it was demonstrated that removal of sialic acids from cells greatly enhanced FECV infections in the enterocyte cultures, even after only 5 minutes absorption time. This shows that terminal sialic acid residues are not receptor determinants and that FECV more efficiently interacts with its (still unknown) receptor after desialylation of the cells. These results are valuable for the future propagation, titration, and study of FECVs in cell cultures.

Two different serotype I FECV strains were used in this study. It was noticed that UG-FH8 behaved more virulent compared to UCD in the enterocyte cultures. In comparison with UCD, UG-FH8 seemed to replicate and/or spread much faster, resulting in up to 31 times

more infected cells 12 h p.i., although inoculated at comparable m.o.i. The impact of this virulence during *in vivo* infections remains to be investigated. However, since FIPVs arise by mutations during FECV infections, strains as UG-FH8 are probably more prone to the introduction of pathotype-switching mutations.

In contrast to betacoronaviruses, both alpha- and gammacoronaviruses lack a receptor-destroying enzyme that keeps the sialic acid binding site free from competitive inhibitors to ensure efficient interaction with cell surface sialic acids. For these viruses, including TGEV, PEDV and IBV, it has been described that their sialic acid binding capacity becomes more pronounced when virions are pretreated with NA (Park *et al.*, 2010; Schultze *et al.*, 1992; Schultze *et al.*, 1996). This masking effect has also been described before for mammalian siglecs such as sialoadhesin (Delputte & Nauwynck, 2004) and CD22 (Razi & Varki, 1998) and was also demonstrated for FECV in the present study. The effect of unmasking the viral sialic acid binding activity on viral infectivity has only been studied for TGEV. Whereas NA treatment of the virus enhanced sialic acid-mediated attachment to cells (Schwegmann-Wessels *et al.*, 2002), it had no effect on the infectivity of the viruses (Schultze *et al.*, 1996). In contrast to TGEV, removal of sialic acids from FECV virions had an enhancing effect on the viral infectivity *in vitro*. By using sialyllactoses as competitive inhibitors, it was shown that this was due to an enhanced sialic acid binding (2,6-α-linked > 2,3-α-linked). By performing competitive inhibition experiments with the highly α2,6-sialylated macromolecule BSM (Tsuji & Osawa, 1986), which in contrast to sialyllactose allows multivalent binding, replication of both untreated and NA-treated viruses was almost completely inhibited at a concentration of 1 mg ml^{-1}. This inhibition was not seen with another mucin, PGM, which especially contains neutral and sulphated oligosaccharides (Nordman *et al.*, 1997). These results indicate that both untreated and NA-treated viruses use sialylated compounds as attachment factors in the enterocyte cultures. However, when sialic acid binding is reduced/avoided by desialylation of cells, the viral receptor can be more efficiently reached, resulting in an enhanced infectivity of both untreated and NA-treated viruses. In addition, viruses become less susceptible to competitive inhibition with sialylated compounds in desialylated cells (sialyllactoses had no effect, and the effect of BSM was less pronounced compared to untreated cells), showing that sialic acid binding is not a prerequisite for the initiation of FECV infections *in vitro*. This can explain why NA treatment of the virus is detrimental in desialylated cells, since its enhanced sialic acid binding activity potentially delays the virus in its receptor engagement by binding to remaining sialic acid residues. This

decoy activity of sialic acids *in vitro* can be explained by the fact that FECV has no receptor-destroying activity mediating its detachment from non-receptor glycoproteins. Although the lack of a receptor-destroying enzyme seems to disadvantage the virus, allowing competitive inhibitors to cover the virus is possibly a major strategy of these viruses to enable intestinal infections. In contrast to non-enveloped viruses, which represent the majority of all enteritis-inducing viruses, coronaviruses are more prone to inactivation by different unfavourable conditions and it is still unclear how the enteritis-inducing coronaviruses survive the harsh conditions (low pH, enzymes and bile salts) in the gastrointestinal tract. Since sialic acids can confer protection against enzymatic degradation (Schauer, 2000), additional covering with sialylated compounds such as mucins can help the virus to survive the unfavourable conditions in the upper part of the gastrointestinal tract (Schultze *et al.*, 1996).

Based on the results of the present study, a hypothetical model for the initiation of FECV infections *in vivo* can be proposed. In addition to the abundant intrinsic glycosylation (Siddell *et al.*, 1983), FECV most probably becomes covered with sialylated compounds encountered during exit from infected host cells and/or during uptake in the oral cavity. This protects the virus and potentially masks the viral sialic acid binding site, allowing the virus to pass the stomach without degradation or distraction by attachment to gastric mucins. However, during this passage viruses are faced with acidic environments and host/bacterial sialidases, which mediate hydrolysis of sialic acids, resulting in the release of sialic acids from the viral surface. This induces the liberation of the virus' sialic acid binding domain, allowing FECV to escape from the intestinal flow by attaching to the mucus and to engage with its functional receptor on the enterocyte membrane. Since FECV lacks its own receptor-destroying enzyme, passage through this mucus layer and efficient receptor engagement most likely depend on intestinal sialidases.

For TGEV, it has been shown that its enterotropism is highly dependent on its sialic acid binding capacity (Krempl *et al.*, 1997). The sialic acid binding activity of FIPV, and the role of sialic acid binding in the enterotropism of FCoVs remain to be investigated. In addition, it remains elusive if this sialic acid binding is also involved in further steps of the pathogenesis and the onset of FIP. In the present study, it was shown that sialylated compounds can act as inhibitors for at least FECV infections. This decoy activity has also been suggested before by Paltrinieri and co-workers, who proposed that the cat's own sialylated acute phase protein AGP can confer protection against the development of FIP (Paltrinieri *et al.*, 2008). Whereas cats with FIP tend to have elevated, but hyposialylated serum AGP concentrations (Ceciliani

et al., 2004), healthy cats in the same environment have hypersialylated AGP (Paltrinieri *et al.*, 2008). This hyposialylation of AGP in FIP cats included both 2,6-α-linked and 2,3-α-linked sialic acids (Ceciliani *et al.*, 2004). However, in contrast to the more pronounced decoy activity of 2,6-α-linked sialic acids observed in the present study, 2,3-α-linked sialic acids on AGP seemed to be more involved in the determination of the outcome of FCoV infections (Ceciliani *et al.*, 2004; Paltrinieri *et al.*, 2008). Since in the present study only FECV was considered, it would be interesting to investigate if changes in the viral sialic acid binding activity occur during the pathotype switch.

In conclusion, this study shows that serotype I FECVs have a sialic acid binding capacity that is partially masked by virus-associated sialic acids. However, binding to terminal sialic acid residues on the enterocyte surface is not a prerequisite for infection, and these sialic acids even seem to hamper efficient receptor engagement during *in vitro* infections. Nevertheless, if the *in vivo* situation is taken into account, the rationale for such a lectin activity is more clear, since it gives the virus advantages in its confrontation with the harsh conditions and mucosal barriers in the intestinal tract. These insights provide new opportunities for antiviral intervention.

Acknowledgements

We thank Ytse Noppe for her excellent technical support. LMBD was supported by a doctoral fellowship of The Research Foundation Flanders (FWO Vlaanderen). IDMR was supported by the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT Vlaanderen).

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Effect of lysosomotropic agents and protease inhibitors on feline enteric coronavirus infections in enterocytes

Summary

Feline enteric coronavirus (FECV) is a worldwide distributed enteropathogen of cats that is important as the parent virus of the fatal feline infectious peritonitis virus. Since there is no information on fusion triggers/proteases required for FECV infections, lysosomotropic agents, serine-, cysteine-, aspartyl-, and metalloprotease inhibitors, and the furin inhibitor decanoyl-RVKR-CMK, were assessed for their effect on serotype I FECV infections in enterocytes. In contrast to feline coronavirus (FCoV) 79-1683 and transmissible gastroenteritis virus (TGEV), which were taken as controls, serotype I FECV did not depend on low pH, cathepsin B and/or furin cleavage for its entry in enterocytes. The serine protease inhibitor AEBSF strongly reduced replication of all FCoVs and TGEV, when it was continuously present before and during replication or added after inoculation. Interestingly, this reduction resulted from the inhibition of a virion-associated serine protease because a similar inhibition was observed when only the virus was treated with AEBSF. As expected, since coronavirus' polyprotein processing occurs by viral cysteine proteases, the cysteine protease inhibitor E64d also inhibited replication, when it was continuously present before and during replication or added after inoculation. Neither AEBSF nor E64d inhibited replication when only the cells were pre-treated. In conclusion, serotype I FECVs do not rely on low pH, cathepsin B or furin cleavage for entry, but the exact fusion trigger remains to be elucidated. In addition, it was shown that FECV and TGEV use a virion-associated serine protease to start the replication.

4.2.1 Introduction

Coronaviruses have been isolated from many animal species and humans and are associated with various diseases, ranging from mild/unnoticed to highly fatal intestinal, respiratory, or systemic infections. Coronaviruses are enveloped viruses that have to release their positive single stranded RNA in the cytoplasm to allow replication. This entry process requires cell attachment and subsequent fusion between the viral envelope and the host plasma- or endosomal membrane. The viral spike (S) protein carries out both steps in the entry process and is an essential determinant of host/tissue tropism and virulence (Belouzard *et al.*, 2012; Rottier *et al.*, 2005). S proteins are class I fusion proteins, possessing common structural features with the fusion proteins of other enveloped viruses such as orthomyxo-, paramyxo-, retro-, filo-, and arenaviruses (Bosch *et al.*, 2003; White *et al.*, 2008). Typically, viral fusion proteins are synthesized as precursor proteins that undergo endoproteolytical cleavage by host proteases, generating a metastable complex of the globular receptor binding (S1 for coronaviruses) and the stalk-like fusion (S2 for coronaviruses) subunit. This brings the protein in a fusion competent state, allowing the rapid dissociation of both subunits and the insertion of the hydrophobic fusion peptide in the host membrane upon receptor binding, low pH exposure and/or, in case of (some) coronaviruses, additional proteolytical cleavage (Belouzard *et al.*, 2012; Dimitrov, 2004; Heald-Sargent & Gallagher, 2012; White *et al.*, 2008). Coronaviruses show great distinctions in their requirement for fusion activating triggers. Some coronaviruses, such as infectious bronchitis virus (IBV), murine hepatitis virus (MHV)-4 and MHV-A59, undergo proteolytical S1/S2 cleavage in virus-producing cells, mediated by furin-like cellular proteases encountered during exocytosis processes. For these viruses, receptor engagement and/or exposure to acid pH had been generally believed to be sufficient to allow genome release in the cell (Chu *et al.*, 2006; Eifart *et al.*, 2007; Gallagher *et al.*, 1991), but it has recently been questioned if these are the only triggers. Indeed, furin cleavage occurs not directly adjacent to the putative fusion peptide, and both MHV-A59 and IBV spikes seem to become additionally cleaved within their S2 subunit during entry (Wicht *et al.*, 2014; Yamada & Liu, 2009). In contrast to IBV, MHV-4 and MHV-A59, most coronaviruses carry uncleaved spikes and indisputably rely on proteolytical activation with non-furin proteases encountered during virus entry to allow infections. MHV-2, severe acute respiratory syndrome (SARS)-CoV, human CoV 229E, and Middle East respiratory syndrome (MERS)-CoV rely on low pH-dependent cathepsins, encountered during endocytosis (Gierer *et al.*, 2013; Huang *et al.*, 2006; Qiu *et al.*, 2006; Simmons *et al.*, 2005). However, it seems that at least some of them have evolved to use multiple cell entry routes, depending on the physiological conditions in the target tissue. Indeed,

SARS-CoV entry becomes completely independent of low pH and cathepsins when exposed to trypsin, thermolysin, or elastase after receptor engagement or by expression of serine proteases such as TMPRSS2 on the cell surface (Belouzard *et al.*, 2010; Bertram *et al.*, 2011; Glowacka *et al.*, 2011; Matsuyama *et al.*, 2005; Shulla *et al.*, 2011). Cleavage with these proteases occurs both at the S1/S2 boundary and within S2, directly N-proximal of the fusion peptide (Belouzard *et al.*, 2009; Belouzard *et al.*, 2012; Matsuyama *et al.*, 2005; Simmons *et al.*, 2004). This mode of entry is about 100 times more efficient than the cathepsin-dependent entry, and can explain the virulence of SARS-CoV in the lung where these transmembrane serine proteases are available (Matsuyama *et al.*, 2005). Consequently, fusion processes seem to largely influence virus tropism and pathogenicity, and can explain why 2 viruses or strains using the same receptor can show such great distinction in cell tropism.

Feline coronaviruses (FCoVs) occur as 2 pathotypes for which the disease-causing potential is determined by their cell tropism. Feline enteric coronavirus (FECV) is an enzootic enteropathogenic virus, replicating in intestinal epithelial cells after oral uptake (Addie & Jarrett, 1992; Herrewegh *et al.*, 1997; Meli *et al.*, 2004; Pedersen *et al.*, 1981). FECV infections mainly manifest subclinically (Hickman *et al.*, 1995; Pedersen *et al.*, 1981; Vogel *et al.*, 2010), but mutations in the viral genome can allow this mutational variant, designated feline infectious peritonitis virus (FIPV), to efficiently replicate in monocytes/macrophages (Dewerchin *et al.*, 2005; Rottier *et al.*, 2005; Stoddart & Scott, 1989), resulting in a fatal and incurable pyogranulomatous phlebitis and serositis. Each pathotype exists as 2 different serotypes (Fiscus & Teramoto, 1987a, b; Hohdatsu *et al.*, 1991). Serotype II viruses are the result of double recombination events between serotype I FCoVs and canine coronaviruses (Herrewegh *et al.*, 1998; Lin *et al.*, 2013). The determinants of the FCoV tropism have fascinated researcher for years, and the S2 fusion subunit of the spike protein is considered of key importance in the FCoV pathotype switch (Chang *et al.*, 2012; Licitra *et al.*, 2013; Rottier *et al.*, 2005). As serotype II viruses readily propagate in cell cultures, entry processes of these viruses have been abundantly studied. Endocytosis has been identified as the main entry route for serotype II FCoVs (Van Hamme *et al.*, 2007). After aminopeptidase N engagement, FIPV 79-1146 undergoes clathrin- and caveolae- independent, but dynamin-dependent endocystosis (Van Hamme *et al.*, 2008). Proteolytical cleavage is mediated by cathepsin B, and is only mildly affected by low pH. In contrast, the avirulent 79-1683 strain depends on both cathepsin B and low pH-activated cathepsin L activity for infection in cell culture. Based on the molecular weight of the cleavage products, it has been suggested that cleavage of serotype II spikes by

cathepsins occurs most probably directly adjacent to the fusion peptide, and not at the S1/S2 boundary (Belouzard *et al.*, 2012; Regan *et al.*, 2008). Whereas serotype II FCoVs have been studied in quite detail, the more prevalent and clinically important serotype I viruses are less well understood, and it remains elusive if results obtained for serotype II viruses can be extrapolated to the real life situation. Therefore, many scientists have redirected their research to serotype I viruses in recent years. Genome analysis have revealed 2 regions in the spike protein that are very often affected by mutations when comparing faecal with tissue strains, one which is located at the S1/S2 boundary comprising the furin cleavage site, and the other which is located in the S2 subunit (Chang *et al.*, 2012; Licitra *et al.*, 2013). Although serotype I viruses have become genetically well characterized, the biology of these viruses is still largely unclarified. Therefore, the aim of this study was to contribute to the complex puzzle of FCoV biology by providing information on fusion triggers and proteases necessary for serotype I FECV infections in enterocytes. The effect of lysosomotropic agents and various protease inhibitors on FECV infection was evaluated for two serotype I FECV strains (UCD and UG-FH8) in feline enterocyte cultures. Since during replication viral proteases are involved in viral polyprotein processing, and these viral proteases can be attractive targets for the development of therapeutics, protease inhibitors were tested for both their effect on cellular and viral proteases. Since entry processes for FCoV 79-1683 and TGEV Purdue have been (partially) characterized, and both viruses can infect feline enterocytes, these strains were included as controls.

4.2.2 Materials and methods

4.2.2.1 Cells and viruses

Feline colonocyte cultures were grown in Dulbecco's modified Eagle's medium (DMEM)/Ham's F12 Nutrient Mixture (1/1) supplemented with 100 U penicillin ml^{-1} (Continental Pharma Inc.), 0.1 mg streptomycin ml^{-1} (Certa), 0.1 mg gentamycin ml^{-1} (Gibco BRL), 5 % FBS (Gibco BRL) and 1 % non-essential amino acids 100x (Gibco BRL). These cells were used to provide a third passage of two serotype I FECV strains, UCD and UG-FH8. The origin of the viruses and cells has previously been described (Desmarets *et al.*, 2013). The FCoV strain 79-1683, purchased from the American Type Culture Collection (ATCC), was grown in Crandell feline kidney (CrFK) cells and a third passage was used. Swine testicular cells were used to obtain a third passage of TGEV Purdue.

4.2.2.2 Infection inhibition assay using lysosomotropic agents

The weak bases ammonium chloride (NH_4Cl) and chloroquine diphosphate, and the ionophore monensin were purchased from Sigma. NH_4Cl was used at a range of 0-30 mM, chloroquine

diphosphate at 0-70 μM, and monensin at 0-20 μM. Monolayers were treated for 1 h with neuraminidase (NA) from *Vibrio Cholerae* (Roche Diagnostics) in Ca^{2+}- and Mg^{2+}-enriched PBS to improve virus infection (Desmarets *et al.*, 2014). Then, cells were pre-incubated for 2 h with FBS-depleted medium (= control) or different concentrations of each pH drop inhibitor diluted in FBS-depleted medium, after which they were inoculated with FECV UCD ($10^{5.8}$ $TCID_{50}$ ml^{-1}), UG-FH8 ($10^{5.8}$ $TCID_{50}$ ml^{-1}), FCoV 79-1683 ($10^{6.8}$ $TCID_{50}$ ml^{-1}), or TGEV Purdue ($10^{5.8}$ $TCID_{50}$ ml^{-1}), in presence of the inhibitor. Subsequently, cells were washed 3 times and further incubated with lysosomotropic agents. Twelve hours p.i., cells were fixed with 4 % paraformaldehyde for 10 min at room temperature (RT) and stained for infection (see below). Viability of the cells was assessed by ethidium monoazide bromide (EMA) staining, ensuring > 99 % viability at the highest concentration of each inhibitor.

4.2.2.3 Infection inhibition assay using serine-, cysteine-, aspartyl-, and metalloprotease inhibitors

All protease inhibitors were purchased from Sigma, except for the cathepsin B inhibitor CA-074Me, which was purchased from Calbiochem. The serine protease inhibitor 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) was tested in a range of 0-100 μM, the aspartyl protease inhibitor pepstatin A between 0 and 0.75 μM, the metalloprotease inhibitor phosphoramidon between 0 and 15 μM, the membrane permeable cysteine protease inhibitor (2S,3S)-trans epoxysuccinyl-L-leucylamido-3-methylbutane ethyl ester (E-64d) between 0 and 8 μM, the serine/cysteine protease inhibitor leupeptin at a range of 0-100 μM, and the cathepsin B inhibitor CA-074Me between 0 and 10 μM. Viability of the cells was assessed by ethidium monoazide bromide (EMA) staining, ensuring > 99 % viability at the highest concentration of the inhibitor. All inhibitors were tested for their inhibiting effect on FECV replication as described for the lysosomotropic agents, namely by pre-incubation for 2 h, followed by continuous incubation during and after inoculation. Inhibitors with effect on viral replication were additionally tested for their potential to inhibit replication by 1) only pre-incubating the cells (2h, 37°C), 2) by treating the inoculum (see below), or 3) by addition of the inhibitor after inoculation (see below). Twelve hours p.i., cells were fixed with 4 % paraformaldehyde for 10 min at room temperature (RT) and stained for infection (see below).

4.2.2.4 Immunofluorescence staining of infected enterocytes

Cells were permeabilized with 0.1% Triton X-100 for 2 min at RT. Then, cells were incubated for 1 h at 37 °C with the monoclonal anti-nucleocapsid antibody 10A12 (produced and characterized in the laboratory) containing 10% normal goat serum, followed by incubation with

goat anti-mouse FITC labelled antibodies (Molecular Probes) for 1 h at 37 °C. Nuclei were stained with Hoechst 33342 (Molecular Probes) for 10 min at RT. Slides were mounted using glycerine-PBS solution (0.9:0.1, vol:vol) with 2.5 % 1,4-diazabicyclo[2.2.2]octane (Janssen Chimica) and analysed by fluorescence microscopy (Leica Microsystems GmbH).

4.2.2.5 Determination of the target protease of AEBSF and E64d

To assess the target protease of AEBSF and E64d, 2 additional experiments were performed. First, virus inoculum, containing FECV UCD or UG-FH8, was treated for 2 h with 0 μM (control) or 100 μM AEBSF / 8 μM E64d at 37°C. Then, these virus-AEBSF/E64d mixtures were used to inoculate NA-treated cells for 5 min, after which inoculum was removed by three washings, and cells were further incubated in medium. Nine hours p.i., cells were fixed and stained as described above. In a second experiment, the time point at which the protease was involved in the replication cycle was assessed. Therefore, NA-treated cells were inoculated with FECV UCD or UG-FH8 at $10^{5.8}$ $TCID_{50}$ ml^{-1} for 5 min, after which inoculum was removed by three washings, and medium was added. At different time points p.i. (5, 30, 60, 90, 120, and 180 min), medium was replaced by medium containing 0 μM (control) or 100 μM AEBSF / 8 μM E64d. Cells were fixed at 9 h p.i. to determine the percentage of infected cells as described above.

4.2.2.6 Assessment of the requirement of furin cleavage for FECV infectivity

The furin inhibitor decanoyl-RVKR-CMK was purchased from Calbiochem. To investigate the effect of furin cleavage during entry, cells were treated with 10 μM 2 h before and during inoculation (1 h). Then, the inoculum was removed by 3 washings and medium without inhibitor was added. Twelve hours p.i., cells were fixed. To study the effect of furin cleavage inhibition on the infectivity of progeny virus, cells were treated for 8 h after inoculation with 10 μM decanoyl-RVKR-CMK, after which both intra- and extracellular virus was collected and infectivity of the virus was assessed by determination of 50% tissue culture infective dose ($TCID_{50}$).

4.2.2.7 Infectivity titration

Monolayers of colonocytes, seeded in collagen I coated 96-well plates, were inoculated with 50 μl of serially diluted (1/10) virus suspensions. After 1 h (37 °C, 5% CO_2), medium was added and the cells were further incubated for 72 h. Then, plates were washed with PBS, air-dried (1 h 37°C) and frozen (-20°C). The 50% tissue culture infective dose was determined by means of immunoperoxidase monolayer assay (IPMA). Therefore, cells were fixed and permeabilized by incubation with PF 4% (10 min, RT), followed by incubation with methanol containing 1%

H_2O_2 (5 min, RT). Then, cells were incubated with PBS containing 10% normal goat serum and 0.1% Tween 80 for 30 minutes at 37°C. Subsequently, cells were incubated with monoclonal antibodies against the N-protein, followed by goat anti-mouse HRP-labelled antibodies. Infected cells were visualized by adding sodium-acetate buffer containing amino-ethylcarbazole (AEC) and H_2O_2 for 10 minutes at RT. The fifty percent end-point was calculated according to the method of Reed and Muench (Reed & Muench, 1938).

4.2.2.8 Statistics

Experiments were independently repeated 3 times, and results were compared with the Mann-Whitney U test. Statistical analysis was performed using GraphPad Prism version 5.0c (GraphPad software, San Diego, CA, USA).

4.2.3 Results

4.2.3.1 Serotype I FECV does not depend on acidic pH for its entry

Figure 4.2.1 shows the effect of the endosomal/lysosomal acidification inhibitors ammonium chloride (NH_4Cl), monensin, and chloroquine on FECV infection. FECV UCD and UG-FH8 were not affected by NH_4Cl or chloroquine, and marginally (but not significantly) affected by monensin. In contrast, FCoV 79-1683 and TGEV, known to depend on acid pH (Hansen *et al.*, 1998; Regan *et al.*, 2008), were clearly affected by all of these pH drop inhibitors. These results imply that serotype I FECVs enter enterocytes via an acid-independent process.

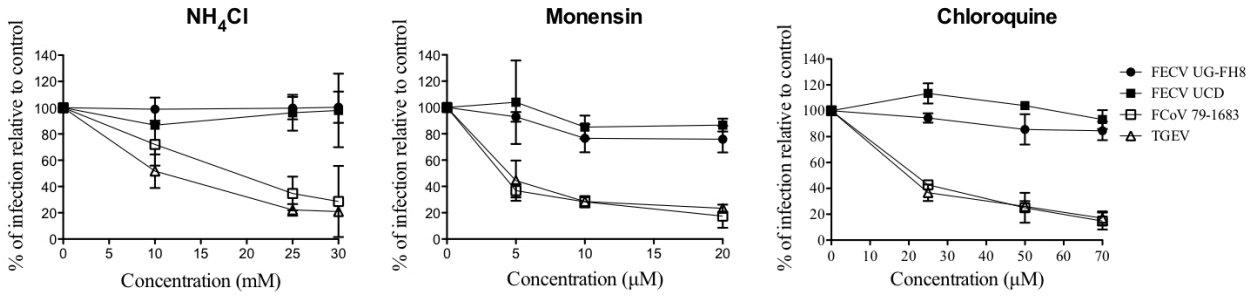

Figure 4.2.1. Effect of lysosomotropic agents on the infection of FECV UCD, FECV UG-FH8, FCoV 79-1683 and TGEV in feline enterocytes. Cells were incubated 2 h before, during and after inoculation with different concentrations of NH_4Cl, monensin or chloroquine diphosphate. Cells were fixed 12 h p.i., and the percentage of infected cells was determined relative to the control.

4.2.3.2 Effect of continuous exposure of cells to various protease inhibitors on the replication of serotype I FECVs, serotype II FCoV 79-1683 and TGEV in enterocytes

Several protease inhibitors were tested for the potential to inhibit serotype I FECV strains UCD and UG-FH8, FCoV 79-1683, and TGEV replication. For all inhibitors, the effect on infectivity was first evaluated by continuous incubation of the inhibitor in the medium before and during

replication, i.e. until 12 h p.i. As shown in Figure 4.2.2, the serine protease inhibitor AEBSF was a very potent inhibitor of all 4 viruses, with almost complete inhibition of infection at a concentration of 100 μM. The cell-permeable cysteine protease inhibitor E64d affected all feline coronaviruses, but not TGEV, which is in consistence with a previous report (Kim *et al.*, 2013). Since serotype II feline coronaviruses are known to depend on cathepsin B, CA-074Me was tested for its inhibiting effect on the serotype I viruses. Serotype II FCoV 79-1683 was affected by cathepsin B inhibitor CA-074Me, but this effect was not as obvious as previously reported in other cell cultures (Regan *et al.*, 2008), as only 71.5 ± 10.2 % infection relative to the control ($p = 0.0636$) was seen in the enterocyte cultures. In contrast to serotype II FCoV 79-1683, no effect was seen with cathepsin B inhibitor CA-074Me on infection of serotype I FECVs and TGEV. None of the viruses was inhibited by leupeptin, pepstatin A, and phosphoramidon. Also bestatin had no effect on the replication in concentrations up to 60 μM (data not shown).

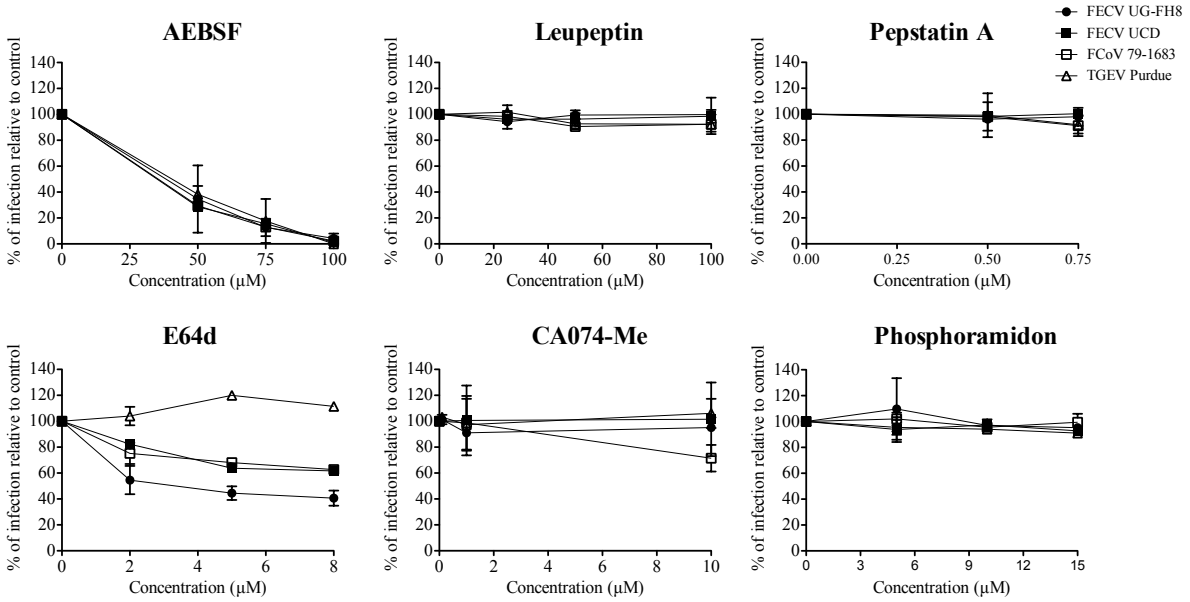

Figure 4.2.2. Effect of various protease inhibitors on the replication of FECV UCD, FECV UG-FH8, FCoV 79-1683, and TGEV. Cells were incubated 2 h before, during and after inoculation with different concentrations of protease inhibitors. Cells were fixed 12 h p.i., and the percentage of infected cells was determined relative to the control.

As inhibitors were continuously present during replication of the virus, these results cannot give a clue on the target protease (host and/or viral) and the stage of the replication cycle that was inhibited by AEBSF and E64d. To assess if cellular serine and/or cysteine proteases were involved in the entry, it was investigated whether only pre-treatment of cells with the inhibitors would similarly decrease the virus replication (Figure 4.2.3). When only pre-incubating with AEBSF, no inhibition was seen for all viruses, whereas E64d affected FCoV 79-1683, but not

serotype I FECVs or TGEV. Also a combination of AEBSF and E64d did not affect serotype I FECV replication. As both AEBSF and E64d are irreversible blockers, these data suggest an effect of both inhibitors on viral rather than on cellular proteases, except for serotype II FCoV 79-1683, which used cellular cysteine proteases.

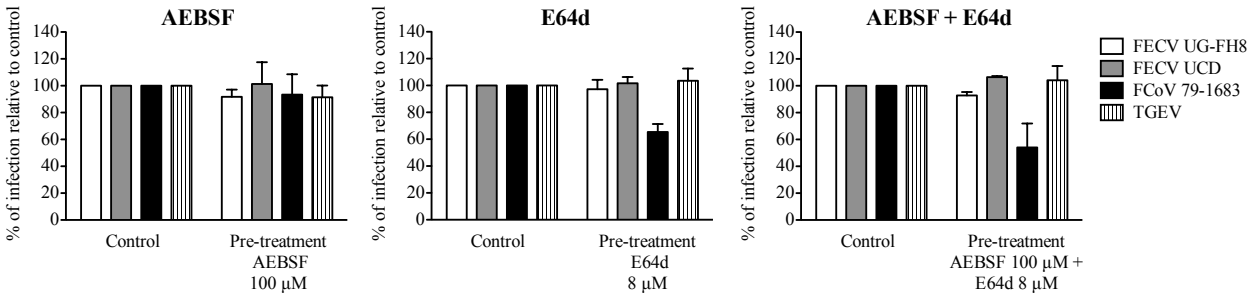

Figure 4.2.3. Effect of pre-treatment of cells with protease inhibitors AEBSF and/or E64d on the replication of FECV UCD, FECV UG-FH8, FCoV 79-1683, and TGEV. Cells were incubated 2 h before inoculation (pre-treatment) with 100 µM AEBSF, 8 µM E64d, or a combination of AEBSF and E64d. Cells were fixed 12 h p.i., and the percentage of infected cells was determined relative to the control.

Taken together, these experiments showed that inhibition of cellular proteases by AEBSF or E64d was not able to reduce serotype I FECV infection, but both reduced viral replication, most probably by inhibiting viral protease activity. In addition, it was demonstrated that AEBSF had a potent antiviral activity against both feline coronaviruses and TGEV.

4.2.3.3 AEBSF inhibits a virion-associated serine protease that works between 60 and 120 min p.i., and E64d targets non-virion associated cysteine protease(s) that also work(s) before 120 min p.i.

Coronaviruses are known to encode for cysteine proteases involved in polyprotein processing, but no coronaviral serine protease activity has been described. To further identify the target protease, AEBSF and E64d were used to either treat the inoculum, or were added at different time points post inoculation to identify which step in the replication cycle was blocked. Figure 4.2.4 shows the results of AEBSF or E64d treatment of the inoculum. Since inhibitors could not be separated from the virus after pre-treatment of the inoculum, it was decided to inoculate the virus-inhibitor mixtures for only 5 min, in order to be sure that AEBSF could not block anything but the virus, and a control was taken by adding this AEBSF-virus mixture for 5 min without any pre-treatment of the virus. Interestingly, if only the inoculum was treated with AEBSF, the same inhibition was seen as when AEBSF was continuously present during the experiment, indicating that the viral protease that is blocked by AEBSF is incorporated in the virion. For E64d, pre-treatment of the inoculum did not inhibit the FECV replication.

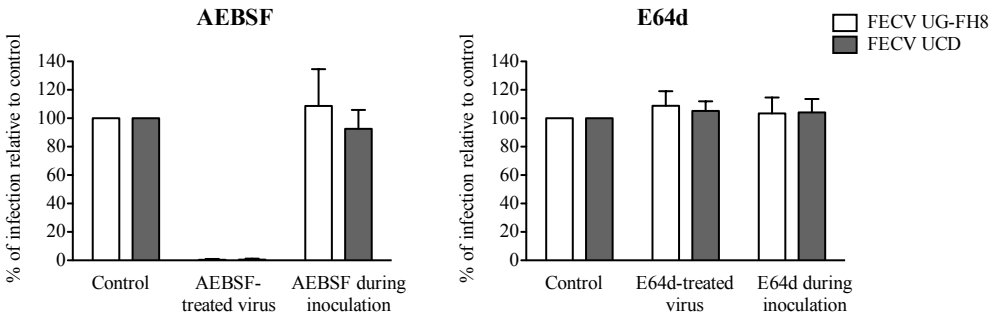

Figure 4.2.4. AEBSF, but not E64d treatment of viruses reduces their replication capacity. FECV UG-FH8 and UCD were treated for 2 h with 100 μM AEBSF or 8 μM E64d or medium (control). Then, virus-inhibitor mixtures were inoculated for 5 min, after which the inoculum was removed by three washings. An extra control consisting of the addition of AEBSF or E64d during 5 min inoculation without pre-treatment was also included. Cells were further incubated in medium and percentage of infected cells was assessed 9 h p.i.

Subsequently, a kinetic study was performed to assess at which time points p.i. this viral serine protease fulfilled its function, and the same was done for E64d to get an idea about the potential role of the protease. Therefore, cells were inoculated for 5 min with the virus, after which the inoculum was removed by three washings and medium was added. At different time point p.i. (5, 30, 60, 90, 120, and 180 min), medium was changed to medium containing 0 μM (control) or 100 μM AEBSF / 8 μM E64d, and the percentage of infected cells was determined at 9 h p.i. Figure 4.2.5 shows that until 1 h p.i., the replication remained blocked by AEBSF addition, indicating that the protease had not yet completed its function by 1 h p.i. From 90 min p.i., viral replication became less affected by the addition of AEBSF, indicating that the viral serine protease fulfilled its function between 60 and 90-120 min after addition of the virus to the cells. E64d had a slight inhibitory effect on the FECV replication when added to the medium at all time points before 120 min p.i., although there was slightly less inhibition when added after 60 or 90 min p.i. compared to 5 or 30 min p.i.

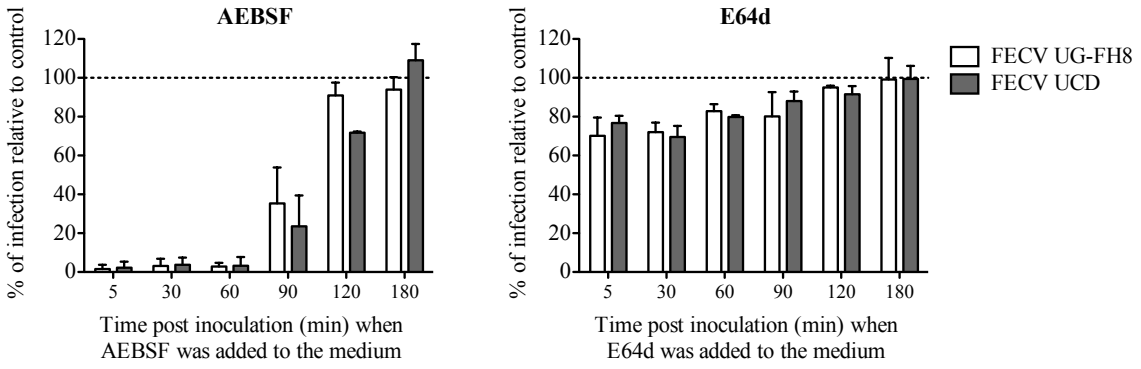

Figure 4.2.5. Effect of addition of AEBSF or E64d at several time points p.i. Cells were inoculated with FECV UG-FH8 or UCD for 5 min, after which inoculum was removed by three washings and medium was added. At different time points post inoculation (5, 30, 60, 90, 120, and 180 min), medium was replaced by medium containing no (control) or 100 μM AEBSF/8 μM E64d and cells were fixed 9 h p.i. Graphs represent the percentage of infected cells relative to the control (dashed line) for each of the assessed time points.

4.2.3.4 Effect of furin inhibitor decanoyl-RVKR-CMK on the entry and infectivity of serotype I FECVs

Serotype I FECVs are known to have a highly conserved furin cleavage site at the S1/S2 boundary (de Haan *et al.*, 2008; Licitra *et al.*, 2013). Therefore, it was investigated if furin cleavage of the FECV spike would be a determining factor in its infectivity for enterocytes. As furin cleavage can occur either during entry or during exit, the effect of both incubation with furin inhibitor before/during inoculation (pre-treatment) and after inoculation (post-treatment) was studied. Figure 4.2.6 shows the relative percentage of infected cells 12 h p.i. after treatment of cells 2 h before and during inoculation (1 h) with 10 μM decanoyl-RVKR-CMK. Neither of the two serotype I FECVs was affected by this treatment, whereas infectivity of serotype II strain 79-1683 and TGEV was reduced to 73.7 ± 3.2 % and 68.4 ± 8.4 %, respectively ($p = 0.0636$). As furin cleavage inhibition had no effect on the entry of serotype I FECVs, the effect of furin cleavage inhibition during production of new progeny virus was investigated. Figure 4.2.7 shows the results of infectivity titration 9 h p.i. after treatment of cells with 10 μM furin inhibitor decanoyl-RVKR-CMK for 8 h p.i. Neither of the two serotype I viruses showed decreased infectivity when assessing both intra- and extracellular titre, and there was also no effect when 25 μM of inhibitor was used or when the furin inhibitor was refreshed at 4 h p.i. (data not shown). These results indicate that furin cleavage is not a prerequisite for FECV infectivity in enterocytes. In addition, viruses released from post-treated cells, which are supposed to have uncleaved spikes, were not more susceptible to chloroquine or furin pre-treatment compared to the control, indicating that these viruses did not started to use furin encountered during entry nor another entry pathway, which could have explained the lack of effect on infectivity (data not shown).

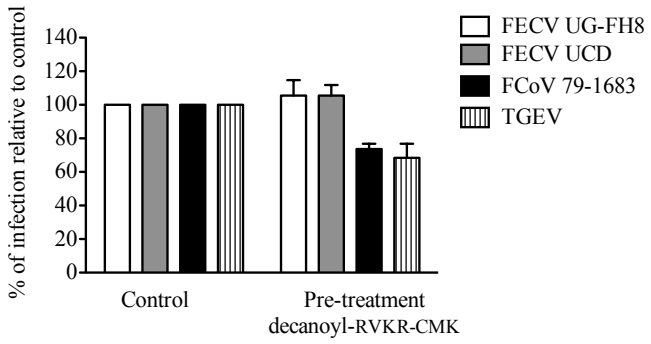

Figure 4.2.6. Effect of pre-treatment of cells with furin inhibitor decanoyl-RVKR-CMK. Cells were treated with 10 μM furin inhibitor decanoyl-RVKR-CMK 2 h before and during inoculation and the percentage of infected cells was assessed relative to control 12 h p.i.

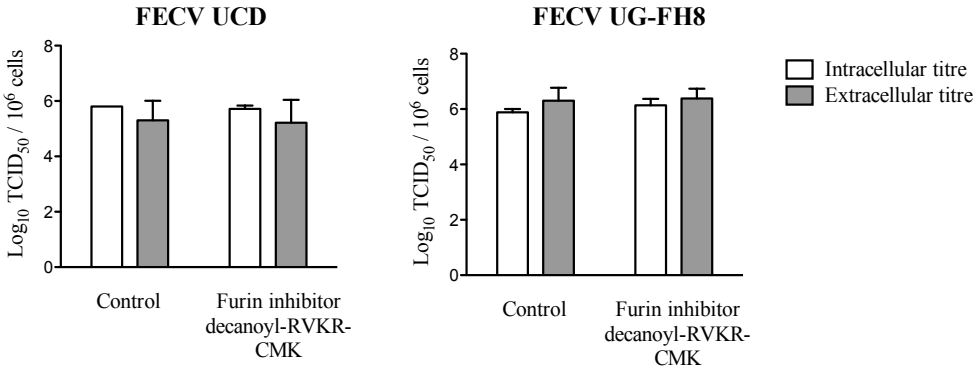

Figure 4.2.7. Effect of furin inhibitor decanoyl-RVKR-CMK on the infectivity of progeny FECV. Cells were inoculated with FECV UCD and FECV UG-FH8 (m.o.i. 0.2). After 1 h, the inoculum was removed by 3 washings and cells were further incubated with 10 μM decanoyl-RVKR-CMK for 8 h, after which both intra- and extracellular infectious titres were assessed.

4.2.4 Discussion

As all enveloped viruses, coronaviruses require fusion processes to deliver their genome into the cytosol. This fusion process is mediated by the spike, a class I fusion protein which protrudes from the viral surface as a homotrimer complex (Bosch *et al.*, 2003). Viral class I fusion proteins are typically synthesized as inactive precursor proteins and require proteolytical activation to acquire their fusion competent state. In addition, other triggers such as low pH and/or receptor binding are needed to finally allow the fusion of the viral envelope and the host membrane (Dimitrov, 2004; White *et al.*, 2008). Depending on the required fusion triggers, genome release occurs at the plasma- or at the endosomal membrane. Fusion triggers and entry pathways differ greatly among coronaviruses, and depend on the virus, strain, or even the cell type a certain virus/strain is faced with (Belouzard *et al.*, 2012; Heald-Sargent & Gallagher, 2012). In addition to the receptor distribution, these differences are of key importance in the determination of virus tropism and pathogenicity. Cell tropism switch is a crucial event in FIP pathogenesis, but so far nothing is known about fusion triggers required for the abundantly present and clinically relevant serotype I FCoVs. In the present study, fusion triggers required for serotype I FECV infection in enterocytes were investigated by using various endosomal acidification- and protease inhibitors. Serotype II FCoV 79-1683 and TGEV Purdue were taken along the experiments as control for the activity of the products, since these viruses can infect feline enterocytes and their fusion triggers have been (partially) characterized.

In contrast to TGEV and FCoV 79-1683 for which we and others (Hansen *et al.*, 1998; Regan *et al.*, 2008) showed that they depend on endosomal acidification, serotype I FECV did not require a low pH step to initiate infection in enterocytes. This indicates that serotype I FECV most probably enters cells via fusion at the plasma- or early endosomal membrane. To elucidate

whether FECV fuses at the plasma- or endosomal membrane, further research using chemical inhibitors of internalization pathways, dominant-negative proteins involved in the internalization and/or co-localization studies need to be performed, as previously described for FIPV 79-1146, amongst others (Van Hamme *et al.*, 2008). Acid-independent entry has been described for MHV-4 and FIPV 79-1146 (Gallagher *et al.*, 1991; Regan *et al.*, 2008). In contrast to FIPV 79-1146 (and other serotype II FCoVs) (Regan *et al.*, 2008), serotype I FECV entry did not depend on the (acid-independent) cathepsin B, or on any other host cysteine protease that could be inhibited by the broad-spectrum cysteine protease inhibitor E64d. However, a reduction of viral replication was seen when E64d was present in the medium until 12 h p.i. The same inhibition pattern (i.e. no effect when cells were only pre-treated, but substantial reduction when the inhibitor was present in the medium during the entire course of the infection) was noticed with the broad-spectrum serine protease inhibitor AEBSF, which, in contrast to E64d, not only affected FCoVs (serotype I and II), but also potently inhibited TGEV infection. Both E64d and AEBSF are irreversible blockers, and hence should block all host proteases when only pre-treating cells before inoculation. Consequently, the inhibitory effect seen when leaving both compounds in the medium during replication was most probably due to the inactivation of viral proteases. Indeed, coronaviruses undergo autoproteolytical processing of 2 precursor proteins, polyprotein (pp) 1a and 1ab, which are directly synthesized from the genome upon genome release. This proteolytical cleavage results in the formation of 16 mature proteins and is mediated by at least 2 or 3 viral protease encoded by nsp3 and nsp5. However, despite the chymotrypsin-like structure of nsp5-encoded proteases, all viral proteases known to be involved in polyprotein processing are cysteine proteases, as the papain-like proteases encoded by nsp3 employ the catalytic cysteine-histidine-aspartic acid triad, and the main proteases encoded by nsp5 the cysteine-histidine catalytic dyad (Hegyi *et al.*, 2002; Lu *et al.*, 1995; Wojdyla *et al.*, 2010; Ziebuhr *et al.*, 2000). Consequently, this viral protease-mediated genome processing could explain the reduction seen with E64d, which is in consistence with previous reports (Kim *et al.*, 1995; Kim *et al.*, 2013), but not the strong reduction induced by AEBSF for all tested viruses. Therefore, it was further determined which stage of the replication cycle was targeted by AEBSF. Interestingly, similar reduction in infection was noticed when only the inoculum was treated with AEBSF or when AEBSF was added to the medium after inoculation at 5, 30, or 60 min p.i. From 90 min p.i., virus replication was less affected by the addition of AEBSF, indicating that the proteolysis had already occurred in a substantial amount of cells by then. So far, inhibition of coronavirus replication with AEBSF has only been reported for IBV and PEDV, but for both viruses, this effect was attributed to inhibition of host serine proteases

during entry (Park *et al.*, 2014; Yamada & Liu, 2009). However, in both studies, AEBSF was present in the inoculum for 1 (PEDV) or 2 hours (IBV), and hence inhibition of a viral protease cannot be excluded in these studies. Results obtained in the present study give an indication that beside cysteine protease activity encoded by the pp1a, coronaviruses also encode a serine protease, which, in contrast to the cysteine proteases, resides in the virion. However, it cannot be excluded that this protease activity results from the incorporation of a cellular serine protease in the virion. Based on the kinetic study that was performed with AEBSF and E64d, this serine protease is most likely involved in the initiation of the replication during a post-entry step, but the identity and function remain to be elucidated. Nonetheless, the fact that all these coronaviruses could be blocked by AEBSF makes this information highly valuable for future coronavirus research and multi-coronavirus drug development.

At least two distinct cleavage sites (CS) for proteolytical activation of coronavirus S proteins have been described. CS1 is located at the S1/S2 boundary, and CS2 is found within S2, adjacent to the putative fusion peptide (Belouzard *et al.*, 2009; Belouzard *et al.*, 2010; Belouzard *et al.*, 2012; Heald-Sargent & Gallagher, 2012; Yamada & Liu, 2009). Some coronaviruses, such as IBV, MHV-A59, MHV-4, and serotype I FECV are believed to carry a pre-cleaved spike by cleavage at CS1 mediated by furin, encountered during the exocytosis process (de Haan *et al.*, 2008). Furin is a Ca^{2+}-dependent serine protease and is one of the mammalian proprotein convertases (PCs). Furin is ubiquitously found in endocytic and exocytic pathways by its circulation from the trans-Golgi network to the plasma membrane, and back via the endocytic pathway (Seidah & Prat, 2012). Typically, furin cleavage preferably occurs after the C-terminal arginine residue in the consensus motif RXR(K)R/, where X represents any amino acid, but cleavage after the motif RXXR/ has also been described (Molloy *et al.*, 1992). This multi-basic motif is found at CS1 in some coronaviruses, including serotype I FECVs, but seems to be absent in most other coronaviruses, and its exact role in *in vivo* coronavirus infections is still not completely clarified. CS1 cleavage by furin is not a determinant of infectivity in cell culture (de Haan *et al.*, 2004; Gombold *et al.*, 1993; Yamada & Liu, 2009), but it does promote cell-cell fusion, and, at least for SARS-CoV, also promotes CS2 cleavage (Belouzard *et al.*, 2009). In addition, cleavage at the S1/S2 boundary occurs too far from the fusion peptide and therefore can potentially not liberate the internal fusion peptide. Consequently, cleavage at CS2, and not at CS1, is considered as the key fusion-determining factor of coronaviruses. The need for CS2 and not CS1 cleavage for virus entry has been clearly demonstrated for IBV Beaudette strain. Indeed, S proteins of IBV Beaudette (and related

strains) have 2 furin cleavage sites, one at amino acid position 531-538 (CS1) and the other at amino acid position 684-692 (CS2). By mutational analysis and the use of the broad spectrum PC inhibitor decanoyl-RVKR-CMK, Yamada and Liu showed that whereas the first cleavage site can promote fusion, only CS2 cleavage is the key determinant for induction of both virus-cell and cell-cell fusion. At that position, FCoV 79-1683, TGEV, CCoV, and bat HKU5-1 also have a furin cleavable motif (RKYR), whereas other coronaviruses have a highly conserved trypsin cleavable site (Yamada & Liu, 2009). In the present study, the requirement for furin-like enzymes has only been studied for virus-cell fusion processes, and not for cell-cell fusion, as the enterocyte cultures are not prone to syncytium formation with any of the viruses used in this study. It was shown that both FCoV 79-1683 and TGEV rely on PC (potentially furin) cleavage during their entry in enterocytes, most probably by cleavage at the CS2 site as suggested by Yamada and Liu (2009), although this was not further confirmed in the present study. In contrast, serotype I FECVs were not at all affected by pre-treatment of cells with decanoyl-RVKR-CMK, indicating that PCs are not involved during entry. According to the ProP server, no PC cleavage site was present at CS2 of FECV UCD (http://www.cbs.dtu.dk/services/ProP/) (Duckert *et al.*, 2004). In addition, viruses released from decanoyl-RVKR-CMK treated cells were not less infectious than those released from non-treated cells, indicating that furin cleavage at the CS1 site is not a prerequisite for FECV infectivity. This is in consistence with all previous reports on other coronaviruses with a furin cleavable CS1 site (de Haan *et al.*, 2004; Gombold *et al.*, 1993; Yamada & Liu, 2009), but should be further confirmed by mutational analysis of this motif in serotype I FECVs. In addtion, none of the other tested protease inhibitors, including AEBSF, E64d, leupeptin, pepstatin A, cathepsin B inhibitor, phosphoramidon, bestatin (data not shown), chymostatin (data not shown), phenylmethylsulfonyl fluoride (PMSF) (data not shown) and tosyl phenylalanyl chloromethyl ketone (TPCK) (data not shown), affected serotype I FECV entry. These inhibitors target the main classes of proteases, although leupeptin was the only inhibitor that could potentially inhibit threonine proteases. Leupeptin works on a wide range of cysteine, threonine and trypsin-like serine proteases and has been shown to affect replication of many coronaviruses, such as SARS-CoV, MERS-CoV, HCoV 229E, and PEDV (Appleyard & Tisdale, 1985; Shirato *et al.*, 2013; Shirato *et al.*, 2011; Simmons *et al.*, 2005). This inhibition had been attributed to both its effect on endosomal cysteine proteases and/or to its effect on type II transmembrane serine proteases. However, although FCoV 79-1683 is known to use endosomal cysteine proteases, no effect of leupeptin was seen in the present study. This can be attributed to the questionable cell permeability of leupeptin and is in consistence with a previous report, showing that leupeptin had no effect on the cathepsin B-dependent FIPV

79-1146 replication (Appleyard & Tisdale, 1985). This observation implies that other threonine protease inhibitors than leupeptin should be tested to reveal whether or not this class of proteases is involved. In addition, it cannot be assured that all proteases within a certain class were targeted with the inhibitors that were used. Consequently, although FECV serotype I entry was not affected by the tested inhibitors targeting the main classes of proteases, this does not exclude that the spike is cleaved by cellular proteases during entry. Indeed, a recent study reporting the use of a conditional biotinylation assay showed that MHV-A59 spike is proteolytically cleaved during entry, but the exact protease could also not be identified, as this process was not susceptible to inhibitors targeting serine-, cysteine-, aspartyl-, and metalloproteases (Wicht *et al.*, 2014). So far, it remains unknown whether serotype I FECV warrants proteolytical cleavage by cellular protease during entry.

In conclusion, present study demonstrated that, in contrast to serotype II FCoVs, serotype I FECVs did not rely on acidic pH or cathepsin B for entry, confirming that care should be taken when extrapolating results obtained with the serotype II viruses to the *in vivo* situation, especially when it concerns viral entry. In addition, the furin inhibitor decanoyl-RVKR-CMK did not affect serotype I FECV entry or infectivity of progeny virus, but did inhibit FCoV 79-1683 and TGEV entry. Moreover, it was shown that serotype I FECVs (and some other alphacoronaviruses) potentially carry a virion-associated serine protease, which fulfils its function between 60 and 120 min p.i., thereby identifying a new target for drug development.

Acknowledgements

LMBD was supported by a doctoral fellowship of The Research Foundation-Flanders (FWO Vlaanderen). MET was funded by a scholarship from the Erasmus Mundus Action 2 WELCOME Project. We thank Ytse Noppe for her excellent technical support.

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In vivo FECV infections: targets for FIP prevention?

5.1 Clinical, virological, and immunological parameters during experimental feline enteric coronavirus infection

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Manuscript in preparation

5.2 Monitoring and control of feline coronavirus infections in two breeding catteries

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Clinical, virological, and immunological parameters during experimental feline enteric coronavirus infection

Summary

Feline enteric coronavirus (FECV) is a worldwide distributed, harmless intestinal virus of cats. However, occasionally, mutations occur that transform FECV into the deadly feline infectious peritonitis virus (FIPV). In order to better understand the feline coronavirus pathogenesis, three specific pathogen free cats were experimentally infected with the serotype I FECV UCD. Virus shedding was quantified in faeces and oral fluid by 2 different RT-qPCRs (one recognising the 3' of all genomic and subgenomic mRNAs (3' qPCR), and the other recognising the ORF1b of the genomic RNA (5' qPCR)), and by virus titration in enterocyte cultures. Blood samples were used to assess viraemia, neutralizing antibodies, and different subsets of leukocytes. The three cats remained clinical healthy during the course of the experiment, although some loss of appetite and slight weight loss was noticed in 2 cats (cat 1 and cat 3) in the beginning of the experiment. In these 2 cats, viral RNA was detected in faeces from day 2 (3' qPCR) or day 4 (5' qPCR) post inoculation (p.i.), and remained detectable for 2 months p.i. Infectious virus was found from day 4 until day 28 p.i. It was shown that the 3' qPCR gave a viral genome overestimation of 3-4.3 $\log_{10}$. Neutralizing antibodies were detectable from day 9 p.i, and a cell-associated viraemia was detected at infrequent time points after the onset of faecal shedding. No abnormal leukocyte numbers were noticed, except for a granulocytopenia in cat 1. Interestingly, the other cat (cat 2) showed a deviating infection pattern, characterized by absence of clinical signs, a delayed faecal shedding (from day 14 p.i.), which was not infectious in cell cultures, a delayed rise in antibody titres (from day 21 p.i.), and a viraemia that was detected far before any intestinal replication. No abnormalities or differences could be seen in leukocyte numbers compared to the other two cats, with the exception of $CD8^+$ regulatory T cells, but if and how these cells played a role remains elusive.

5.1.1 Introduction

Feline coronaviruses (FCoVs) occur as two pathotypes, associated with either enteric or systemic diseases in cats. Feline enteric coronavirus (FECV) is an enteropathogenic virus that is ubiquitously present throughout the cat population worldwide (Addie & Jarrett, 1992; Pedersen *et al.*, 1981b). The mild enteritis caused by its intestinal replication can be manifested by transient mild anorexia, weight loss and/or diarrhoea, but this is often too mild to be noticed (Hickman *et al.*, 1995; Pedersen *et al.*, 1981b; Vogel *et al.*, 2010). Feline infectious peritonitis virus (FIPV) arises most likely by mutation from FECV in individually infected cats (Chang *et al.*, 2011; Chang *et al.*, 2010; Herrewegh *et al.*, 1995; Pedersen *et al.*, 2009; Pedersen *et al.*, 2012; Poland *et al.*, 1996; Vennema *et al.*, 1998). These yet unknown mutations provide the virus with tools to productively replicate in monocytes/macrophages, causing a highly fatal systemic disease (FIP) characterized by a diffuse vasculitis, polyserositis and severe lymphopenia (Addie *et al.*, 2009; Horzinek & Osterhaus, 1979; Kipar *et al.*, 1998). Both pathotypes occur as 2 serotypes. Serotype II viruses arise by double recombination events between serotype I FCoVs and canine coronaviruses but represent only a minority of all strains worldwide (Addie *et al.*, 2003; Benetka *et al.*, 2004; Herrewegh *et al.*, 1998; Hohdatsu *et al.*, 1992; Kummrow *et al.*, 2005; Lin *et al.*, 2009; Vennema, 1999).

In vivo experiments are indispensable to study pathogenesis events. Due to its pathogenicity, most studies have been done with FIPV in order to investigate its complex epidemiology, pathogenesis and its interplay with the host's immune system. Although FECV is the source of every FIPV and consequently an important target in the control of FIP, experimental studies with FECV are rather scarce (Meli *et al.*, 2004; Pedersen *et al.*, 2008; Pedersen *et al.*, 1981b; Poland *et al.*, 1996; Vogel *et al.*, 2010). These studies mainly focussed on disease causing potential, faecal excretion patterns, and humoral immune responses during FECV infections. However, up until now, very little is known about the oral shedding of the virus, the viral infectivity of oral and faecal excretions, the presence of neutralizing antibodies, and the dynamics of the several leukocyte subsets during FECV infections. In addition, information on the relationship between PCR-assessed shedding and infectivity is scarce, as this has only been investigated once by infecting specific pathogen free (SPF) cats with either PCR-negative, weak-positive or strong-positive faecal samples (Foley *et al.*, 1997). Quantification of infectious virus and its correlation to RT-qPCR results have never been investigated, as there had been no susceptible cell cultures available. The recently established feline intestinal cell cultures (Desmarets *et al.*, 2013) can further shed light on these missing

links. Therefore, this study aimed at further broadening our knowledge on FECV pathogenesis, by providing information on viral infectivity, neutralizing antibody titres, and the dynamics of different leukocyte subsets during FECV infection by inoculation of 3 SPF cats with FECV UCD. For all cats, clinical, virological, and immunological parameters were followed during 3 months after inoculation. Surprisingly, 1 of the cats showed an aberrant excretion pattern compared to the other cats. Differences and potential causes for this abnormality are discussed.

5.1.2 Materials and methods

5.1.2.1 Virus

A faecal suspension containing an unknown titre of the FECV strain UCD (originally isolated at UC Davis, (Pedersen *et al.*, 1981b)) were kindly provided by Dr. P. Rottier (Utrecht University, The Netherlands). This suspension was diluted 1/10 in phosphate buffered saline and stored at -70 °C until use. The RNA content was determined using an RT-qPCR based on SYBR Green detection (see below). The suspension was centrifuged at 16200 g for 10 min to remove bacterial or host cells, and animals were infected with the suspension supernatant.

5.1.2.2 Inoculation and monitoring

Three 14 to 18 months old SPF cats (feline leukaemia virus-, feline immunodeficiency virus-, and FCoV-negative) (Harlan laboratories, Indianapolis, IN, USA) were orally infected with 800 µl of faecal suspension supernatant, containing $10^{11.3}$ viral RNA copies, while stimulating the swallowing reflex. Cats were housed in the same room but were separated from each other to avoid any physical contact between the animals. Additionally, precautions were taken to prevent exposure to any source of contaminating coronavirus. Briefly, with each handling, sterile clothing and footwear was ensured while litter trays, food trays and water bowls were cleaned and decontaminated daily. To ensure no contamination could arise from the litter being used, fine sand was washed extensively and autoclaved to serve as litter. The cats were monitored each day during the first week after infection and subsequently on day 9, 14, 21, 28, 56, and 84. Each time, the rectal temperature was measured, lymph nodes were palpated, an oral swab was taken and faeces were collected. If faeces were not available, faecal shedding was monitored by inserting a cotton tipped swab (Copan diagnostics, CA, USA) into the rectum. Swabs were suspended in 1 ml DMEM supplemented with 1000 U ml^{-1} penicillin (Continental Pharma Inc., Puurs, Belgium), 0.4 mg ml^{-1} gentamycin (Gibco BRL, Merelbeke, Belgium) and 10% fetal bovine serum (FBS, Gibco BRL). Faeces were diluted 1:5 (w:v) in the same medium. Suspensions were centrifuged at 2000 g for 10 min and supernatant was

frozen (-70°C) until determination of the viral load. Additionally, on day 0, 3, 5, 7, 9, 14, 21, 28, 56 and 84, cats were weighed, and 5 ml blood was taken from the *vena jugularis* in heparin (15 U ml^{-1}) (Leo, Zaventem, Belgium).

5.1.2.3 One step RT-qPCR for the quantification of the viral RNA load

5.1.2.3.1 RT-qPCR for the detection of total viral RNA

RNA was extracted from the faecal suspension using the QIAamp Viral RNA Mini Kit (Qiagen, Benelux BV, Belgium). A one step real-time RT-PCR based on SYBR Green detection was performed with primers described by Gut *et al.* (1999), targeting a 102 bp fragment at the 3' end of the genome (Gut *et al.*, 1999). A 15 µl PCR mixture was used per reaction and contained 0.3 µl Superscript™ III RT/ Platinum® Taq Mix, 7.5 µl 2x SYBR® Green Reaction Mix with ROX (Superscript™ III Platinum® SYBR® Green One-Step qRT-PCR Kit with ROX, Invitrogen), 0.5µM forward primer FCoV1128f, 0.5 µM reverse primer FCoV1229r and 3 µl FECV UCD RNA or diluted standard RNA (see below). A reverse transcription step of 20 min at 50°C and a denaturation step at 95°C for 5 min were followed by 45 cycles each 15 s at 95°C and 30 s at 60°C. A first-derivative melting curve analysis was performed by heating the mixture to 95°C for 15 s and then cooling to 60°C for 1 min and heating back to 95°C at 0.3°C increments. Reverse transcription, amplification, monitoring and melting curve analysis were carried out in a Step One Plus™ real-time PCR system (Applied Biosystems, Life Technologies Corporation, Carlsbad, CA, USA).

Synthetic RNA standards were generated by extracting RNA from FECV 79-1683 using the QIAamp Viral RNA Mini Kit (Qiagen). The RNA was reverse-transcribed into cDNA using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen). Briefly, 250 ng RNA was incubated for 5 min at 65°C with 2 µM reverse primer FCoV1229r and 10mM dNTP mix. Afterwards, an equal volume of cDNA synthesis mix, containing 10x RT buffer, 25mM $MgCl_2$, 0.1 M DTT, 40 U $µl^{-1}$ RNase OUT and 200 U $µl^{-1}$ Superscript III RT was added and incubated for 50 min at 50°C. The reaction was terminated at 85°C for 5 min. RNA was removed by incubation with RNase H for 20 min at 37°C. The 50 µl PCR mixture for the amplification of the cDNA contained 5x Herculase II reaction buffer, 25mM dNTP mix, 200 ng DNA template, 0.25µM forward primer FCoV1128f modified with a T7 promotor sequence at its 5' end, 0.25µM reverse primer FCoV1229r and 0.5 µl Herculase II fusion DNA polymerase (Agilent Technologies Inc., Santa Clara, CA, USA). After a denaturation step for 1 min at 95°C, 30 cycli of amplification, each 20 s at 95°C, 30 s at 60°C and 1 min at 68°C, were followed by a terminal elongation of 4 min at 68°C. cRNA standards were

transcribed by inbubation for 1 h at 37°C with 10x transcription buffer, 500μM rNTPs and 20 U T7 RNA polymerase-Plus Enzyme Mix (Applied Biosystems). Transcription reactions were DNase treated and the amount of RNA was determined using the Nanodrop 200 system. Ten-fold serial dilutions were made over a range of 6 log units (10^{10}-10^{5}) for the generation of the standard curve.

5.1.2.3.2 RT-qPCR for the detection of genomic RNA

RNA was extracted from the faecal suspensions using the QIAamp Viral RNA Mini Kit (Qiagen, Benelux BV, Belgium). Primer design and PCR conditions have previously been described (see chapter 3) (Desmarets *et al.*, 2013).

5.1.2.4 Infectivity titration

Monolayers of colonocytes, seeded in collagen I coated 96-well plates, were inoculated with 50 μl of serially diluted (1/10) faecal suspensions (ranging from 10^{0} to 10^{-7}). After 1 h (37°C, 5% CO_2), medium was added and the cells were further incubated for 72 h. To avoid cell loss due to faecal toxicity, undiluted suspensions were removed from the wells 1 h p.i., and the cells were washed 2 times before they were further incubated in medium. Then, plates were washed with PBS, air-dried (1 h 37°C) and frozen (-20°C). The 50% tissue culture infective dose ($TCID_{50}$) was determined by means of immunoperoxidase monolayer assay (IPMA). Therefore, cells were fixed and permeabilized by incubation with PF 4% (10 min, RT), followed by incubation with methanol containing 1% H_2O_2 (5 min, RT). Then, cells were incubated with PBS containing 10% negative goat serum and 0.1% Tween 80 for 30 min at 37°C. Subsequently, cells were incubated with monoclonal antibodies against the N-protein (produced and characterized in the lab), followed by goat anti-mouse HRP-labelled antibodies. Infected cells were visualized by adding sodium-acetate buffer containing amino-ethylcarbazole (AEC) and H_2O_2 for 10 min at RT. The fifty percent end-point was calculated according to the method of Reed and Muench (Reed & Muench, 1938).

5.1.2.5 Determination of neutralizing serum antibody titres

Sera were incubated at 56°C for 30 min to inactivate complement. Two-fold serial dilutions of the sera were mixed with an equal volume of a virus suspension containing 100 $TCID_{50}$ FECV UCD and incubated for 1 h (37°C, 5% CO_2). Then, colonocytes were added and further incubated with the virus-serum suspensions for 3 days. Infection was visualized by means of IPMA as described for the assessment of the infectious titre. The virus neutralizing titres were expressed as the reciprocal of the serum dilution that neutralized infection in 50% of the monolayers.

5.1.2.6 Leukocyte isolation

Blood mononuclear cells were separated on Ficoll-Paque (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). After isolation, cells were counted and frozen. Briefly, maximum 2 x 10^7 cells ml^{-1} were resuspended in RPMI supplemented with 30% fetal bovine serum (FBS), 100 U penicillin ml^{-1}, 0.1 mg streptomycin ml^{-1}, and 10% dimethyl sulfoxide (DMSO). Subsequently, cells were frozen by lowering the temperature with 1°C min^{-1} until -30°C, followed by a 15 min incubation period at -30°C and finally lowering the temperature to -150°C at a rate of 1°C s^{-1} (PTLPD81, Orthodyne, Alleur, Belgium). After freezing, cells were stored in liquid nitrogen.

5.1.2.7 Antibodies used for leukocyte staining

Monoclonal antibodies against the epsilon chain of feline CD3 (NZM1) and against feline CD56 (SZK1) were kindly provided by Dr. Yorihiro Nishimura (Tokyo University, Japan) (Shimojima *et al.*, 2003). Monoclonal antibodies FE5.4D2, and CA2.1D6 recognizing feline CD8β, and canine CD21, respectively, were purchased from AbD Serotec (Dusseldorf, Germany). A monoclonal antibody (FJK-16s), directly conjugated with Alexa fluor 647 (AF647) and crossreacting with feline Foxp3 was purchased from eBioscience (San Diego, USA). Monoclonal antibody CAT30A against feline CD4 was purchased from Veterinary Medical Research and Development (VMRD, Pullman, USA). Conjugated secondary antibodies [Molecular Probes (Invitrogen, Carlsbad, USA)] were goat anti-rat Alexa Fluor 488, goat anti-mouse IgG R-Phycoerythrin (R-PE), goat anti-mouse IgG2a Alexa Fluor 488, goat anti-mouse IgG1 Alexa Fluor 647 and goat anti-mouse IgG3 fluorescein isothiocyanate (FITC). When primary antibodies from the same IgG1 isotype were used, one primary antibody was labeled with Zenon Alexa Fluor 488 Mouse IgG1 (Invitrogen, Carlsbad, USA).

5.1.2.8 Leukocyte staining

Phenotyping of cells from all compartments was performed simultaneously. All analyzed cells were first stored in liquid nitrogen, facilitating analysis workflow. Several precautions were taken in order to preserve immunophenotypic properties as was done in previous research (Vermeulen *et al.*, 2012). Briefly, cells were frozen directly after isolation, they were stored at -196°C for the entire storage period and viability of thawed cells was routinely 80-90%. A minimum of 1×10^6 of frozen cells were stained for phenotypic analysis in RPMI supplemented with 1 mM Ethylenediaminetetraacetic acid (EDTA). Cells were incubated for 20 min at 4°C while gently shaking, both with the primary and dye-conjugated secondary antibodies. Cells were washed with cold RPMI containing EDTA and centrifuged at 300 g for

10 min at 4°C. During regulatory T cell staining, surface molecules were first stained, after which cells were fixed with the fixation/permeabilization kit optimized for staining of intracellular Foxp3 (eBioscience, San Diego, USA). Cells were then stained with anti-Foxp3 antibody, directly conjugated with AF647. Analysis was done on a FACSCanto flow cytometer using FACSDiva software (BD Biosciences, Mountain View, California, USA). After singlet gating, a minimum of 2×10^5 events was analyzed.

5.1.2.9 Animal welfare

This study was performed according to animal welfare guidelines. Under the application EC2012/043, this research was positively evaluated by the Ethical Committee of the Faculty of Veterinary Medicine, Ghent University.

5.1.3 Results

5.1.3.1 Clinical signs, viral shedding, and viraemia in 3 cats inoculated with FECV UCD

Mild clinical symptoms were seen in 2 of the 3 cats (cat 1 and cat 3) during the first week p.i. (Figure 5.1.1). Symptoms consisted of a diminished appetite and moderate weight loss, to 95.4 and 88.4% of the initial weight for cat 1 and 3, respectively. Cat 1 also showed an increased body temperature at day 4 (39.5 °C) and day 6 (39.7°C) p.i. No diarrhoea or changes in faeces consistency were noticed. From day 9, both cats started to recover and reached their original weight at 21 days p.i. Cat 2 showed no signs of loss of appetite, weight loss or abnormal faeces consistency during the entire infection course, but slightly swollen submandibular lymph nodes were noticed at day 3 p.i., and a slightly raised temperature (39.3°C) at day 7 p.i.

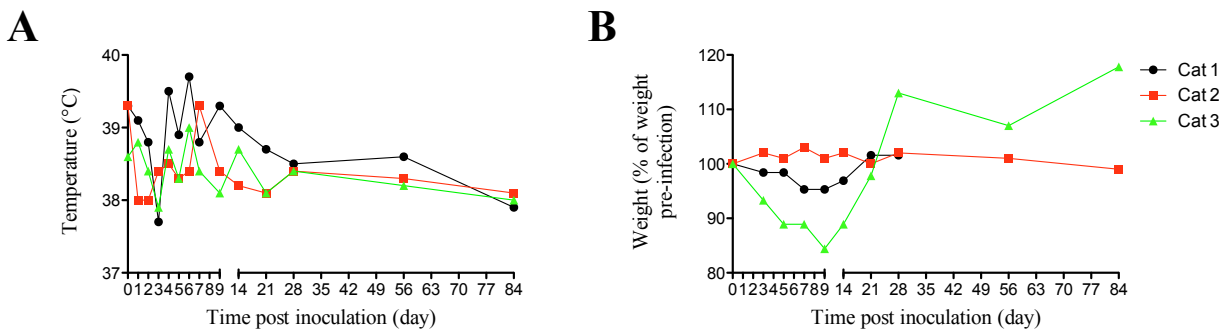

Figure 5.1.1. Clinical parameters followed during the entire FECV UCD infection course. (**A**) Rectal temperature was monitored daily during the first week, and on day 9, 14, 21, 28, 56, and 84 p.i. (**B**) Body weight was measured at day 0, 3, 5, 7, 9, 14, 21, 28, 56, and 84, and expressed relative to the weight before inoculation.

Oral and faecal shedding were quantified in all animals by 2 different RT-qPCRs, and by virus titration in feline enterocyte cultures (Figure 5.1.2). These 2 RT-qPCRs were taken along to assess the overestimation of virus particles by the generally used 3' qPCR (Gut *et al.*, 1999), as this qPCR detects not only genomic RNA, but also all subgenomic mRNAs.

Directly after inoculation, only a fraction of the viral inoculum was found in the mouth, indicating that all cats had swallowed most of the inoculum. The next day, viral RNA was still detected in oral fluid of cat 2 and 3 with the 3' qPCR, whereas only in cat 3 genomic RNA (5' qPCR) and infectious virus were detected. Total RNA (3' qPCR) remained detectable for cat 2 and 3 at day 2 p.i., whereas genomic viral RNA was detected for cat 1 and 3. During the remainder of the infection course, oral viral RNA load and days of detection varied considerably. Overall, the amount of oral RNA nearly always approached detection levels, which can explain the inconsistency seen between both qPCRs. Infectious virus could not be found in oral fluid, except for cat 3 at day 0 and day 1.

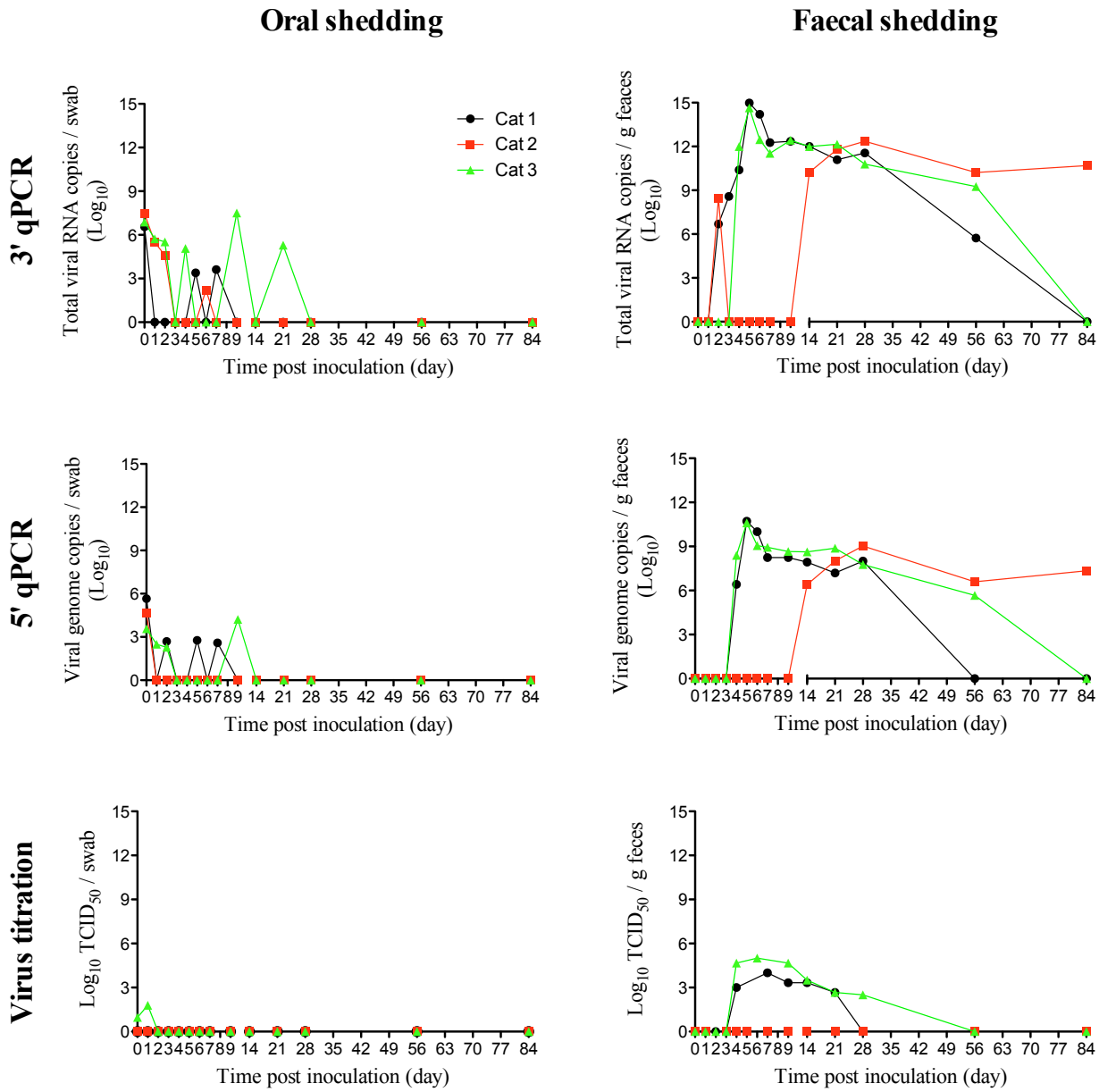

Figure 5.1.2. Quantification of oral and faecal shedding by 2 different real time RT-PCRs and virus titration. Oral swabs and faeces (or faecal swabs if no faeces were available) were taken at regular time points p.i., and the total amount of viral RNA was quantified by RT-qPCR using either primers targeting the 3' part of the genome and subgenomic mRNAs (3' qPCR) or primers against the ORF1b to detect only genomic RNA (5' qPCR). The amount of infectious virus was determined by titration in feline enterocyte cultures.

Faecal RNA shedding was detected for cat 1 and 3 from day 2 (3' qPCR) or day 4 (5' qPCR) p.i. onwards. For these 2 cats, faecal shedding peaked at day 5 p.i., whereupon shedding slightly dropped but remained at high levels until 28 days p.i. Thereafter, virus shedding dropped and both cats had ceased shedding by day 84 p.i. Infectious virus was found in cell culture from day 4 until day 21 (cat 1) or day 28 (cat 3) p.i. In contrast to cat 1 and 3, an aberrant excretion pattern was found in cat 2. At day 2, viral RNA was detected with the 3' qPCR, but not with the 5' qPCR. Thereafter, viral RNA excretion disappeared and was not

detected anymore until day 14 p.i. From then, faecal RNA shedding remained high during the remainder of the experiment. However, infectious virus could not be detected for this cat at any of the time points.

Concerning the faecal shedding, the 3' qPCR gave viral RNA quantities that were 3-4.3 $\log_{10}$ higher than the 5' qPCR, indicating that only 1/1000 to 1/20000 of all copies detected with the 3' qPCR are viral genomic RNA copies. This can explain why total RNA copies detected with the 3' qPCR were 6-8 $\log_{10}$ higher compared to the infectious virus titre. Overall, the 5' qPCR gave total viral titres that were more correlated with the results of infectious virus titres. Indeed, the amount of infectious particles was typically 3-4 $\log_{10}$ times lower compared to the total amount of particles in the first week p.i. From day 9 p.i., infectivity titres progressively declined to undetectable levels, whereas PCR titres remained high for an additional 1-2 months.

Both cell-free and cell-associated viraemia were assessed at regular time points for all cats, using the 5' qPCR (Table 5.1.1). A cell-associated viraemia was detected at infrequent time points for all cats. In contrast to cat 1 and 3, viraemia in cat 2 was detected before the onset of faecal shedding (day 3 and 5 p.i.), and no longer thereafter.

Table 5.1.1. Detection of viraemia during the entire infection course.

	Cat 1		Cat 2		Cat 3	
Day p.i.	**Plasma**	**Cell-associated**	**Plasma**	**Cell-associated**	**Plasma**	**Cell-associated**
0	-	-	-	-	-	-
3	-	-	-	+	-	-
5	-	-	-	+	-	+
7	-	+	-	-	-	-
9	-	+	-	-	-	+
14	-	+	-	-	-	+
21	-	-	-	-	-	-
28	-	-	-	-	-	+
56	-	-	-	-	-	-
84	-	-	-	-	-	-

5.1.3.2 Immunological parameters

5.1.3.2.1 Neutralizing antibody response

Figure 5.1.3 shows the amount neutralizing antibodies detected in the serum of the three cats during the infection experiment. For cat 1 and 3, which displayed an active intestinal replication during the first week p.i., neutralizing antibodies were detected from day 9 p.i. and peaked at day 21 (cat 3) or day 28 (cat 1) p.i. In cat 2 with the delayed shedding pattern,

similar signs of seroconversion occurred only after the onset of intestinal replication, with the first detectable antibodies appearing at day 21 p.i. In all cats, antibody titres remained at high levels during the remainder of the experiment.

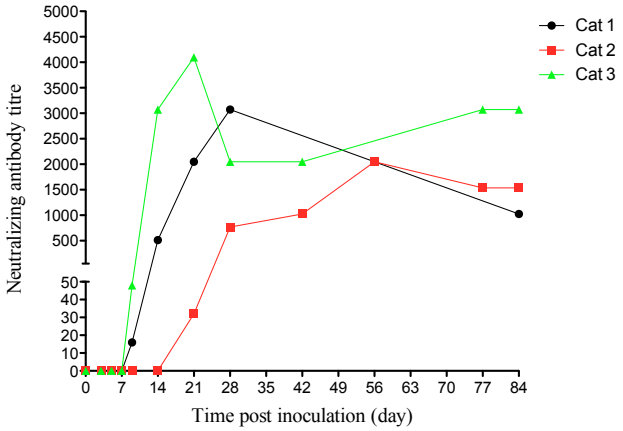

Figure 5.1.3. Neutralizing serum antibody responses during FECV infection. Neutralizing antibody titres were assessed in the serum on day 0, 3, 5, 7, 9, 14, 21, 28, 56, and 84 p.i. by virus neutralization assay in enterocytes using FECV UCD.

5.1.3.2.2 Dynamics of leukocyte subsets during FECV infection

Figure 5.1.4 shows the absolute number of T cells, B cells, monocytes, and granulocytes determined in blood taken at regular time points p.i. No abnormal leukocyte numbers were noticed in any of the three cats, except for a depletion of peripheral granulocytes in cat 1 during the first 3 weeks p.i. For each cat, T and B cell numbers followed a similar trend. All cats showed a small decrease in lymphocyte numbers, which started to resolve from day 21 p.i., but this recovery phase was much more pronounced in cat 1 and 3 compared to cat 2. Indeed, whereas lymphocyte numbers remained at pre-infection levels for cat 2, both cat 1 and 3 showed a considerable rise in lymphocyte numbers, characterized by a slight lymphocytosis, which coincided with cessation of the shedding in both cats. Monocytes of cat 1 and 2 slightly declined to raise back to pre-infection levels at day 28 p.i., but numbers always remained within the normal limits.

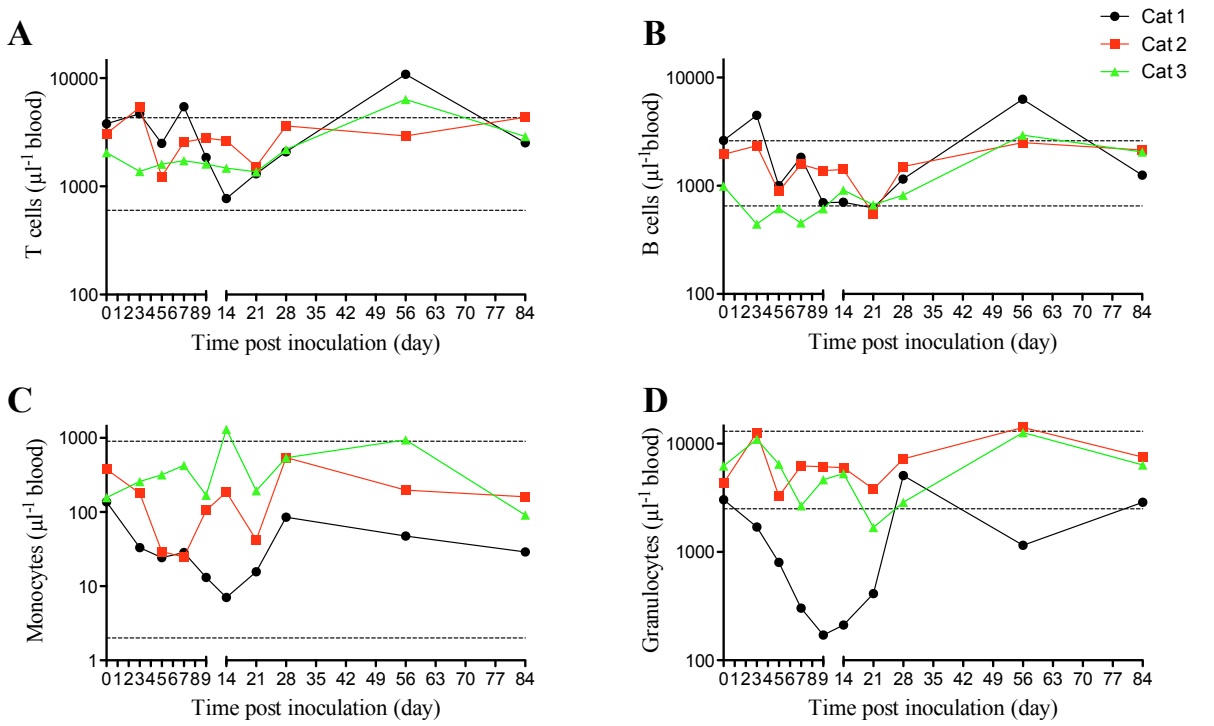

Figure 5.1.4. Absolute quantity of different leukocyte subsets during FECV infection. Analysed cells were **A)** T cells, **B)** B cells, **C)** monocytes, and **D)** granulocytes. Two horizontal dashed lines represent reference values in healthy animals.

Quantification of regulatory leukocytes (natural killer (NK) and regulatory T cells (Tregs)) is shown in Figure 5.1.5. As for other leukocytes, no abnormal high or low NK cell- or Treg numbers were noticed during the infection course. However, some trends were visible. In all cats, NK cells slowly declined with the lowest amount at 14 or 21 days p.i., whereupon NK cell count rose again to pre-infection level at day 56 p.i. Treg counts similarly declined and rose in all cats. When analysing a subset of Tregs ($CD8^+$ Tregs), which has been associated with suppression of gut immune responses, it was noticed that the delayed shedder had higher numbers of $CD8^+$ Tregs, which increased until day 7 p.i., whereas the number of $CD8^+$ Tregs was slightly decreased during the first week for the other 2 cats.

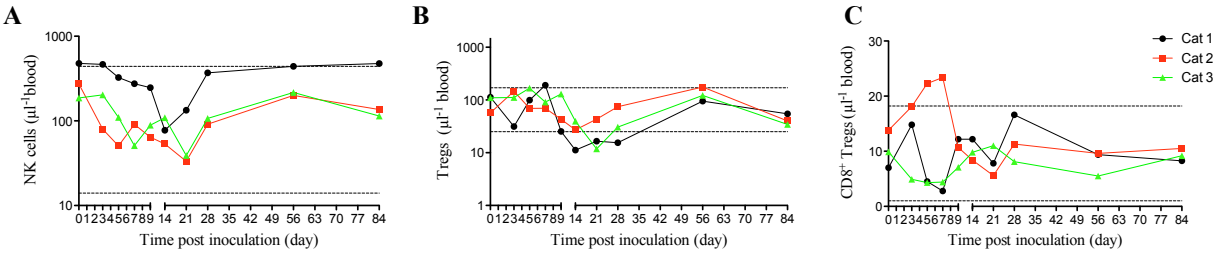

Figure 5.1.5. Number of regulatory cells during FECV infection. Analysed cells include: (**A**) NK-cells, (**B**) Tregs, and (**C**) $CD8^+$ Tregs.

5.1.4 Discussion

Due to its pathogenic behaviour, FIPV has received considerable attention, and clinical, virological and immunological parameters during both natural and experimental FIPV infections have frequently been studied. The last decade, comprehensive studies on the FIPV parent virus, FECV, have extensively contributed to our current understanding of epizootiology and pathogenesis (Addie *et al.*, 2003; Meli *et al.*, 2004; Pedersen *et al.*, 2008; Vogel *et al.*, 2010), but too many unidentified parameters have hampered the unravelling of the highly complex FCoV pathogenesis so far. The aim of this study was to contribute to the current understanding of FECV infections by filling some of the missing links, such as viral infectivity of oral and faecal excretions, the generation of neutralizing antibodies, and the dynamics of the several leukocyte subsets during experimental FECV infections.

In accordance with previous reports on experimental FECV infections (Meli *et al.*, 2004; Pedersen *et al.*, 2008; Vogel *et al.*, 2010), two of the three cats started shedding between day 2 and 4 p.i. Faecally shed RNA peaked at day 5 p.i., showed a plateau until day 28 p.i., and then diminished to become undetectable at day 84 p.i. Both cats showed mild clinical signs, including loss of appetite and mild weight loss, but diarrhoea was never observed. In these cats, infectious virus was found from day 4 until day 21 (cat 1) or day 28 (cat 3) p.i. The antibody response was detectable from day 9 p.i., which is also in accordance with earlier reports (Pedersen, 1995; Pedersen *et al.*, 1981a; Vogel *et al.*, 2010). However, the neutralizing capacity had never been determined due to the lack of an appropriate cell line to perform the neutralization assay on. By using feline enterocyte cultures, it was shown in the present study that serum antibodies mounted during FECV infections are highly neutralizing. Interestingly, one of the infected cats (cat 2) showed an infection pattern that deviated from the other cats. In contrast to cat 1 and 3, no anorexia or weight loss was noticed during the entire study. In addition, shedding in cat 2 was remarkably delayed (until day 14 p.i.), as was the onset of the antibody response, which started to become detectable from 21 days p.i. onwards. A delay in faecal shedding and seroconversion has been described before in 1 study, reporting no faecal shedding before day 10 p.i., and a detectable seroconversion only after 21 days (Foley *et al.*, 1997). In that study, this infection pattern was typically seen in cats infected with weak-positive faecal extracts. However, in the present study all cats were infected with a high dose ($10^{11.3}$ RNA copies), and a previous study reported the successful inoculation of cats with FECV UCD at a dose as low as $10^{5.7}$ RNA copies, without noticing this delay (Vogel *et al.*, 2010). Another possible explanation for this pattern is that the

original inoculation failed, and that this cat became infected later on by inadvertent transmission of the virus shed by one of the other cats. However, this explanation seems also very unlikely, as 1) cats were housed separately and precautions were taken to avoid inadvertent transmission, 2) FCoV RNA was found in oral secretions until 2 days after inoculation, 3) viral RNA was found in faeces at day 2, indicating passage of the virus without any further infection, and 4) a cell-associated viraemia was detected in this cat at day 3 and 5 p.i. Notably the latter observation raises the presumption that FECV could have reached the intestine via the blood. How this can be achieved remains enigmatic, but based on the swollen submandibular lymph nodes at day 3 p.i., it can be hypothesized that FECV was potentially taken up by permissive (most likely monocytic) cells in the oral cavity from which the virus could further spread systemically to finally reach the intestine, from which subsequent shedding occurred. Interestingly, despite this systemic infection, the immunological response was delayed until the intestinal replication was detected, indicating that only intestinal replication succeeded to create sufficient antigenic mass to activate the immune response. This is in accordance with what can be seen during natural infections, as it has been shown that not all viraemic cats have antibodies (Gunn-Moore *et al.*, 1998). The alternative route of infection seems plausible, since it is known that intraperitoneal inoculation of FECV can occasionally result in faecal shedding, which has been attributed to the circulation of FECV-loaded monocytic cells from the periphery to the intestine (Foley *et al.*, 1997; Pedersen *et al.*, 2012). Surprisingly, virus that was shed by this cat was no longer infectious in enterocyte cultures *in vitro*, suggesting that during its circulation the virus has changed. Full genome sequencing of the excreted viruses is currently being performed to reveal if this lack of infectivity has a genetic background. Another possible explanation for the lack of *in vitro* infectivity is that intestinal shedding in this cat occurred from non-enterocytes, which could have changed the virus glycosylation and in that way tropism for enterocytes, but this needs to be further assessed.

In the present study, shedding was quantified by two different RT-qPCR assays, and by infectious titration in previously established enterocyte cultures (Desmarets *et al.*, 2013). The 3' qPCR is commonly used for monitoring of faecal shedding and targets a conserved region at the 3' end of the viral genome, which is also present in all subgenomic mRNAs (Gut *et al.*, 1999), whereas the 5' qPCR recognises a conserved region within ORF1b, that is only present in full genome RNA molecules. Comparison of both qPCRs revealed that virus quantification in faeces was 3-4.3 $\log_{10}$ times overestimated if the 3' qPCR was used. In addition, shedding

quantities and patterns observed with the 5' qPCR correlated much better to what was found on infectivity titration, making the latter qPCR more valuable for management purposes. Infectivity titres were typically 3-4 $\log_{10}$ times lower during the first week p.i., which is reasonable, taken into consideration that a proportion of all viruses will be defective and that infectious titration of faecal samples can give an underestimation due to toxicity in cell culture. In addition, all titrations were performed before it was noticed that neuraminidase treatment could enhance FECV infectivity in the cell cultures (Desmarets *et al.*, 2014), which can also contribute to underestimation. As infection progressed, the difference between total genomic RNA copies and infectious titre even further increased. Since this coincided with the onset of neutralizing antibodies, it is possible that neutralizing antibodies in faeces caused an increased underestimation of infectious virus in cell culture, but the presence of intestinal neutralizing antibodies was not further investigated. Whereas faecal RNA was detected from day 2 p.i. with the 3' qPCR, faecal shedding was never noticed before day 4 p.i. with the 5' qPCR or in enterocyte cultures. In addition, faecal RNA was also found at day 2 p.i. in cat 2 with the 3' qPCR, but no signs of active intestinal replication were seen in this cat until day 14 p.i. These observations raise the question whether the early detection with the 3' qPCR results from active shedding or rather from the shedding of the remainder of the (by then degraded) inoculum. Oral shedding was noticed at inconsistent time points, which is in accordance with what is observed in natural infections (Addie & Jarrett, 2001), and depended on the cat and on the PCR that was used. Whether this oral RNA resulted from active replication in the tonsils, as previously proposed for FIPV (Stoddart *et al.*, 1988), or was rather the result from the licking behaviour of the cats is unknown. However, at least some time points cannot be explained by the latter as oral RNA was found for cat 2 and 3 before any faecal shedding was noticed, making a restricted replication of FECV in the throat possible at these time points.

When analysing general peripheral leukocyte subsets, no major changes were noticed, except for a granulocytopenia in cat 1 during the first 3 weeks p.i, and a slight T and B cell lymphocytosis at day 56 p.i. for cat 1 and 3. Concerning the regulatory cells, FECV infection was characterized by a transient NK cell reduction in peripheral blood, which was most probably the result of migration of NK cells to the intestine or associated lymphoid tissue, since NK cells had an elevated CD11b and CD62L expression (data not shown). FECV infection also appeared to be characterized by a transient lowered amount of peripheral Tregs, which can most probably also be explained by specific trafficking to the gut or associated

lymphoid tissue. Acute or chronic virus infections are very often associated with an increase in peripheral Treg frequency or function, a feature that was not noticed in the present study. However, gut immunology seems to differ quite a lot from systemic immunity, notably given the fact that the gut has regulatory systems in place to induce tolerance against commensal bacteria and food antigens, systems where Tregs play a vital role. Manipulation of Tregs through accumulation or activation at sites of infection can also cause immune tolerance against pathogenic micro-organisms, as exemplified by protozoan (*Leishmania Major*), nematodic (*Heligmosomoides polygyrus*) and bacterial (*Helicobacter pylori)* infections (Belkaid, 2007; Bilate & Lafaille, 2012). Whether these cells also contribute to the long-lasting or persistent shedding of FECV remains to be investigated. In addition, cat 2 showed a deviating pattern in peripheral circulating $CD8^+$ Tregs compared to the other cats. This subset has recently gained a lot of interest in the context of gut immunity to colorectal cancer, graft-to-host disease and rectal HIV/SIV infection, where they are associated with suppressed immunity (Beres *et al.*, 2012; Chaput *et al.*, 2009; Nigam *et al.*, 2010). However, if and how these cells played a role in the aberrant infection pattern of this cat remains elusive, as not much is known about the exact function of these cells.

In conclusion, present study reports the simultaneous assessment of different virological (presence of viral RNA and/or infectious virus in faeces, oral fluid, and blood), and immunological parameters (neutralizing antibodies and several leukocyte subsets) during experimental FECV infections in three cats. Two of the three cats showed mild clinical signs; a faecal shedding that started within a few days p.i. and was infectious *in vitro* for 3-4 weeks; a cell-associated viraemia and oral shedding at inconsistent time points; highly neutralizing antibody responses from day 9 p.i.; and no abnormalities in leukocyte subsets. Remarkably, a deviating infection pattern was noticed in one cat, characterized by an early cell-associated viraemia (day 3 and 5 p.i.), a delayed faecal shedding (starting from day 14 p.i) and antibody response (detectable from day 21 p.i.), and a loss of infectivity of the excreted virus in enterocytes cultures. No differences could be seen in leukocyte numbers compared to the other cats, with the exception of $CD8^+$ regulatory T cells, but their role remains elusive so far. Consequently, whether this deviating infection pattern was due to mutational variants or rather was a cat-dependent factor requires further investigation.

Acknowledgements

We are grateful to Dr. P. Rottier and Dr. N. Pedersen for supplying the FECV UCD strain. This work was funded by a doctoral fellowship of The Research Foundation-Flanders (FWO Vlaanderen) (LMBD), and by a doctoral fellowship of The Agency for Innovation by Science and Technology (IWT) (BLV). Special thanks go to Ytse Noppe for excellent technical assistance.

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Monitoring and control of feline coronavirus infections in two breeding catteries

Summary

In multi-cat environments, feline infectious peritonitis (FIP) is a major cause of financial and emotional losses by killing up to 12% of the young cat population. FIP is the result of mutations occurring in the viral genome during common FECV infections, which reside in virtually all multi-cat households. To date, adapting the management/housing to restrict these FECV infections is the only way to deal with FIP. However, reports that describe how the management can be adapted using currently available diagnostic PCR techniques and, more importantly, if these efforts are feasible and cost-effective in the prevention of FIP, are still lacking. Therefore, 2 breeding catteries (<15 cats) were followed for 3 years (household 1), and 1 year (household 2). In both households, faecal shedding of individual cats was regularly monitored, and cats were grouped according to the results in order to avoid FECV transmission. Grouping was performed by housing cats in separate rooms, without any other restrictions concerning handling of cats, clothing or people movement, in order not to compromise practical feasibility. In household 1, all but one of the cats had ceased shedding 9 months after the start of the monitoring. The cattery was even totally cleared from shedders after removal of this persistently shedding cat, and two negative litters were raised. As a result of the regular import of new cats, this cattery could not be kept negative, but due to the grouping, it was prevented that the virus spread again throughout the complete cat population. In household 2, grouping of cats was also successful to make a non-shedding population, but in contrast to household 1, 7/14 cats were still shedding 1 year after the start of the monitoring. It was concluded that making an FECV-negative population is practically feasible by regularly monitoring of shedding and grouping of cats, but that cost-effectiveness of this strategy depended on the household.

5.2.1 Introduction

In the sixties, feline infectious peritonitis (FIP) was first named and described as a new viral disease entity in cats (Holzworth, 1963; Wolfe & Griesemer, 1966; Zook *et al.*, 1968). In 1970, the causative agent of FIP was found to be coronavirus, designated feline infectious peritonitis virus (FIPV) (Ward, 1970). However, whereas many healthy cats tended to have anti-FIPV antibodies, and FIPV seemed to be highly infectious upon experimental inoculation, only a minority of all cats actually succumbed to FIP. This inconsistency between seroprevalence and disease was solved in the eighties, when Pedersen and colleagues discovered that many healthy cats shed a seemingly harmless coronavirus in their faeces, and that FIPV most probably arose by mutation from this feline enteric coronavirus (FECV) (Pedersen *et al.*, 1984; Pedersen *et al.*, 1981). This mutational pathotype switch was later on confirmed, and it is now widely accepted that the majority of all FIP cases are the consequence of mutations arising in the viral genome during a common FECV infection (Chang *et al.*, 2010; Pedersen *et al.*, 2009; Pedersen *et al.*, 2012; Poland *et al.*, 1996). With the observations that infection with the easily transmittable FECV precedes the development of FIP, it became rapidly clear that managing the faecal-oral transmission in multi-cat environments is of key importance in FIP prevention. Indeed, recommendations from the workshops on FCoV control in catteries that were held at the first and second international feline coronavirus/feline infectious peritonitis symposium stated that control of FIP must preferably be directed at the control of the underlying FECV infections, and should that fail, at the FIPV itself (Addie *et al.*, 2004; Pedersen *et al.*, 1995).

FECV is found in virtually all multi-cat environments worldwide (Addie & Jarrett, 1992; Pedersen *et al.*, 1981), except for the Falkland Islands, which have remained FCoV seronegative so far by extensive testing of incoming cats (Addie *et al.*, 2012). Faeces from shedders are highly contagious, resulting in a very fast and efficient faecal-oral transmission of the virus to susceptible cats, which in turn start to shed high amounts of FECV in their faeces within one week after uptake (Pedersen *et al.*, 2008; Pedersen *et al.*, 2004; Pedersen *et al.*, 1981; Vogel *et al.*, 2010). Infected cats shed the virus for many weeks, months, or in case of persistent shedders, years. These cats are a continuous source of infection or reinfection of negative cats, the latter being the result of the readily declining local immunity after the infection has been cleared (Addie & Jarrett, 2001; Addie *et al.*, 2003; Pedersen, 2009; Pedersen *et al.*, 2008). Therefore, if one wants to prevent FECV infection, the most important measure to be taken is to prevent any contact between shedders and naive animals. In the past,

this separation policy has been successfully applied to either completely eradicate FCoVs (Hickman *et al.*, 1995), or to control transmission to the most susceptible population in breeding catteries, which comprise the several weeks old kittens after they have lost their protection from maternal immunity (Addie & Jarrett, 1992). Hickman and colleagues reported the introduction of FECV in a closed specific pathogen free (SPF) facility, which had been unnoticed until several cats started to die from FIP. As these cats were of high value, the researchers decided to completely eradicate FCoVs from the colony by serological testing and grouping based on antibody titres, since no diagnostic PCR tests were available at that time. Only seronegative animals were kept to create offspring, and cats that remained seropositive were removed from the colony. By regular testing and strict quarantine measures, the researchers were able to recreate a FCoV-negative SPF population (Hickman *et al.*, 1995). In practice, however, this method has many drawbacks and is hardly feasible. Indeed, by using PCR to measure faecal shedding, many researchers reported inconsistent conclusions on the correlation between antibody titre and shedding, indicating that it is very difficult to reliably isolate shedders from non-shedders based on their serum antibody titre (Addie *et al.*, 2003; Foley *et al.*, 1997; Harpold *et al.*, 1999; Pedersen *et al.*, 2008). In addition, eradication was highly simplified in the study of Hickman and colleagues by the fact that only few animals were saved and kept for further breeding programs, and by the availability of high quality isolation facilities to prevent any further transmission of the virus. Consequently, complete eradication of FCoVs based on antibody titre is hardly feasible in practice. In addition, even if this would succeed, it is quite challenging to remain FCoV-negative, as FCoV infections are present in virtually all multi-cat environments from where new cats are frequently imported. For these reasons, work groups recommended to direct the control of FIP towards the control of transmission from shedders to the highly susceptible young animals (Addie *et al.*, 2004; Pedersen *et al.*, 1995). FIP is typically seen in kittens in the post-weaning period and most kittens are protected by maternally derived antibodies until 5-10 weeks of age (Addie *et al.*, 2009; Foley *et al.*, 1997; Harpold *et al.*, 1999; Pedersen *et al.*, 2008). Therefore, it has been recommended to control the transmission of FECV from shedders to the kittens by isolating the queens 2-3 weeks prior to parturition, taking the kittens away from their mother at 4-6 weeks of age (= early weaning) and raise them in complete isolation (Addie *et al.*, 2004). Although clearly demonstrated that early weaning can strongly decrease the incidence of FIP (Addie & Jarrett, 1992), the success rate is variable and depends on the isolation procedure and the shedding state of the queen (Addie *et al.*, 2004). When kittens are faced with a high infection pressure, viruses break through the maternal immunity, and kittens can become

infected as soon as 2 weeks of age (Lutz *et al.*, 2002). In addition, early weaning has been questioned by its negative impact on the socialisation of kittens, and is therefore not regularly applied in practice.

FECV remains enzootic by continuous transmission of the virus from shedders to susceptible animals, and FECV shedding can be easily detected with currently available high sensitive PCR techniques (Addie *et al.*, 2003; Foley *et al.*, 1997; Pedersen *et al.*, 2008). Consequently, grouping of cats based on their shedding state has been proposed to avoid (re)infections, but no reports on the successful isolation of shedders from non-shedders have yet been published. However, this strategy would allow the prevention of FECV exposure to kittens without the need for early weaning and complete isolation, as this would allow selection of negative animals for breeding and/or socialisation of kittens. In the present study, the feasibility of this strategy was evaluated in 2 breeding catteries. These catteries were followed over a period of 3 years (household 1) or 1 year (household 2). Faecal shedding was monitored every 2-4 months, and after every testing, decisions were made for grouping of animals according to the test results. The practical feasibility, advantages, and costs (time and money) are discussed.

5.2.2 Materials and methods

5.2.2.1 Households

Household 1 represents a Siamese and Oriental Shorthair breeding cattery, established in a normal two-storey house, in which cats were held in groups at both floors. We were contacted by this cattery at the end of 2011, since a lot of FIP deaths had occurred in their kitten population. Indeed, from August 2011, 8 out of 21 kittens born in that cattery in 2011 finally succumbed to FIP (6 pathologically confirmed cases, 2 highly suspected cases). From October 2011, faecal shedding was monitored regularly over a period of 3 years in order to control the FECV transmission. Table 5.2.1 gives an overview of the cattery cats and their fate from June 2010 until August 2014. Kittens are indicated by the letter of the litter, followed by a number (e.g. A1 = cat 1 from litter A), and the identity of the parents is given in between brackets (tomcat x queen). In this cattery, cats were regularly imported and sporadically exported for breeding.

Household 2 represents a British Shorthair and Selkirk Rex breeding cattery, established in a normal two-storey house, in which cats only had access to the ground floor. In contrast to household 1, this cattery had never had FIP cases in the kitten population, but had had 4 adult cats that had succumbed to FIP in 2 years. After the fourth FIP case (2013), the owner started to fear for FIP in any future newborn litter and started to monitor shedding of cats to control

transmission of FECV. Table 5.2.2 gives an overview of the cattery cats and their fate from 2007 until August 2014. Litters are indicated with a letter (A to M), and kittens that were kept in the cattery are identified with a letter and a number (e.g. A1). The identity of the parents is given in between brackets (tomcat x queen). In this cattery, breeding occurred with in-house animals.

5.2.2.2 Sampling

For evaluating individual faecal shedding of group-housed cats, cotton tipped swabs (Copan diagnostics, CA, USA) were inserted about 5 cm in the rectum. Subsequently, swabs were suspended in 1 ml transport medium, consisting of Dulbecco's modified Eagle's medium (DMEM) containing 100 U ml^{-1} penicillin (Continental Pharma Inc., Puurs, Belgium) and transported at 4°C to the lab. For sampling of kittens or individually housed animals, faeces were collected/pooled and upon arrival, 20% suspensions were made in the same transport medium.

5.2.2.3 Assessment of the viral RNA load

After centrifugation (10 min, 2000 g) of the sample, viral RNA was extracted from the supernatant using the viral RNA mini kit (Qiagen, Benelux BV, Belgium). Assessment of the viral load was done by RT-qPCR against the viral ORF1b, as previously described (Desmarets *et al.*, 2013). In this study, the amount of viral genomic copies was only semi-quantitatively assessed, as absolute quantification is too expensive for routine follow up of cattery cats. Therefore, shedding of each cat was expressed as a C_q value. Since C_q value 37 represents <1 RNA copy in our reactions, all signals rising after $C_q = 37$ were considered as negative.

5.2.2.4 Grouping of cats

Cats were grouped in the house according to their shedding state, generating separated non-shedding- and one or more shedding groups. Precautions taken to avoid inadvertent transmission from the shedding to the non-shedding group included daily cleaning of litter trays, thereby first cleaning the litter trays of the negative group, and regular cleaning and disinfecting of surfaces. As owners wanted to continue normal daily tasks in their house, no special precautions were taken considering people movement, handling of cats, or clothing.

Table 5.2.1. Overview of cats present in household 1 and their fate from 2010 until 2014.

Cat id	Sex	Birth date	2010	2011	2012	2013	2014
1	M	03/2006					
2	M	05/2006		11/2011 † no FIP			
3	F	01/2006		12/2011 Adopted			
4	F	08/2008		12/2011 Sold			
A1(1x4)	F	02/2010					
A2(1x4)	F	02/2010					
A3(1x4)	F	02/2010					
A4(1x4)	M	02/2010	† no FIP				
5	M	05/2006	Import 12/2010 † FIP				
6	M	05/2008	Import		01/2012 †Amyloidosis		
7	F	06/2006	Import				
8	F	03/2009	Import				
9	F	06/2008	Import				
B1(5x7)	M	01/2011					
B2(5x7)	F	01/2011					
B3(5x7)	F	01/2011		08/2011 †FIP suspected			
B4 (5x7)	F	01/2011		09/2011 †FIP			
C1-5(5x4)		02/2011		03/2011 † FHV-pneumonia			
C6 (5x4)	M	02/2011					
D1(5x9)	M	02/2011		04/2011 † no FIP			
D2(5x9)	M	02/2011		10/2011 †FIP			
D3(5x9)	F	02/2011					
D4(5x9)	M	02/2011		11/2011 †FIP			
E1(6x8)	M	02/2011		10/2011 †FIP suspected			
E2(6x8)	M	02/2011		08/2011 †FIP			
E3(6x8)	M	02/2011		11/2011 †FIP			
E4(6x8)	F	02/2011		08/2011 †FIP			
E5(6x8)	F	02/2011					
E6-7(6x8)		02/2011		† no FIP			
F1(A1xB1)	M	03/2013					
F2-5 (A1xB1)		03/2013					
10	F	01/2013				Import	
G1(B1x8)	F	05/2013					
G2-6(B1x8)		05/2013					
G7(B1x8)	M	05/2013				08/2013 † no FIP	
H1-4(B1x8)		02/2014					
I1(B1xA1)	F	02/2014					07/2014 †FIP
I2(B1xA1)	M	02/2014					
I3-6(B1xA1)		02/2014					
11[a]	F						Import
12[a]	F						Import
13[a]	M						Import
14[a]	F						Import
15[a]	M						Import
16[a]	M	02/2014					Import

Abbreviations and symbols: † = death, M = male, F = female, FHV= feline herpes virus. Dark shading represents absence in cattery at the given time point.

[a] Cat 11-16 represent British Shorthair cats that joined the cattery from March 2014.

Table 5.2.2. Overview of cats present in household 2 and their fate from 2007 until 2014.

Cat id	Sex	Birth date	2007	2008	2009	2010	2011	2012	2013	2014
1	M	02/2006						01/2012 † FIP		
2	F	07/2004	Import						† no FIP	
3	F	07/2004	Import							
4	F	03/2007	Import							
A(1x3)		09/2007								
A1	F	09/2007								
5	M	04/2007		Import					04/2013 † FIP	
B(1x2)		02/2008								
6	F	05/2008		Import						
7	F	08/2005		Import		Temporary adoption				
C(5xA1)		12/2008								
8	M	05/2008			Import	01/2010 † FIP				
D(1x3)		04/2009								
9	F	05/2009			Import	11/2010 † FIP				
E(5x7)		08/2009								
F(8x4)		01/2010								
G(8xA1)		02/2010								
10	F	07/2005				Import				
11	F	02/2010				Import				
H(15x9)		09/2010								
H1(15x9)	F	09/2010								
H2(15x9)	F	09/2010								
I(5x3)		09/2010								
12	M	11/2010					Import			
J(5x3)		08/2011								
K(15xA1)		03/2012								
K1 (15xA1)	F	03/2012								
L(12xH1)		04/2012								
L1(12xH1)	M	04/2012								
M(12xH2)		10/2012								
13[a]	F	?							Import	
14[a]	M	?							Import	
15	M	07/2009							† no FIP	

Abbreviations and symbols: † = death, M = male, F = female, FHV= feline herpes virus. Dark shading represents absence in cattery.

[a] Cat 13 and 14 are European Shorthair cats that were found outside and were imported in the cattery.

5.2.2.5 Serum antibody titre

As there is still some controversy on the correlation between antibody titres and faecal shedding, serum antibody titres were assessed in household 1 from October 2011 until February 2013. Therefore, porcine respiratory coronavirus-infected swine testicular cells, seeded in 96-well plates, were incubated with serial dilutions of the serum samples (50 µl/well). After 1 h (37°C), cells were washed with PBS containing 0.0025% Tween 80, followed by incubation with HRP-labeled rat anti-cat antibodies (1 h, 37°C). Visualization was done by adding sodium-acetate buffer containing amino-ethylcarbazole (AEC) and H_2O_2

for 10 minutes at RT. The antibody titer was the highest dilution at which infection could still be visualized.

5.2.3 Results

5.2.3.1 Household 1

In October 2011, the faecal shedding of all cats present in the house was monitored for the first time. At that time, most of the kittens born in 2011 had been sold, and 8/21 died from FIP in their new home. Two of the three affected litters had the same father (cat 5) that died of FIP itself in December 2010, but had a different mother (cat 7 and 9 for litter B and D, respectively). Litter E was the results of the mating of queen 8 with tomcat 6. At the first sample taking, 9 cats were still present in the house, and both faecal shedding and antibody titres were assessed for each of them (Figure 5.2.1).

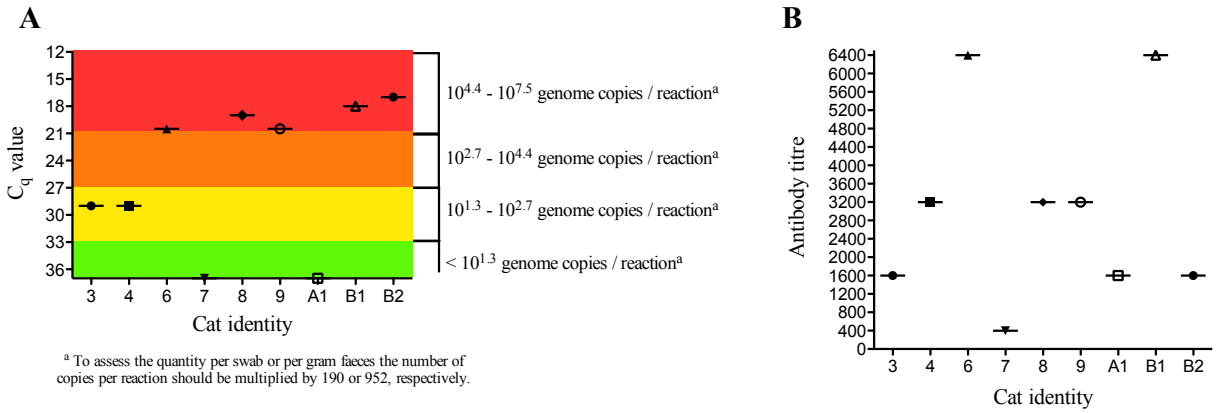


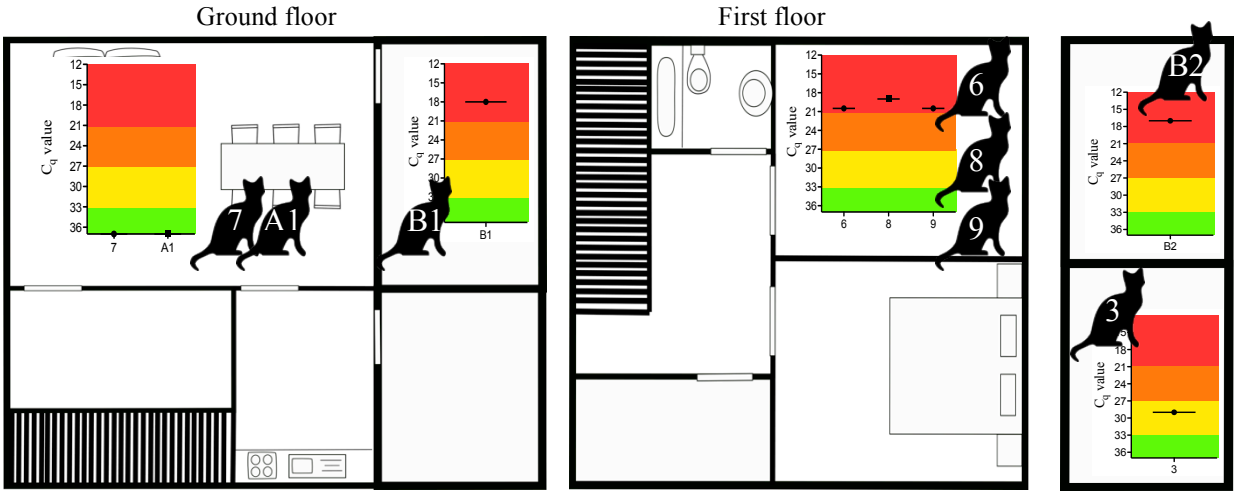
Figure 5.2.1. Semi-quantitative assessment of faecal shedding (A) and antibody titres (B) of all cats present in household 1 at the first sampling (October 2011).

Although all cats had serum antibodies, two cats (cat 7 and cat A1) did not shed FECV. Two other cats (cat 3 and cat 4) showed a moderate shedding, whereas the remainder of cats shed more than 4 million copies per swab. Cat 7 had the lowest antibody titre (400), followed by cat 3, A1, and B2 with a titre of 1600; cat 4, 8, and 9 with a titre of 3200; and cat 6 and B1 with a titre of 6400. Consequently, antibody titres did not correlate with the amount of faecal shedding, and would not have been a good parameter for grouping. Indeed, if grouping would have been based on antibody titre, the negative cat A1 could have been put together with the moderate shedding cat 3 and/or the high shedder B2.

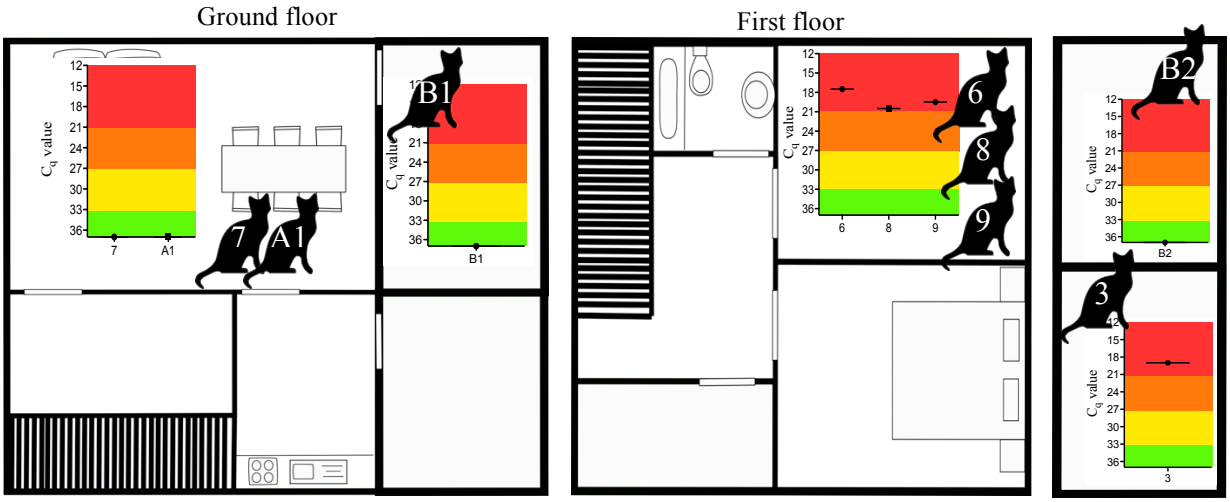
After having received the test results, the owner decided to lower infection pressure as fast as possible by removing some of its less valuable cats from the cattery. Therefore, cat 4 was sold, and cat 3 and B2 were given for adoption to family members, one of which was a

sporadic caretaker of the cats in household 1. Cat 3 and B2 remained available for further testing of shedding and antibody titres. The other cats were grouped in the house as shown in Figure 5.2.2. In January 2012, shedding was monitored again. The negative group (cat 7 and A1) was still negative, and cat B1 and B2 had become negative. All cats from the positive group (cat 6, 8, and 9) at the first floor were still shedding, as was cat 3. Cat B1 is a tomcat, and hence this cat could not be grouped together with the negative (female) cats. Consequently, all groups remained as they were. Shortly after the second sampling, cat 6 developed severe disease, characterized by waves of lethargy, anorexia and icterus. This cat finally succumbed, but whereas it was highly FIP suspected, pathological examination diagnosed the cat with hepatic amyloidosis, and not FIP.

October 2011

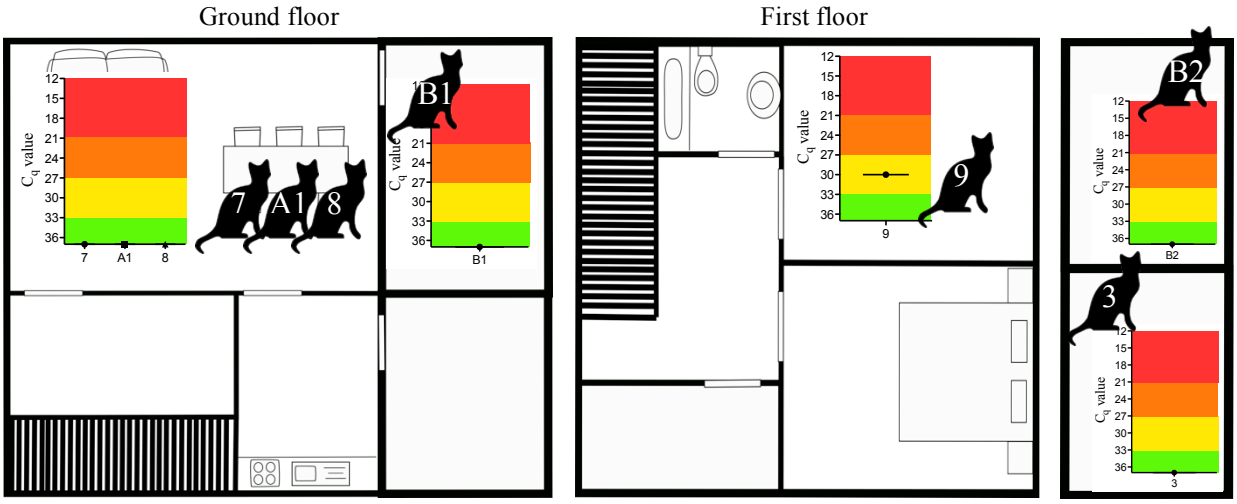


January 2012



April 2012

July 2012



October 2012

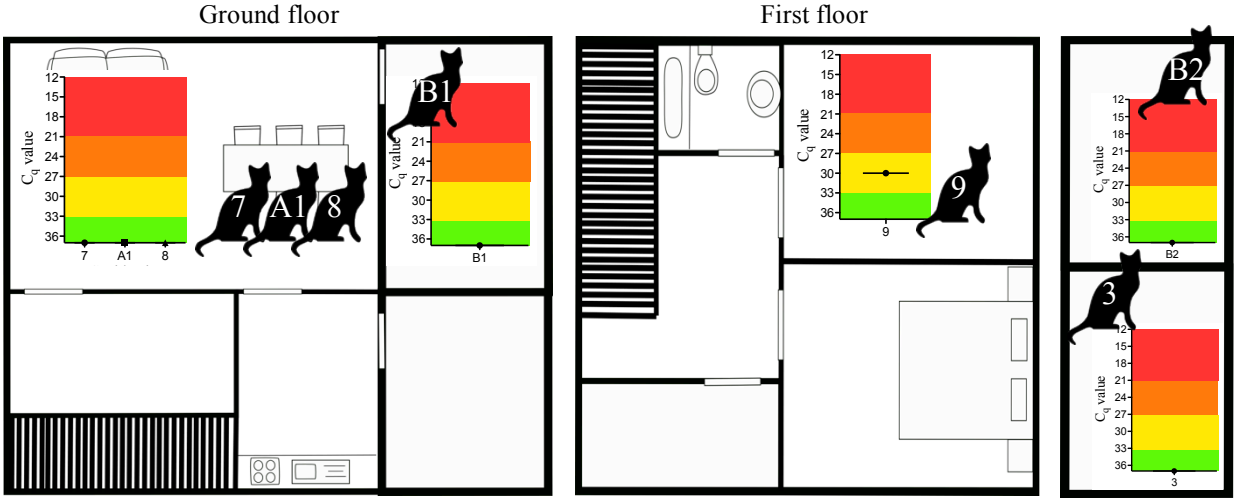


Figure 5.2.2. Shedding and grouping of cats in household 1 from October 2011 until October 2012. Figures represent the grouping of the cats after the shedding was monitored at the indicated time points.

In April 2012, all cats, except for the 2 remaining cats of the positive group, had become negative, and groups remained as they were. In July 2012, cat 8 had ceased shedding and was removed from the positive group to the negative group. Cat 9 was the only cat that remained shedding, and this was still the case in October 2012, one year after the first sampling. Consequently, this cat was identified as a persistent shedder and was kept isolated from the other cats.

As most of the cats were now non-shedders, breeding program was taken up again, and queen A1 was mated with tomcat B1 in January 2013. In February 2013, the next sampling was performed to make sure that all cats had indeed remained negative before the kittens would be born, as there was still a continuous risk for inadvertent transmission of FECV from the persistent shedder to the other cats (since no special precautions were taken to avoid such transmissions). All cats remained negative, except for the persistently shedding cat 9, which continued to shed the virus at moderate levels (Figure 5.2.3).

February 2013

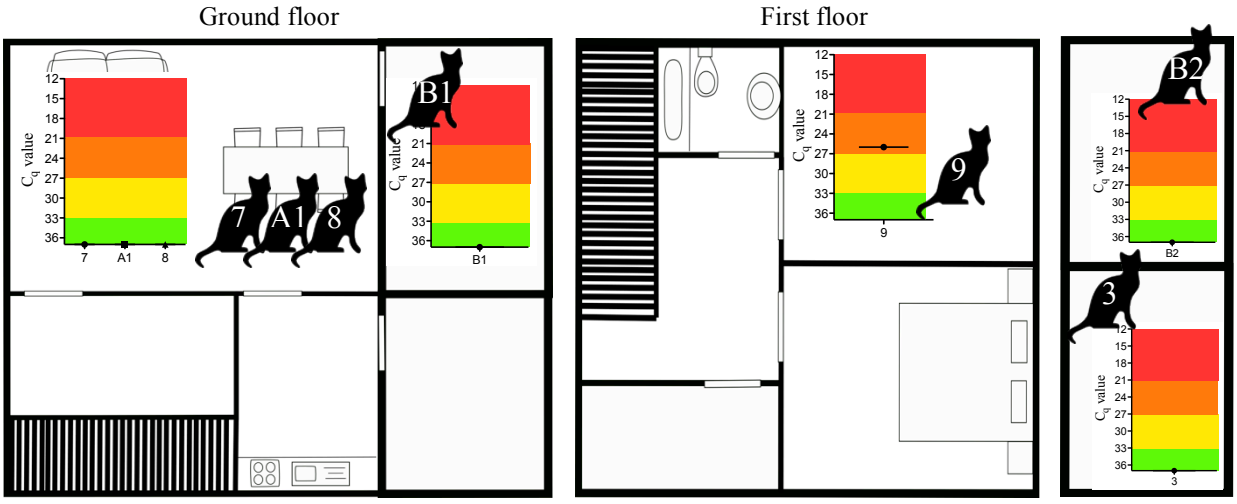


Figure 5.2.3. Shedding and grouping of cats in household 1 in February 2013. Figures represent the grouping of the cats after the shedding was monitored.

In addition to faecal shedding, antibody titres of the cats were assessed from October 2011 to February 2013. After the sampling in February 2013, it was decided to stop monitoring these antibody titres, as it became rapidly clear that this rather invasive parameter did not add any value in the management of FCoVs. Figure 5.2.4 overviews the evolution of both shedding and antibody titres for all seven remaining cats from October 2011 until February 2013. Whereas there was a clear correlation between low/absent antibodies and lack of shedding, higher antibody tires were much more difficult to interpret, as cats with higher antibody titres were not necessarily shedders. In addition, antibody titres declined much slower compared to

shedding (except for cat B2), clearly demonstrating the advantage of monitoring shedding over serum antibody titres. Despite the absence of shedding in cat A1, antibodies showed a small rise in titre in July 2012. Whether this is the result from restricted systemic replication, or rather results from variation of the antibody test, was not further determined.

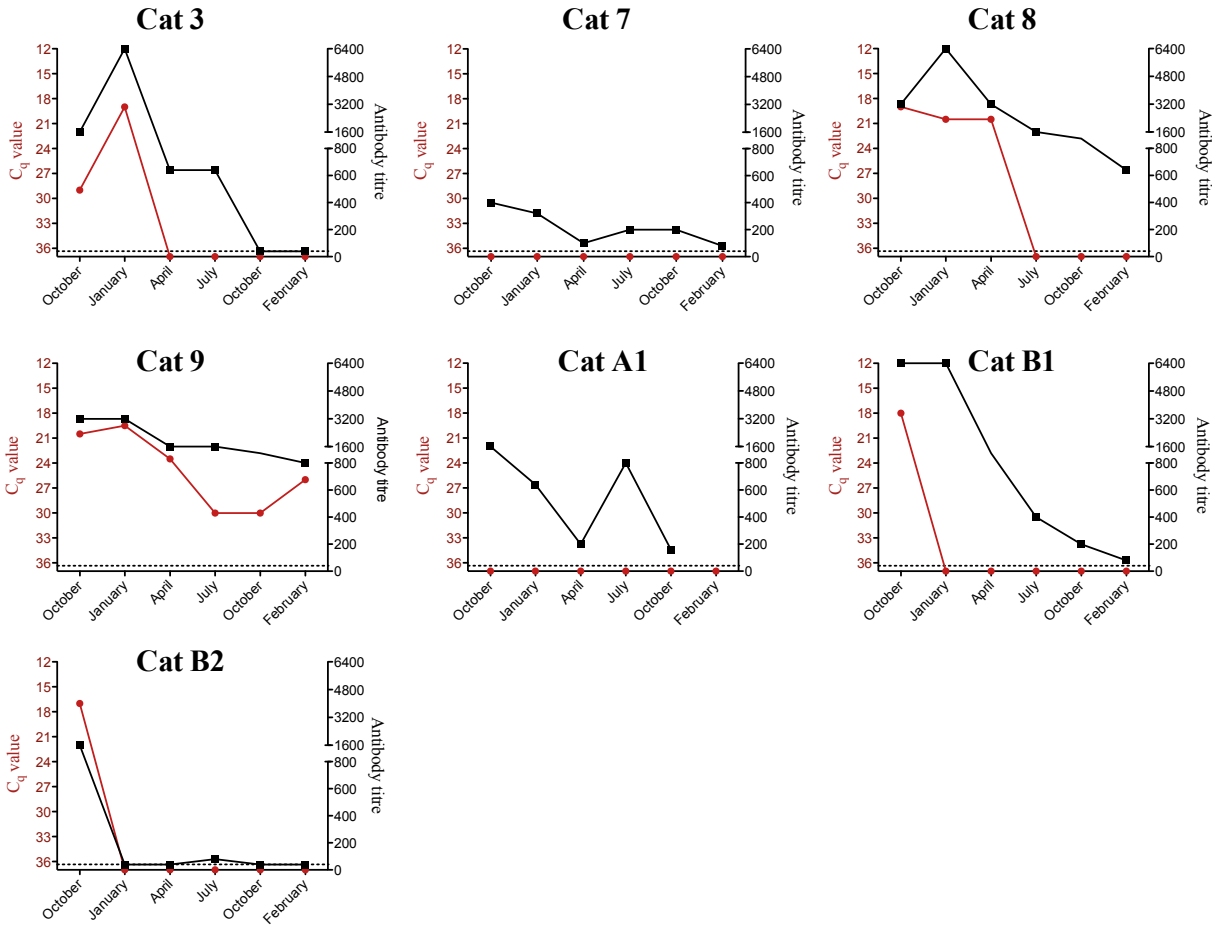


Figure 5.2.4. Evolution of shedding (red line) and antibody titres (black line) in 7 cats from household 1.

By the end of February, cat A1 was isolated for parturition, and in March 2013, the first litter (F-litter) was born since the start of the study. From April 2013, when kittens were 4 weeks of age, shedding of all cats, kittens inclusive, was monitored every 1-2 weeks to assure their negative status. At the time of sampling, kittens were housed in one room together with their mother and cat 7. Cat 8 and B1 were housed together for mating, and the persistently shedding cat 9 was still housed separately. All cats, except for cat 9, were negative. April 16th, a new cat (cat 10) was imported in the cattery. This cat was immediately isolated and tested for shedding, together with the remainder of the cattery cats. As expected given the widespread distribution of FCoVs, cat 10 was positive, and it was decided to house this cat with the persistent shedder for the welfare of the animals (Figure 5.2.5). During two

samplings in April, cat 7 showed a very weak positive signal, but this was so low that is was decided to keep this cat within the negative group, and this had indeed no further consequences, showing that this cat was not infectious for other cats (and kittens).

April 16 2013

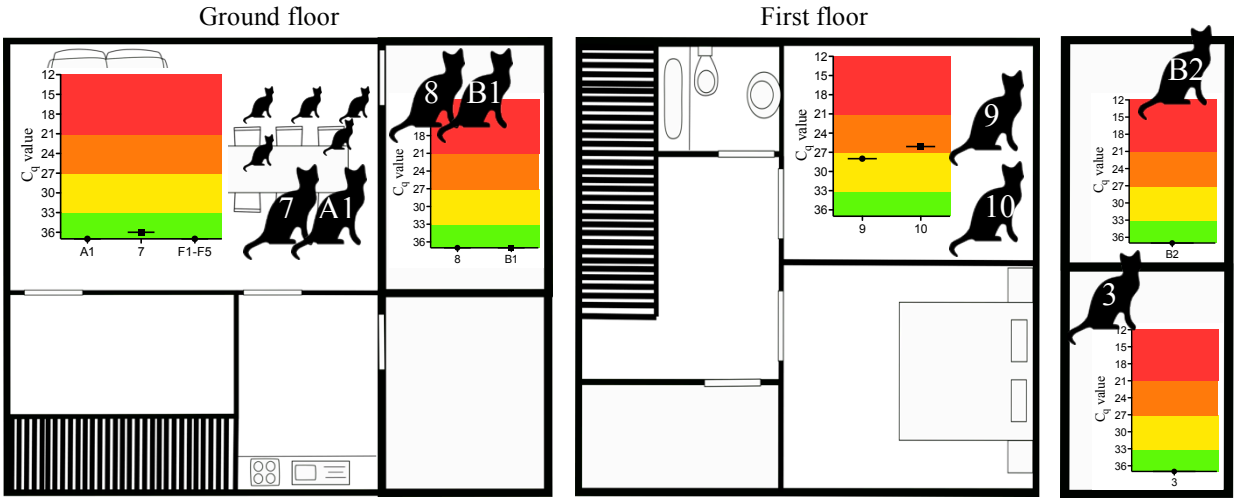


Figure 5.2.5. Shedding and grouping of cats after the introduction of new cat (10) in household 1. The figure represents the grouping of the cats after the shedding was monitored.

At the end of May, a new litter (G-litter) was born, resulting from the mating of cat 8 with tomcat B1. These kittens were born in a bench in the same room as cats A1, cat 7 and the F-litter, and were finally allowed to mix with each other (after having confirmed that all cats in the room were still negative), resulting in a total of 12 kittens and 3 adults in that room in June 2013. At that time, cat 9 and 10 were still shedding (Figure 5.2.6).

June 2013

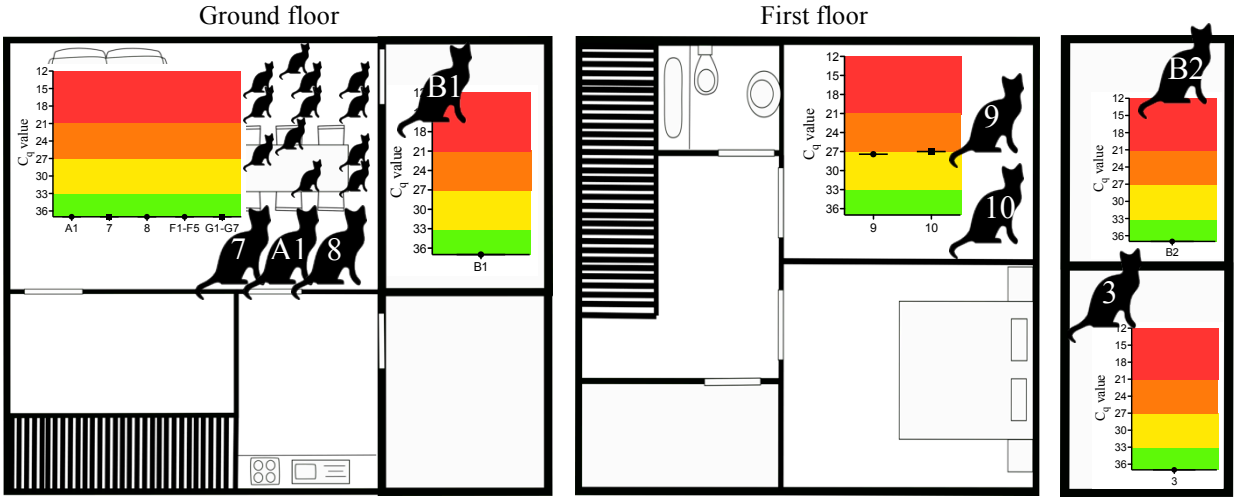


Figure 5.2.6. Shedding and grouping of cats of household 1 in June 2013. The figure represents the grouping of the cats after the shedding was monitored.

By the end of July 2013, 4 kittens of the F-litter had been sold, and all of them were negative before they went out and are all healthy at this time. One male kitten of the F-litter was kept for future breeding and housed together with the other tomcat B1. By then, cat 10 had become negative and was separated from the persistent shedder. As there were still kittens in the room of the negative group, cat 10 was kept in a separate room for 1 additional week, whereafter its faeces was monitored again for shedding to be absolutely certain that this cat was indeed negative. After having confirmed its negative status, this cat was allowed to mix with the negative group (Figure 5.2.7).

August 2013

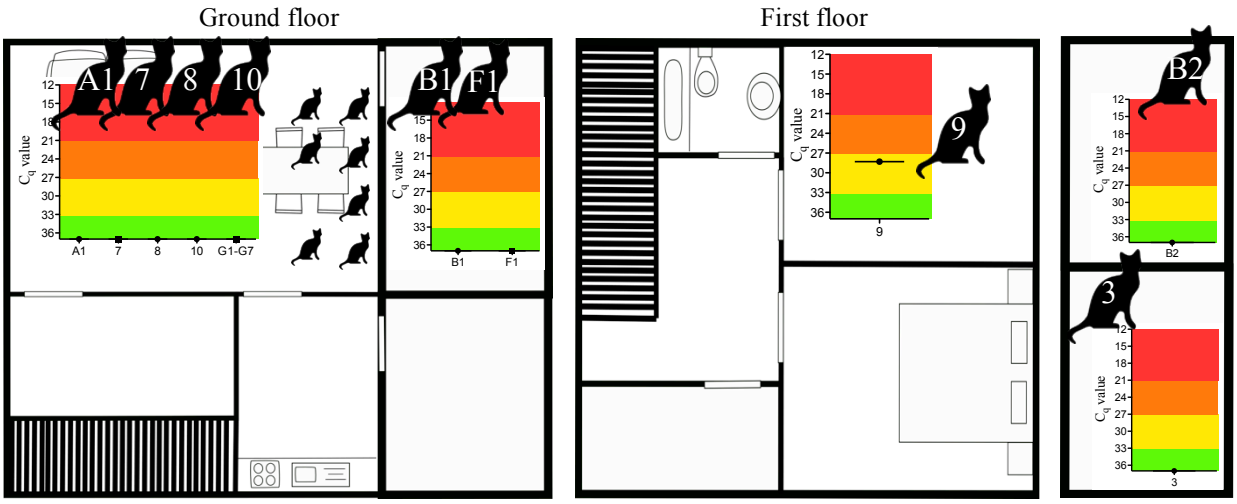


Figure 5.2.7. Shedding and grouping of cats of household 1 in August 2013. The figure represents the grouping of the cats after the shedding was monitored.

In August 2013, there was an outbreak of gastroenteritis, including anorexia, diarrhoea, and vomiting in all kittens, and one of those kittens finally succumbed to it. Faeces of all cats were still negative for coronavirus, but despite many other parasitological, bacteriological, and virological tests, the exact aetiology could not be discovered. In September 2013, queen A1 was exported for mating to another cattery. When she was imported again, she was isolated from the other cats for 4 weeks, in order to avoid outbreak of various infectious diseases. Upon arrival and after 4 weeks, she was tested for FECV shedding. Since she was negative, she was allowed to mix again with the others. All kittens of the G-litter, except for 1, were sold, and were negative when they went out, and are still healthy at this time. The other female kitten (G1) was housed with the negative cats. In November 2013, the persistent shedder died due to chronic kidney failure (which was not caused by FIP). Since the external mating of cat A1 had not been successful, she was mated again in the cattery with tomcat B1

in November 2013, as was cat 8. At that time, the entire cat population in household 1 was negative.

In February 2014, 2 litters of a total of 10 kittens were born from these matings. Unfortunately, 1 of the 2 queens (cat A1) did not survive the caesarean. As cat 8 also had 4 kittens (H-litter), it was decided to place 4 of the kittens from cat A1 (I-litter) with cat 8, and the other 2 (cat I1 and I2) were raised with an adoption mother that had just 3 new kittens in a British Shorthair cattery. In March 2014, when the kittens were 4-5 weeks of age, both catteries were screened for FCoV shedding in all cats. Whereas all cats, kittens inclusive, were negative in household 1, two adult cats from the adoption cattery (inclusive the adoption queen) were moderate shedders, one was shedding at low levels and the other was negative. More importantly, due to the shedding of the mother, kittens had already become positive before they were 5 weeks of age and were shedding enormous amounts (> 5 x 10^9 copies / gram, determined on pooled faeces of all kittens) of FCoV (Figure 5.2.8). At that time, kitten I1 suffered from anorexia and did not gain normal weight, but this resolved after 1 week.

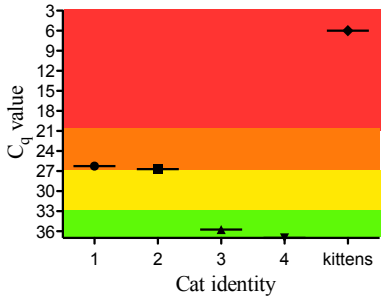


Figure 5.2.8. Shedding of cats and kittens in the adoption cattery in March 2014.

In March 2014, household 1 fused with another cattery, which consisted of 4 adult British Shorthair cats. The 4 cats (cat 11-14) were housed in a room at the first floor, and were monitored for shedding, together with the other cats at the ground floor. At that time, cat 7 and 8 were housed together with the kittens, and cat 10 and G1 in another room, as were tomcats B1 and F1. All cats at the ground floor were still negative, whereas all British Shorthair cats were positive, with 1 cat shedding at high levels, and the other 3 at low levels (Figure 5.2.9).

March 2014

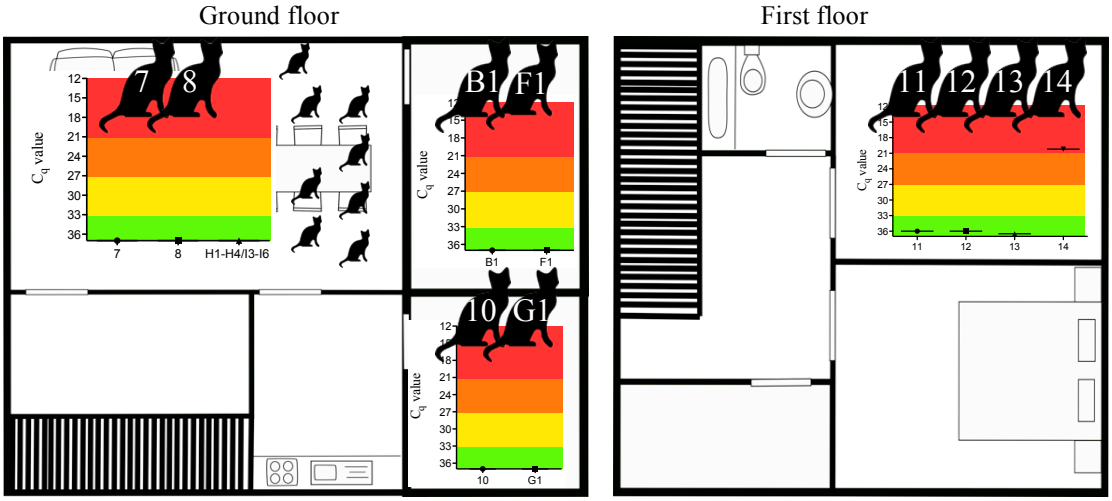


Figure 5.2.9. Shedding and grouping of cats and kittens in household 1 in March 2014. The figure represents the grouping of the cats after the shedding was monitored.

In April 2014, the 2 kittens (I1 and I2) were withdrawn from the adoption cattery, but since there was no more room available in household 1, they were kept in the house of a family member where also cat 3 was housed, but kittens were kept in a separate room. Both kittens were still shedding, although at substantial lower level compared to the initial screening (Figure 5.2.10). Kitten I1 suffered again for about 1 week from anorexia and weight loss at the age of 7 weeks (April 2014) and 11 weeks (May 2014), and nothing but coronavirus could be diagnosed in the faeces (bacteriological and parasitic examinations were negative).

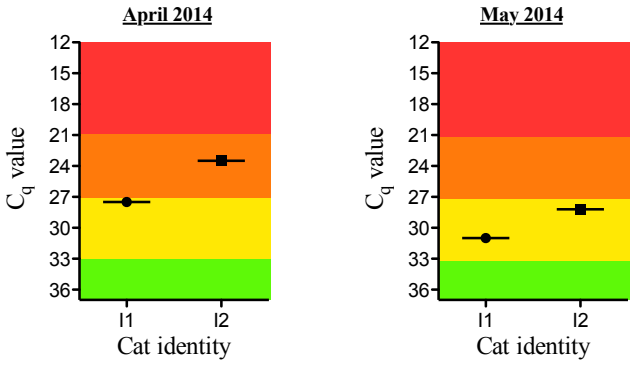


Figure 5.2.10. Shedding of the 2 positive kittens withdrawn from the adoption cattery in April and May 2014.

In the beginning of May 2014, new sampling was done for all cats in household 1. All cats at the ground floor were still negative, and 3 out of 4 cats at the first floor had become negative. Therefore, the only remaining shedder, cat 14, was separated from the others (Figure 5.2.11). At that time, a new puppy was introduced in the house. In addition, although kittens I1 and I2 were still shedding, it was decided to mix them with cat 3 for their socialisation.

May 2014

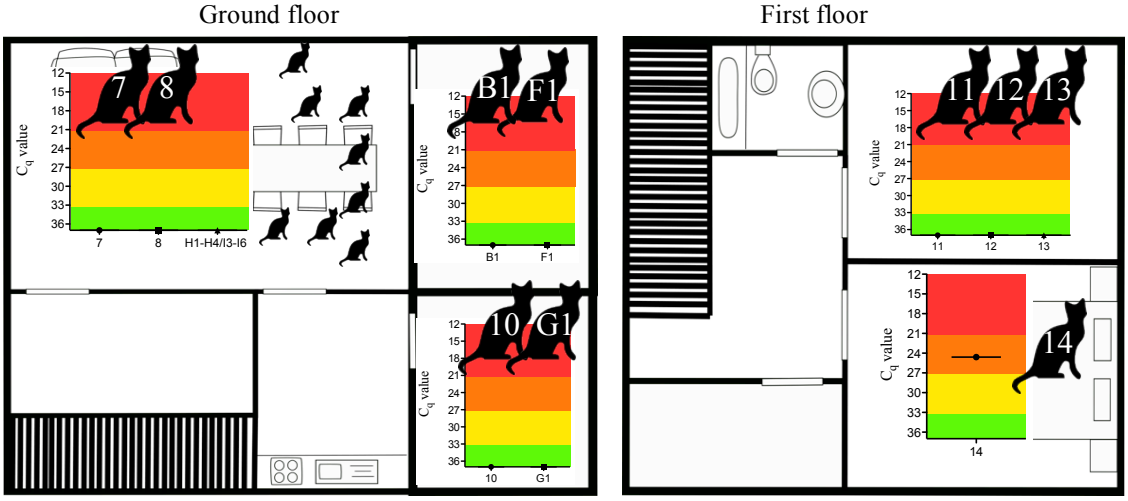


Figure 5.2.11. Grouping and shedding of cats in household 1 at the beginning of May 2014. The figure represents the grouping of the cats after the shedding was monitored.

At the end of May, 2 new British Shorthair cats (cat 15 and 16) were imported in household 1, and cat 13 was removed. The two new cats were kept separated from each other and from the other cats until they were tested. Cat 15 was a male adult cat and the other was a male kitten of around 14 weeks. The adult cat was housed at the first floor, and the kitten at the ground floor. Due to this import, all cats were tested again at the end of May (Figure 5.2.12). The new adult male cat (cat 15) was negative, but the new kitten (cat 16) was shedding moderate amounts of virus. Unfortunately, all kittens of the H-and I litter (then aged 13 weeks) and the two adult cats that were housed in the same room had also become positive, indicating an inadvertent transmission of the virus from 1 room to the other, for which kitten 16 had most probably been the source (based on the melting curve analysis). All the other cats at the ground floor that were housed separately from the kitten-group remained negative. Once the test results were known, owners realized that most probably the new puppy was the reason for the inadvertent transmission, since this dog had been allowed to cross the two rooms, comprising the kitchen and the living room, and had lately been playing around with the kittens and in the litter trays of both the new kitten and the negative kitten-group. This can also explain why the other groups remained negative, since the dog was not allowed to enter the rooms where these cats were housed. All of those kittens are still healthy to date. In contrast, 1 of the kittens that was infected at the age of 4 weeks in the adoption cattery, started to develop signs of FIP, including anorexia, weight loss, fever, and icterus shortly after it was sold. This cat was euthanized at the end of July 2014, and FIP was confirmed on necropsy.

End of May 2014

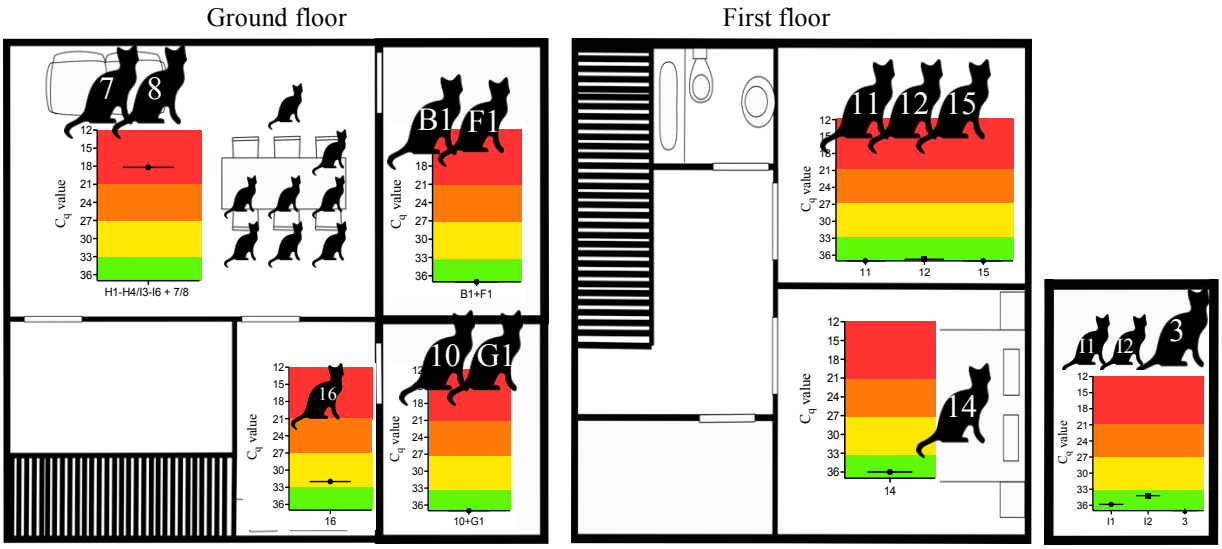


Figure 5.2.12. Grouping and shedding of cats in household 1 at the end of May 2014. The figure represents the grouping of the cats after the shedding was monitored.

5.2.3.2 Household 2

The first sampling in household 2 was done in June 2013 (Figure 5.2.13). At that moment, the cattery consisted of 10 female adults, 2 intact males (cat 12 and L1), and 1 neutered European Shorthair male (cat 14). All female cats were housed together in 1 room, the 2 intact males in another room. The neutered male was allowed to cross both rooms.

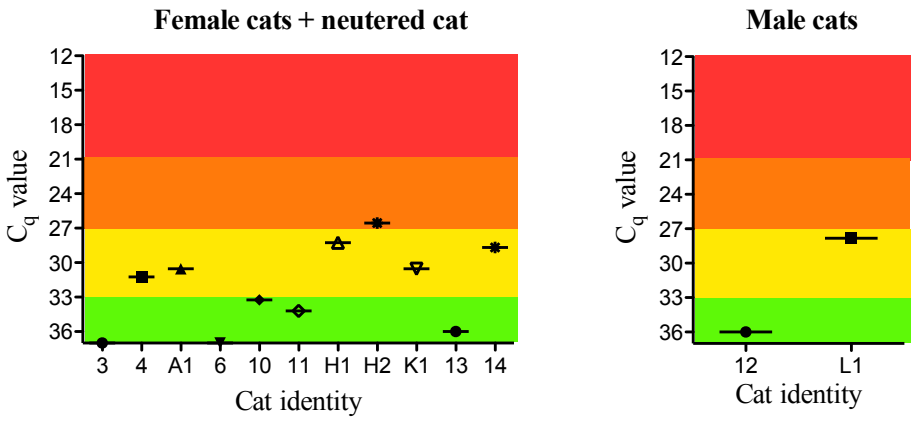


Figure 5.2.13. Shedding of cats at the first sampling in household 2 in June 2013.

Based on these results, cats were housed in 3 groups: the tomcat group, the negative group (composed of cat 3 and 6) and the positive female cat group (Figure 5.2.14). Cat 14 was allowed to cross the rooms of the tomcat- and the positive female cat group.

June 2013

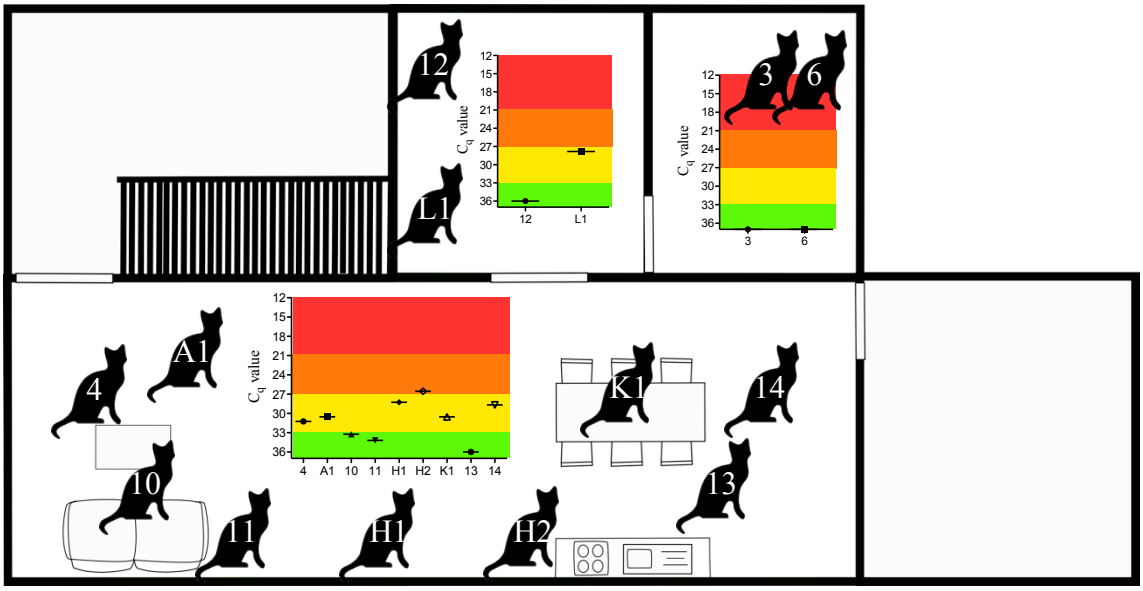
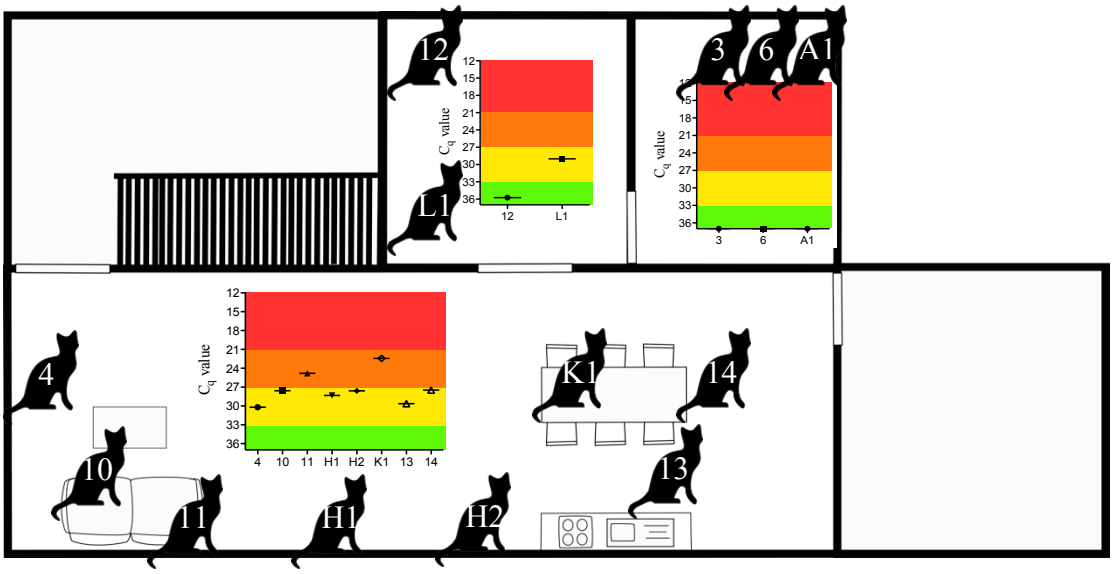


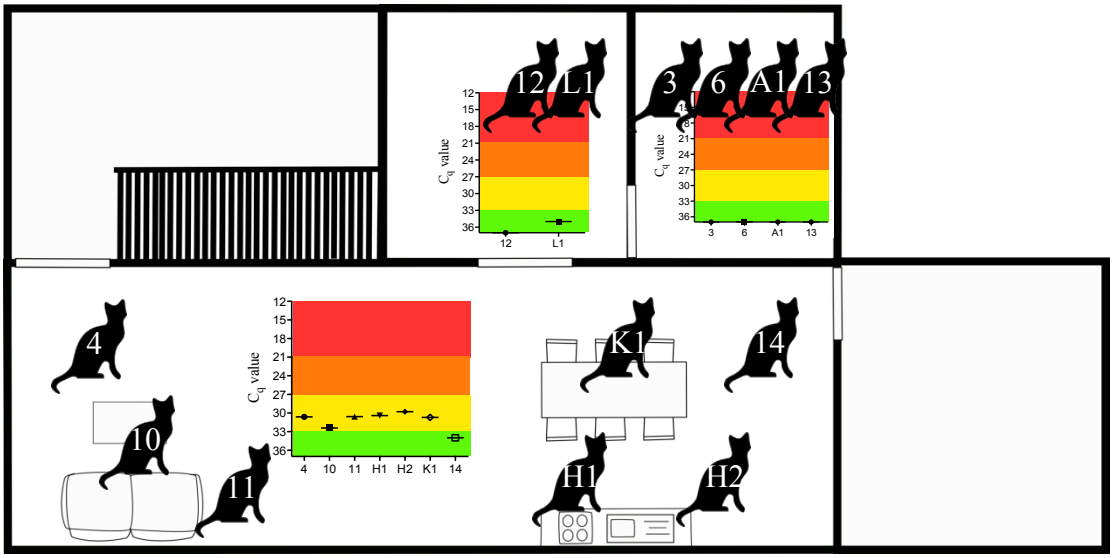
Figure 5.2.14. Shedding and grouping of cats in household 2 in June 2013. The figure represents the grouping of the cats after the shedding was monitored.

Thereafter, cats were tested regularly every 2-5 months and groups were adjusted based on the shedding state (Figure 5.2.15). By the end of august 2013, 1 additional cat (cat A1) had become negative and was grouped with the other 2 negative cats. By December 2013, 1 extra female cat (cat 13) had ceased shedding and was switched from group. Tomcat 12 had become negative, and tomcat L1 was shedding only very low amounts of virus. In March 2014, a new cat (cat 7) was imported in the cattery. This cat had been removed from the cattery in the past due to behavioural problems and had been housed in a single cat environment since then. As she was negative, she was allowed to mix with the negative group. By March 2014, both tomcats had become negative and the neutered male (cat 14) was no longer allowed to mix with these cats, since he was still shedding. After the sampling in March, the owner decided to switch the housing of the groups, since the positive group started to become too big to house in the room they were initially in. Therefore, the positive group was divided into 2 subgroups, and separated over 2 rooms, and the negative group became housed in the room where the positive cats had been housed. By July 2014, nothing was changed in the shedding of all cats, implying that half of the cats in household 2 were still shedding the virus 1 year after the first sampling. This is in sharp contrast to household 1, where all cats but the persistent shedder had ceased shedding within 9 months. As all young breeding animals were in the positive group, the owner decided to postpone the breeding program with the animals. Further follow up is necessary to find out which cats will eventually stop shedding, and which of them are persistently infected.

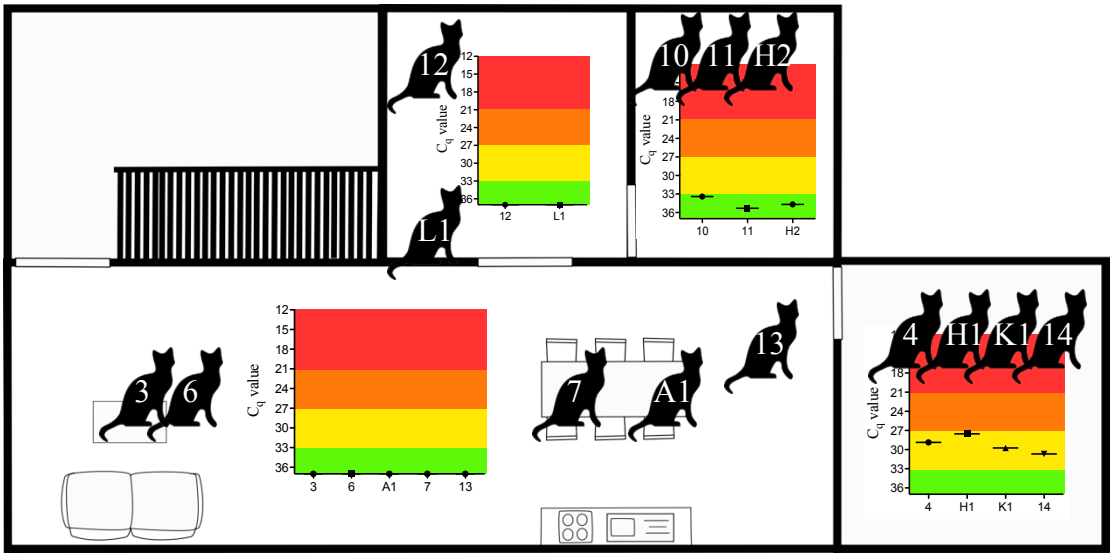
August 2013



December 2013



March 2014



July 2014

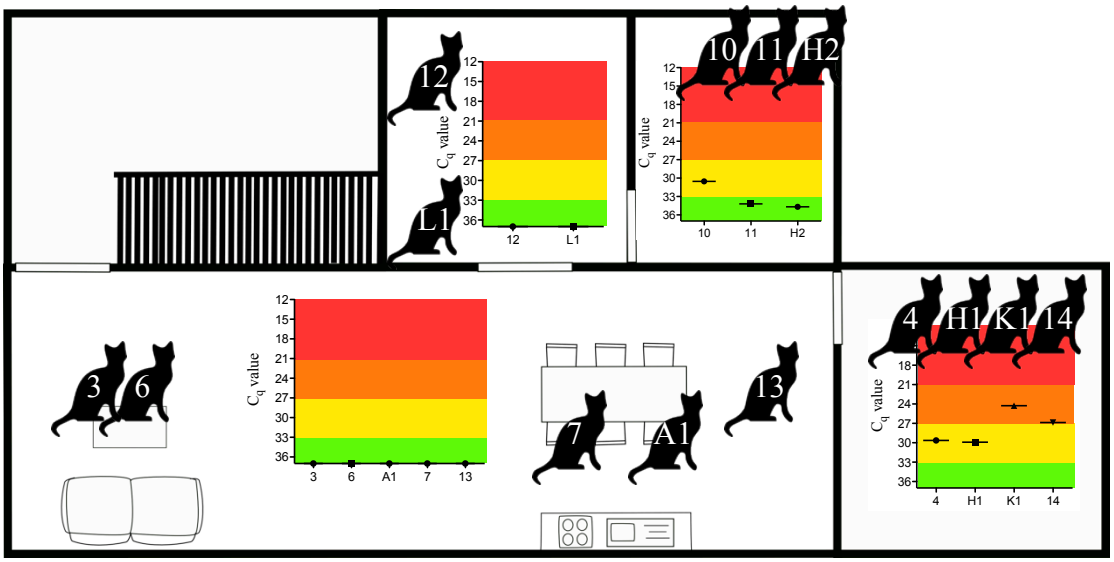


Figure 5.2.15. Shedding and grouping of cats in household 2 from August 2013 until July 2014. Figures represent the grouping of the cats after the shedding was monitored at the indicated time points.

5.2.4 Discussion

Despite the enormous progress made on epizootiology and pathogenesis since its discovery 5 decades ago, FIP has remained one of the few insurmountable and highly feared cat diseases to date. It is widely accepted that the majority of all FIP cases are the consequence of mutations arising in the viral genome during a common FECV infection (Chang *et al.*, 2010; Pedersen *et al.*, 2009; Pedersen *et al.*, 2012; Poland *et al.*, 1996), although this internal mutation theory has been questioned during infrequently observed epizootics of FIP, i.e. when FIP deaths greatly exceed the normally encountered 5-12% of all seropositive cats (Addie & Jarrett, 1992; Kipar & Meli, 2014; Pedersen, 2009; Wang *et al.*, 2013). The huge FECV infection pressure in multi-cat environments together with the often genetically predisposition of those pure bred cats favour the development of FIP, especially in the young cats. Due to the high demand for control measures to avoid these enormous financial and emotional losses, the aim of this study was to evaluate if it is practically feasible and cost-effective to control faecal-oral transmission of FECV by regularly monitoring of the faecal shedding and grouping of cats.

Two medium sized catteries (<15 cats) that faced FIP deaths in the past were followed for 3 years (household 1) or 1 year (household 2). In 2011, household 1 had faced over 30% FIP deaths in its kitten population. Two out of three affected litters (B- and D-litter) had the same father, which died of FIP itself at the age of 4.5 years (cat 5). Although the other affected litter (E-litter) had a completely different genetic background, also 4/7 kittens finally

succumbed to FIP. Consequently, the high incidence of FIP at that time was most probably the unfortunate coincidence of the high infection pressure (16 kittens were present at the same time and allowed to mix), combined with the higher genetic predisposition to develop FIP. In order to control the infection pressure, it was decided to breed only with negative animals in the future, as this seemed reasonably feasible to obtain in this cattery, which does not necessarily has to export/import cats for mating. By grouping of cats based on their shedding status, all but one of the cats had ceased shedding by July 2012, which is 9 months after the start of the monitoring in October 2011. By then, 4 samplings had been performed, with a total cost of €510 (VAT exclusive) to test all cats 4 times. At that time, the owner could have started the breeding program, since all but one of the female cats and the tomcat were negative. However, he decided to wait in the hope that the last cat would also cease shedding, taking away any further threat for the kittens. Unfortunately, this cat was still positive at the next sampling and remained persistently shedding the virus during the remainder of the follow up. In January 2013, 2 years after the birth of the last litter, breeding program was taken up again, as all cats but the persistent shedder remained negative. By assuring that all breeding and in contact animals were negative before the kittens were born, all 12 kittens from the 2 litters did not have to be isolated from the other cats and remained FCoV-negative until they were sold at the age of 13-14 weeks. If they became infected thereafter was not investigated. For many cattery owners the question arises whether the effort to avoid exposure in their cattery is worthwhile, as kittens can indeed be infected later in life when rehomed. Many kittens raised in catteries are sold to private owners and will no longer be housed in a multi-cat environment, making the risk that they will ever develop FIP almost negligible. In addition, for kittens that are sold to other catteries, one should take into consideration that delaying FECV infection decreases the FIP incidence, since older kittens have lower replication rates and a more mature immune system to combat the mutant viruses if these arise, which is the reason why it is worthwhile and highly recommended to delay exposure as much as possible (Addie *et al.*, 2004; Pedersen *et al.*, 2008). Indeed, it was shown in this study that 1 of the 2 kittens that were exposed at 4 weeks of age developed FIP, whereas the other 4 kittens from the same litter, and 4 kittens from another litter with the same father that were exposed at 12-13 weeks remained healthy to date, although all these kittens were potentially prone to the development of FIP due to the extensive FIP history in the fathers line. The exposure of these kittens at the age of 12-13 weeks was the result of an inadvertent transmission of FECV from the adjacent room where a newly imported positive kitten was housed to the room of the negative kitten group. Inadvertent transmission of FECV has

previously been described as an event that readily occurs from 1 room to another by pieces of litter on the body, clothing, or shoes, and has been recognized as a major problem in the management of FCoVs (Hickman *et al.*, 1995; Pedersen *et al.*, 1981). However, inadvertent transmission had never been noticed in the present study in neither of the 2 households, although no special precautions were taken considering clothing, people movement and handling of cats, and despite the fact that caretakers were running back and forth from positive and negative animals within and outside (including cat 3, B2, I1, and I2 of household 1) the cattery. As the inadvertent transmission noticed at the end of May 2014 in household 1 had never occurred before and was confined to only 1 room, it was thought not to result from people movement, but had been attributed to the dog, for which owners later realized that this puppy had been playing around with the kittens and in the litter trays of both groups. In addition, melting curve analysis revealed identical melting peaks for the strain of the newly imported positive kitten and the other kittens, suggesting that this new kitten was indeed the source of the inadvertent transmission. Whether or not the dog was indeed the reason for the transmission remains unknown, but these data emphasize that care should be taken at any time to avoid such accidental infections. Import cats are often only a few months of age and it is known that young animals shed very high amounts of virus (Pedersen *et al.*, 2008). Consequently, these cats are highly contagious and a major risk for such inadvertent transmissions. Therefore, it is recommended to thoroughly monitor the shedding of every imported cat and to separate these cats as far as possible in the house from negative cats, as was done in the present study with cat 10 in household 1. This separation included the housing in a non-adjacent room, which was sufficient to maintain a negative kitten population without the need for unfeasible quarantine measures. In contrast, by housing the newly imported kitten (cat 16) in the adjacent room, transmission of the virus to the negative kittens could not be avoided. Fortunately, the exposure of the kittens was postponed until the age of 12-13 weeks, and has so far not had any consequences, as all 8 kittens are still healthy. In addition, there was no further spread from the kittens to other animals in the cattery (except for 2 adults cats that were housed with the kittens), which will make it possible for the owners to continue breeding with negative animals.

The present follow up studies showed that it is practically feasible to restrict the FECV transmission, and hence FIP incidence, in multi-cat environments by regularly monitoring and grouping of cats. In household 1, the complete 3 years follow up had a total cost of around €1500 (VAT exclusive), which was certainly worthwhile the effort and cost since the FIP

losses in 2011 succeeded more than €4000 of costs on top of the fact that kittens of this cattery became less wanted. This cattery had the advantage that the management procedures were highly feasible by the availability of many rooms (and family that wanted to take care of some cats if they could not be housed in the cattery for some time), and by the fact that taking negative cats for breeding was highly facilitated, given that no import or export was needed for mating, which certainly could have complicated the whole procedure. Indeed, exporting queens/importing tomcats for mating increases the risk that queens become (re)infected, and should be accompanied by screening the shedding state of the tomcat if one wants to prevent this. Other efforts that had to be considered were especially hygienic ones, such as daily cleaning and disinfecting of litter trays, and regular cleaning and disinfecting surfaces in the house, thereby first cleaning the room of negative cats. No special precautions were taken considering people movement, handling of cats or clothing, which made the grouping procedure practically feasible according to the owners. For household 2 on the other hand, which never faced FIP deaths in the kitten population, this management procedure has been less cost-effective so far, as only half of the population ceased shedding after 1 year of sampling, which has cost €730 so far to screen all 14 cats 4 times. Extreme long-lasting shedding (> 1 year) occurred both in purebred cats as in a European Shorthair cat in this household, and could be the result of the strain (Addie *et al.*, 2003), or the infection dose (Vogel *et al.*, 2010), as previously reported for natural and experimental infections, respectively. Whether this persistent infection is also the cause of the many FIP problems in the adult cats in household 2 remains elusive. Nevertheless, it is clear that the major costs are determined by the duration of shedding, which is known to be cat- and strain-dependent and unfortunately cannot be predicted at the start of the control program. Generally taken, one should count for at least 1 year to obtain sufficient negative breeding animals. In addition, feasibility of grouping highly depends on the number of animals and the available rooms in the household, and will be less feasible and even much more time- and money-consuming in large multi-cat households (>20 animals) compared to the households discussed in the present study. This further supports the need to invest in the development of efficient tools for the prevention or treatment of FCoV infections.

In consistency with previous reports, no clinical signs were seen in cats that were infected with FECV (Meli *et al.*, 2004; Pedersen *et al.*, 2008; Pedersen *et al.*, 1981; Vogel *et al.*, 2010). Even most kittens from the adoption cattery that were shedding enormous amounts of FECV at the age of 4-5 weeks remained healthy. However, one of those kittens showed bouts

of anorexia and weight loss/lack of weight gain, first noticed at 4 weeks of age, and later seen at 7 and 11 weeks of age. This cat finally succumbed to FIP when she was less than 6 months, so it remains unknown whether these waves of illness were due to the enteric infection or rather were the result from the gradual development of systemic disease, which is known to occur in waves (de Groot-Mijnes *et al.*, 2005). Nonetheless, the swift development of FIP in this cat allows us to assume that the FIPV strain can only be generated from the initial enteric infection, and full genome sequencing will allow genetic comparison of this FECV/FIPV tandem. In addition, as faecal shedding from this kitten and her healthy brother was monitored several times before she succumbed to FIP, it would be very interesting to investigate whether or not viruses found in the faeces of these two cats show any difference, and how these enteric viruses evolved over time. Clinical signs of gastroenteritis were noticed in FECV-negative kittens, and one of the kittens even succumbed to it. Despite many efforts to identify bacterial, parasitological, and other viral aetiology, no diagnosis could be made. In any other cattery where FCoV viruses are endemic, this diarrhoea would probably have been falsely dedicated to the intestinal coronavirus infections, implying that it is often hard to reliably identify the real aetiology of diarrhoea in cats in FCoV endemic environments.

It has been stated that false negative results due to intermittent shedding of the cats can raise problems in grouping of animals, and that 5 consecutive negative monthly tests are necessary to confirm the cats' negative shedding state (Addie & Jarrett, 2001). In the present study, intermittent shedding was indeed noticed in a minority of the cats (cat 7 and 12 in household 1), but only in cats that were recovering from FECV infection, which is in consistence with a previous report (Herrewegh *et al.*, 1997). These cats were shedding such low amounts of virus that this could have been missed, even if it was assured that swabs were taken deep enough in the rectum and a highly sensitive RT-qPCR was used. However, this 'intermittent shedding' did not at all hamper the grouping, as these cats were no longer infectious to other cats and even kittens. This implies that these cats can be safely grouped with the non-shedders, provided that sampling has been correctly performed and PCR test are highly validated. This is in agreement with a previous study, reporting that low-level positive faecal samples may not be infectious (Foley *et al.*, 1997). However, if one wants to add a new cat in a negative group where very young animals reside, which should be avoided as much as possible, it is indeed recommended to additionally confirm that no false negative results were generated by the sampling or the test, as was done in this study for cat 10 in household 1. Indeed, it was noticed in the present study that shedding in the positive cats could fluctuate between low and

high during different samplings. If for reasons stated above (bad sample taking or inadequate PCR) the low shedding would have been missed, this could indeed have lead to improper grouping of the cats.

Since the discovery that serum antibody tests cannot distinguish between FECV infections and FIP, the value of these tests has been questioned (Pedersen, 1995). Their use for management purposes has also been questioned, but this is still regularly performed in practice, notably prompted by the occurrence of FIP cases. In accordance with previous reports, the present study confirms that antibody tests are a waste of money if performed for the management of FCoV infections in catteries, since they cannot be properly interpreted. Indeed, low/absent antibody titres appear in cats that recovered from FECV infection, but have also been seen in cats that were infected within 10 days before testing (see chapter 5.1), and hence shed enormous amounts of virus. In addition, very high antibody titres are seen in both healthy shedders and non-shedders, making them not at all useful for grouping of animals, and certainly not for the prediction of FIP development. If one wants to spent money on FIP prevention, present study shows that it is certainly more worthwhile to consider grouping based on shedding instead of antibody titres. The motivation for this grouping is that FCoV transmission to kittens can be effectively avoided if one makes sure that the queen and all in contact animals are negative before the kittens are born. To obtain such a non-shedding population, it is recommended to monitor the faecal shedding of all cats every 2-3 months, and to group them in at least one positive and a negative group in separate, and if possible non-adjacent rooms. Care should be taken that swabs are inserted deep enough into the rectum (to avoid false negative testing), and that inadvertent transmission of the virus to the negative group is minimalized (e.g. by regular cleaning/disinfection (sodium hypochlorite), thereby first cleaning the litter trays/room of the negative group), but no strict quarantine measures are needed. In addition, the shedding of every newly imported cat should be thoroughly screened before decisions are made on the housing of the cat.

In conclusion, present study reports the successful control of FECV transmission from shedders to naive animals by regularly monitoring of shedding and grouping of cats in small to medium-sized catteries (<15 animals), without the need for strict measures concerning people movement, clothing, or handling of cats. Cost-effectiveness of this strategy depended on the household, showing that especially in multi-cat environments with major FIP problems it is worthwhile to thoroughly monitor FECV transmission, as this will prevent enormous financial losses by kitten mortality. Although effective, monitoring is (and will always be) a

time- and money-consuming approach, due to the worldwide distribution, the long-lasting shedding and the highly contagious character of FECV. This further supports the need to invest in the development of efficient tools for the prevention and/or treatment of FCoV infections.

Acknowledgements

We gratefully thank the owners for their cooperation to the study. Special thanks goes to Ytse Noppe for her excellent technical assistance. LMBD was supported by a doctoral fellowship of The Research Foundation-Flanders (FWO Vlaanderen).

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Chapter 6

General discussion

Coronaviruses have been reported as a serious threat in many animal species since the mid twentieth century, but only began to gain particular public and scientific interest when threatening the human species during the severe acute respiratory syndrome (SARS) outbreak in 2002-2003. This was further strengthened when a new fatal coronavirus, Middle East respiratory syndrome virus, from camel origin emerged in humans in 2012. But despite this increased research interest, it has remained quite challenging to find effective curative and/or preventive measures to combat coronaviruses, and FCoVs are undoubtedly one of the most fascinating examples of this failure. About 5 decades of research have led to several dozen potential vaccines/treatments, which all have been proven unsuccessful so far (Pedersen, 2009, 2014). Coronaviruses are known to be prone to recombination and mutation events in their RNA genome, and both features seem to have indirectly hampered feline coronavirus (FCoV) research. Recombination events between FCoVs and canine coronaviruses (CCoVs) have led to the classification of FCoVs in two serotypes, serotype II viruses being the result of recombination between original feline serotype I viruses and CCoVs (Fiscus & Teramoto, 1987a, b; Herrewegh *et al.*, 1998; Hohdatsu *et al.*, 1991; Lin *et al.*, 2013). These serotype II viruses represent only a minority of all FCoVs (Addie *et al.*, 2003; Benetka *et al.*, 2004; Hohdatsu *et al.*, 1992; Kummrow *et al.*, 2005; Lin *et al.*, 2009; Vennema, 1999), but have been quite extensively used in the search for effective vaccines/antivirals, as these have been the only viruses that could be easily propagated and studied *in vitro*. However, some of the genes, including the gene encoding for the tropism- and immunity-determining spike protein, are considerably different from the more clinically relevant serotype I FCoVs, which can be one of the reasons for the lack of *in vivo* efficacy of at least some of the generated treatments/vaccines, especially when they target the entry process. With this in mind, many scientists have redirected their research to serotype I viruses in recent years. Genome analysis and comparison have aimed at identifying the other genetic event that readily occurs in feline coronavirus genomes, namely the mutational switch from an avirulent enteric virus (FECV) to the deadly systemic FIPV (Chang *et al.*, 2010; Chang *et al.*, 2012; Licitra *et al.*, 2013; Pedersen *et al.*, 2009; Pedersen *et al.*, 2012; Porter *et al.*, 2014). However, this search has been seriously hampered by the fact that, due to high mutation rate, FECV exists in so many different strains, characterized by distinct single nucleotide polymorphisms and insertion/deletions, and by the fact that (almost) every FIPV has its own specific mutations as it originates *de novo* during infection with FECV (Pedersen, 2009). This makes comparison between FIPV strains and the search for the pathotype-switching mutations very difficult. In addition, as long as serotype I viruses cannot be properly grown and studied *in vitro*, it will

remain difficult to assess if mutations/deletions are indeed the cause of the pathotype switch, and to generate appropriate antiviral measures.

Due to its low pathogenic character and the inability to grow the virus *in vitro*, FECV has not yet received much attention in the search for effective preventive measures or antivirals. However, given that FECV infection precedes the development of FIP, this virus is an attractive target in the fight against FIP. Therefore, this thesis aimed to focus on this root of all troubles, by providing cell cultures to grow and study (serotype I) FECVs, by extending the knowledge on FECV pathogenesis and enterocyte interactions, and by providing some (future) directions to combat this virus in the fight against FIP.

6.1 The initiation of feline coronavirus infections: what about the intestinal phase?

It is widely accepted that most FIP cases are the consequence of mutations arising in the viral genome during a common intestinal FECV infection (Chang *et al.*, 2011; Chang *et al.*, 2010; Pedersen, 2009; Pedersen *et al.*, 2012; Poland *et al.*, 1996). FECV is easily transmitted between cats by the faecal-oral route, and maintained within a cat population by continuous transmission from shedders to susceptible animals, the latter including all cats that have lost local, passively (=maternal) or actively acquired immunity (Addie *et al.*, 2009; Addie *et al.*, 2003; Foley *et al.*, 1997; Pedersen *et al.*, 2008). Experimental infections and *in vivo* follow-up studies have added considerable understanding of how FECV infections are established and maintained within a cat population (Addie & Jarrett, 1992; Addie *et al.*, 2003; Meli *et al.*, 2004; Pedersen *et al.*, 2008; Pedersen *et al.*, 1981; Poland *et al.*, 1996; Vogel *et al.*, 2010), but many insights are still lacking to completely understand how this virus interacts with its target cell and its host. To address some of these missing links, both *in vitro* studies and experimental FECV infections were conducted.

6.1.1 Establishment of intestinal epithelial cell cultures to propagate serotype I FECVs and study FECV-enterocyte interactions (Chapter 3)

At the start of this project, neither a representative *in vitro* model nor FECV strain was available to study FECV-enterocyte interactions. Therefore, the first part of this thesis focussed on the establishment of enterocyte cultures that would allow the propagation of the clinically relevant serotype I viruses and the study of FECV-cell interactions. A technique was optimized for the isolation and cultivation of primary intestinal epithelial cells from the ileum (= ileocytes) and colon (= colonocytes), as both intestinal segments are known to sustain FECV replication (Herrewegh *et al.*, 1997; Kipar *et al.*, 2010; Meli *et al.*, 2004; Pedersen *et al.*, 1981; Vogel *et al.*, 2010). Isolation of primary intestinal epithelial cells is

often complicated by induction of programmed cell death after detachment from the extracellular matrix (designated as anoikis), the uncontrolled contamination with stromal cells and/or bacteria, and the still unknown homeostatic components needed to maintain these cultures (Kaeffer, 2002). To avoid anoikis during isolation of primary enterocytes, intestinal epithelial cells were isolated in cell clusters, still attached to each other and to the laminin part of the basement membrane. This was obtained by using a combination of dispase and collagenase, which digest the fibronectin and collagen but not the laminin layer of the basement membrane. Another advantage of the isolation of cells in clusters is that contaminating mesenchymal cells, which occur as single cells, can be separated from the much larger epithelial cell clusters based on their density, which was done in the present thesis by using 2% sorbitol solution to wash the digested mucosa several times. This protocol allowed the successful isolation of primary ileocytes and colonocytes, characterized by the expression of the epithelial cell marker cytokeratin, and absence of the mesenchyme cell marker vimentin.

Primary epithelial cell cultures are ideal tools to reliably investigate virus-host interactions. However, their isolation is labour-intensive, the cultures are often contaminated with a various amount of mesenchymal cells, and the yield is variable and rather low. In addition, primary feline intestinal epithelial cells underwent no (ileocytes) or only a restricted number of replications (colonocytes) to finally end up in replicative senescence, making these primary cells useless for future long-term, standardized studies. To overcome this barrier of replicative senescence, primary ileocytes and colonocytes were immortalized by inducing SV40 T-antigen- and hTERT-expression. The epithelial nature of the generated continuous cell lines was confirmed by the cobblestone morphology, dome formation and cytokeratin expression. However, in contrast to the primary cells, these cultures were characterized by a co-expression of both cytokeratin and vimentin, a feature that is typically seen in dedifferentiated epithelial cells (Baer & Bereiter-Hahn, 2012). Therefore, the reliability of these cultures as *in vitro* model for enteric FCoV infections was investigated. This was done by comparing replication capacity of the serotype II FCoV WSU 79-1683 and FCoV WSU 79-1146, since these strains were the only viruses that could be propagated at that time, and hence were available to perform standardized, comparative studies between enteric and systemic FCoVs. FCoV WSU 79-1683 is an enteritis-inducing strain, but is believed to rather be an avirulent FIPV than a real FECV, as this strain shows a 3c deletion, a genetic hallmark that has so far only been observed in FIPVs (Chang *et al.*, 2010; Pedersen, 2009; Pedersen *et al.*, 2012). In

contrast, FCoV WSU 79-1146 is one of the most virulent FIP-inducing strains described so far. Two major conclusions were drawn from these experiments: 1) the avirulent, enterotropic FCoV strain 79-1683 infected enterocytes much more efficient compared to the FIP-inducing strain 79-1146, and 2) both viruses infected enterocyte cultures similarly to what was observed for primary cells, making these continuous cell cultures reliable as *in vitro* model to study enteric coronavirus infections.

After having established this *in vitro* model, it was investigated whether we would also be able to propagate and study field strains in order to make future research more reliable. By then, cultivation of an enteric strain had never been achieved, and only few serotype I FIPV strains had been adapted to grow in continuous cell cultures. Seventeen faecal samples from healthy cats (1 including the prototype FECV strain UCD (Pedersen *et al.*, 1981)), and faecal and tissue samples from 4 FIP cats were collected, and the amount of FCoV genomic RNA was quantified by RT-qPCR. For each sample, the presence and amount of enterotropic infectious virus was determined by immunofluorescence and IPMA, respectively. Of the 14 positive faecal samples collected from healthy cats, 8 were found to contain virus that was able to infect the enterocyte cultures. Of the 7 positive samples collected from FIP cats, only 1 faecal sample was found to contain enterotropic virus. A previous report mentioned that faeces from FIP cats is no longer infectious for other cats, as it could neither induce FIP nor intestinal infections upon inoculation of SPF cats (Pedersen *et al.*, 2012). This prompted us to sequence a part of the genome (ORF 3 and 7) and compare the sequence with the strain found in the affected tissues of that FIP cat, showing that the enteric strain and the tissue strain were completely different. This observation indicated that this cat was not shedding an FIPV strain, but rather an FECV strain by which this cat had became superinfected in the shelter in which it was housed. Two viruses with the highest initial infectious titre, including the American FECV UCD strain and the own isolated UG-FH8 strain, were further propagated in the cell cultures to increase their titre, and were used in all subsequent experiments at passage 3.

Based on the data generated in chapter 3, it was concluded that 1) FIPVs were clearly restricted in their replication in enterocytes, giving an explanation why FECV is the only pathotype circulating amongst cats; 2) the established enterocyte cultures provide a new, reliable *in vitro* model to propagate and study feline enteric coronaviruses.

6.1.2 Initiation of enteric infections by FECV-sialic acid interactions (Chapter 4.1)

Enteric viruses have to overcome two important barriers before they can reach their target cell, the enterocyte. Firstly, they have to survive the harsh digestive environment, including low pH, proteases, lipases, bile salts, and carbohydrases that are normally involved in the break down of proteins, lipids, and sugars in food. In contrast to non-enveloped viruses, which represent the majority of all enteritis-inducing viruses, coronaviruses are highly prone to inactivation by different unfavourable conditions, and it is still unclear how the enteritis-inducing coronaviruses survive the harsh conditions in the gastrointestinal tract after they have been swallowed. In addition, intestinal epithelial cells are covered by an enormous layer of mucus, a gellish network mainly formed by heavily glycosylated glycoproteins, called mucins, which act as a second barrier that has to be overcome by the virus to reach the cell surface. Since the carbohydrate moieties of the mucins are the first to be encountered during mucosal infections, it is not surprising that many viruses have evolved to interact with sugars at the mucosal surfaces of the host (Van Breedam *et al.*, 2013). In addition, recognition of a specific glycan composition often determines tropism and pathogenicity of pathogens (Bomsel & Alfsen, 2003). As for other viruses, also many coronaviruses, such as transmissible gastroenteritis virus (TGEV), bovine coronavirus (BCoV), and infectious bronchitis virus (IBV) attach to sugars, notably sialic acids, to initiate their infections (Schultze & Herrler, 1992; Schultze *et al.*, 1996; Schwegmann-Wessels & Herrler, 2006; Schwegmann-Wessels *et al.*, 2011; Winter *et al.*, 2006). In contrast to BCoVs and IBV, TGEV solely relies on a protein receptor for its entry, but uses its sialic acid binding capacity to interact with a mucoglycoprotein (Schwegmann-Wessels *et al.*, 2003; Schwegmann-Wessels *et al.*, 2011). Although dispensable in cell culture, this sialic acid binding capacity is a key-determining factor for the enterotropism of TGEV, potentially by allowing the virus to interact with and pass through the intestinal mucus layer (Bernard & Laude, 1995; Krempl *et al.*, 1997). All these observations prompted us to unravel if FECV can also interact with sialic acids/mucins, and how these interactions are involved in the initiation of FECV infections.

The role of sialic acids during FECV infections was studied *in vitro* by investigating the effect of 1) neuraminidase (NA) treatment of enterocyte cultures, 2) NA treatment of the virus, and 3) the addition of sialylated compounds to the inoculum, on the infectivity of FECV. It was demonstrated that desialylation of cells spectacularly enhanced (up to 72 times) FECV infectivity, showing that sialic acids on the cell surface can hamper efficient FECV-enterocyte interactions *in vitro*. This observation also clearly affected the propagation and titration of

FECV *in vitro*, as both FECV UCD and UG-FH8 were grown to titres of around 10^7 $TCID_{50}$ /ml when cells were desialylated before inoculation (data not shown), instead of around 10^5-10^6 as initially reported in chapter 3. This also implies that infectious titres described in chapter 3 were potentially slightly underestimated. Nonetheless, these data clearly show that in contrast to BCoVs and IBV, sialic acids are no receptor determinants during FECV infections (at least not the terminal sialic acid residues, as internal sialic acids residues are not affected by the neuraminidase used in our studies). Given that neuraminidase treatment of viruses can unmask sialic acid binding capacity (Park *et al.*, 2011; Schultze *et al.*, 1992; Schultze *et al.*, 1996), it was subsequently investigated if neuraminidase treatment of the virus would impact its infectivity in cell cultures, showing that desialylated virus infected the cells up to 7 times better compared to untreated virus. This increased infectivity was reduced/lost if these viruses were pre-incubated with sialyllactose, for which the effect was more pronounced when using α2,6- than α2,3-sialyllactose. Based on these experiments, two major conclusion were made: 1) FECV has a sialic acid binding capacity (α2,6- over α2,3-linked) that is (partially) masked by virion associated sialic acids, and 2) unmasking the sialic acid binding capacity can give the virus advantages in its interaction with enterocytes, although sialic acids were clearly shown not to be receptor determinants.

The hypothesis that sialic acids might probably serve as 'attachment' factors could also explain the seemingly conflicting data on their involvement in FECV infections that were obtained when evaluating the infectivity of NA-treated viruses in desialylated cells (Figure 6.1). Indeed, if cell-associated sialic acids can help the virus to more efficiently stick to the cell surface, it is logical that this can result in an increased number of infected cells if viruses have a more pronounced sialic acid binding capacity obtained by NA treatment of the virus (Figure 6.1; 1). On the other hand, it is also logical that if the virus can no longer stick to these sialic acids by treatment of the cells, that the virus-receptor interaction is even more efficient, as there is less chance that viruses get trapped by non-receptor glycoproteins. The latter can explain both the increase in infectivity upon desialylation of cells (Figure 6.1; 2a and 2b), and the reduced infectivity of virus with an increased sialic acid binding capacity (NA-treated viruses) in these cells (Figure 6.1; 3). Indeed, the less attachment to sialic acids that can occur *in vitro*, the less viruses will get 'trapped' by non-receptor glycoproteins or glycolipids, and hence the better they can interact with the receptor.

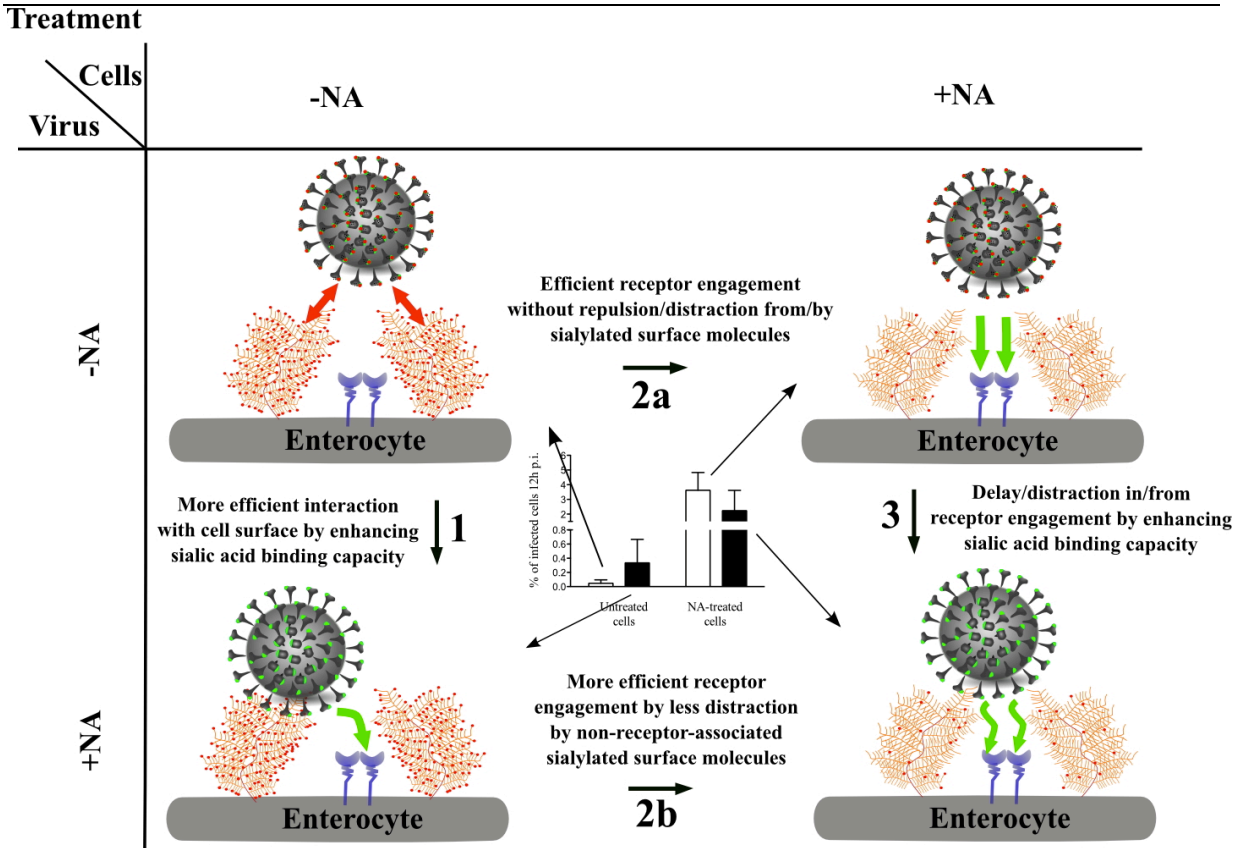

Figure 6.1. Effect of neuraminidase treatment of cells and/or FECV *in vitro*. 1) Enhancement of infection by NA treatment of FECV. Removal of sialic acids (red dots) from the viral surface liberates a sialic acid binding domain (green domain) that allows the virus to more efficiently interact with the sialylated (red dots) glycocalyx. **2**) Enhancement of infection by NA treatment of cells for both untreated (2a) and NA-treated viruses (2b). Removal of sialic acids (red dots) from the cell surface results in less repulsion by/distraction from the sialylated glycocalyx, and hence leads to a more efficient infection. **3**) Reduction of infection by NA treatment of virus in NA-treated cells. Allowing more attachment to sialic acids by the liberation of the viral sialic acid binding domain (green domain) results in more distraction by non-receptor-related sialic acids compared to the non-treated virus, and hence leads to less efficient infection.

These data suggest that FECV resembles TGEV in its requirement for sialic acids in the initiation of infection, and hence that sialic acid binding can be a strategy for this virus to attach to highly sialylated mucins. The FECV-mucin interaction was studied by performing competitive inhibition experiments with 2 different, commercially available mucins, porcine gastric mucin (PGM) and bovine submaxillary mucin (BSM). Whereas PGM could not affect FECV infectivity at concentrations up to 50 mg/ml, BSM potently inhibited FECV infections from 0.5 mg/ml onwards. The fact that this effect was much less pronounced on desialylated cells confirmed previous conclusions that sialic acid binding occurs in non-treated cell cultures, but is not a prerequisite for the initiation of FECV infections *in vitro*. In contrast to sialyllactoses, also non-treated viruses had a mucin binding capacity, which can be explained by the multivalent binding to mucins compared to sialylactoses. These data show that FECV is capable of interacting with mucins, and that it prefers mucins rich in (α2,6-linked) sialic acids (such as BSM), and not other mucins such as PGM, which is mainly composed of

neutral and sulphated oligosaccharides. In addition to these experiments, we have also performed experiments with feline saliva and found that this could also reduce FECV infectivity (data not shown). This interaction with salivary mucins raised the hypothesis that the sialic acid binding capacity is probably not only a way to allow attachment and subsequent passage through the mucus layer, but also a way to deal with the harmful digestive juices the viruses are faced with. This hypothetical model is depicted in Figure 6.2. Indeed, if FECV can cover itself with sialylated compounds/mucins, the virus can be protected against digestion, which can explain how these enveloped viruses manage to reach the intestine. During its passage, the virus is faced with acidic pH and bacterial and/or cellular sialidases, which will finally desialylate the virus. This desialylation will liberate the sialic acid binding domain of the virus, allowing it to escape from the intestinal flow by attachment to the intestinal mucus layer. The need for this 'digestion' would also explain why the proximal part of the duodenum seems not to become infected by the virus (Pedersen *et al.*, 1981).

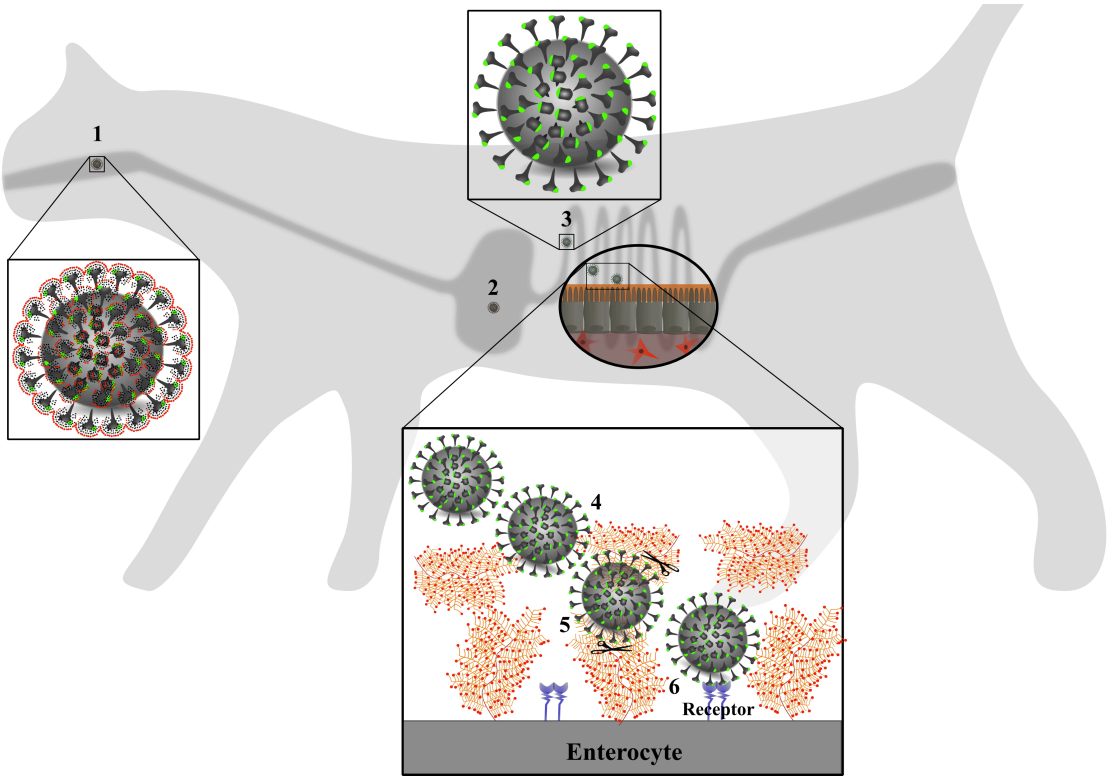

Figure 6.2. Hypothetical model for the initiation of intestinal FECV infections. (**1**) Virion protected by a carbohydrate/mucin shell, formed by intrinsic glycosylation and additional binding to sialylated compounds/mucins, encountered during exit from infected cells or during uptake in the oral cavity (e.g. salivary mucins). (**2**) This protects the virus from degradation when faced with the unfavourable conditions (enzymes, pH) in the uppert part of the gastro-intestinal tract. (**3**) Exposure of the virions to low pH and/or bacterial/cellular sialidases liberates the viral sialic acid binding site (green domain), (**4**) allowing the virus to attach to sialic acids (α2,6- > α2,3-linked sialic acids) in mucus. (**5**) Passage through the mucus layer, most probably facilitated by intestinal sialidases, finally allows FECV to engage with its functional receptor (**6**) expressed on the enterocyte membrane.

Taken together, chapter 4.1 demonstrated that FECV has a sialic acid binding capacity, but that attachment to sialic acids is not a prerequisite for the initiation of *in vitro* infections, and even hampers efficient enterocyte infections. Nonetheless, it seems reasonable for the virus to possess this property, as it can give the virus advantages in its confrontation with the harsh conditions and mucosal barriers during its passage through the intestinal tract. However, passage through the stomach seems not to be the only way by which FECV can reach the intestine…

6.1.3 Alternative route to initiate enteric infections? (Chapter 5.1)

In an attempt to learn more about *in vivo* FECV infections by experimental inoculation of cats, it was found that one of the cats showed a remarkably different infection pattern compared to the other cats. This infection pattern was characterized by a delayed faecal shedding (starting from day 14 instead of day 2-4 p.i.), absence of infectivity in enterocyte cultures of the excreted virus, a delayed raise in antibody titres, and viraemia that was detected far before any intestinal replication. The only reasonable explanation that could link the early viraemia with the delay in intestinal shedding was that FECV could have reached the intestine by the systemic route. The initiation of intestinal infections by the systemic route has been reported before, when it was noticed that intraperitoneal FECV inoculation of cats can result in faecal shedding (Foley *et al.*, 1997; Pedersen *et al.*, 2012). However, in contrast to our results, intraperitoneal inoculation of FECV gave no aberrant excretion pattern, as onset and level of shedding were comparable to the orally inoculated cats, indicating that FECV was carried very fast from the periphery to the intestine in that study. In our study, the oral RNA shedding during the first 3 days and the swollen submandibular lymph nodes at day 3 p.i. raised the hypothesis that FECV could have encountered susceptible, most probably monocytic cells, in the tonsils, from where subsequent viraemia occurred. For FIPV, infection of monocytic cells in tonsils has been described upon oral inoculation, making this site of infection quite plausible (Stoddart *et al.*, 1988). As a result of the viraemia, these infected cells should then have extravasated in the intestinal mucosa, explaining the sudden detection of shedding in that cat at 14 days p.i. However, although this alternative route for the initiation of enteric FECV infections can link the oral shedding, swollen submandibular lymph nodes, and early viraemia with the delayed shedding, it cannot give an explanation for the lack of infectivity of this virus. Indeed, the virus shed by this cat was no longer infectious for enterocytes, at least not *in vitro*, as no additional inoculation studies were done with the faecal suspensions of this cat to confirm this feature. Nevertheless, the huge differences in

infectivity compared to the virus excreted from the other 2 cats raised the question whether this virus was indeed shed by enterocytes, and not by another cell type (from the monocytic lineage?) residing in the intestinal mucosa. Two possible explanations can be considered for this feature. First, it is possible that viral genetics have changed by its adaptation to/replication in this cell type, which would give the virus a more FIPV-like phenotype, for which we showed that they replicate very inefficient in enterocytes (chapter 3). Full genome sequences of the excreted viruses of all cats and the inoculum are currently being assessed to find any of those genetic differences. If these would not reveal any differences, another explanation for the lack of enterotropism would be that by its adaptation to/replication in cells of the monocytic lineage, the glycosylation of the virus has extensively changed, as it is known that cell-specific post-translational glycosylations can determine the cell tropism of the progeny virus (Dejnirattisai *et al.*, 2011; Lin *et al.*, 2003). However, coronaviruses are known to use their sugar coat to bind to lectins expressed on monocytes, but so far there are no indications that cell surface carbohydrate-binding molecules are also used for infection of enterocytes. If the glycosylation of the virus has indeed been changed, a reasonable explanation for the lack of infectivity in enterocyte cultures would be the steric hindrance of the sugar coating to allow efficient spike-receptor interactions. Further experiments with glycosidases should reveal whether or not this is indeed the case. Figure 6.3 gives an overview of the hypothetical model for the initiation of intestinal infections and the sources of FECV shedding.

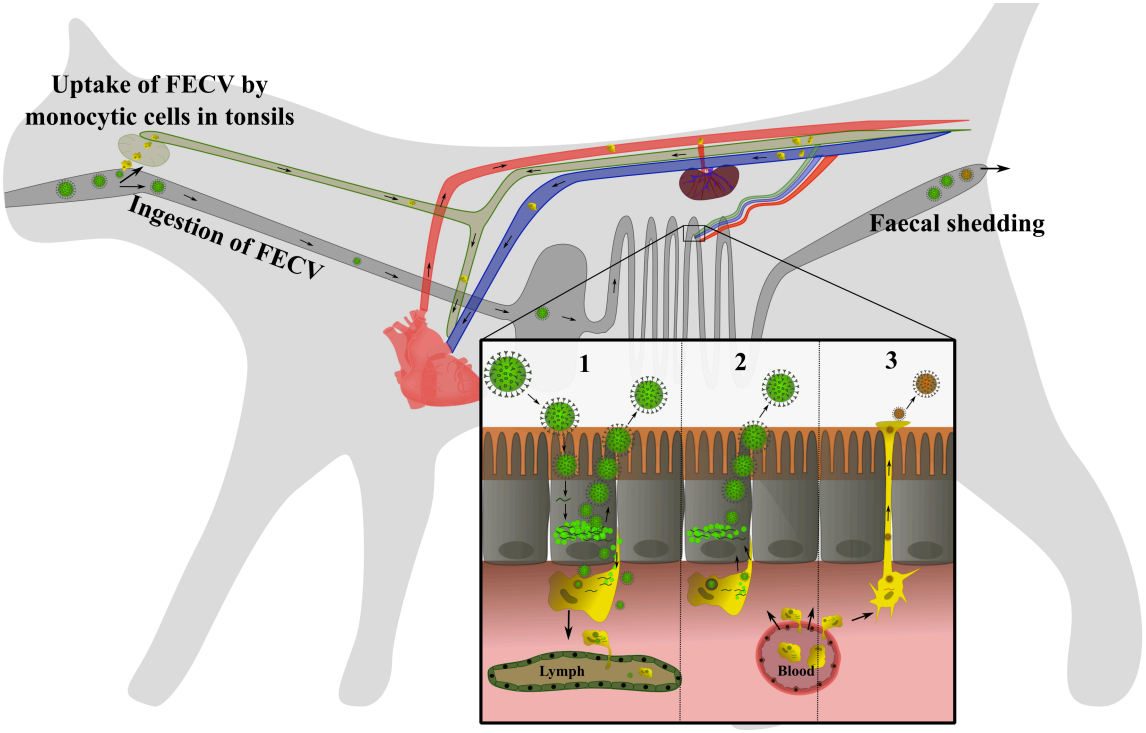

Figure 6.3. Alternative route for intestinal infections and possible sources of FCoV particles found in faeces. 1) After oral uptake, FECV can pass the stomach, to finally encounter its target cell, the enterocyte, in the intestine, resulting in subsequent shedding from the infected enterocytes. **2, 3)** Possibly, FECV is also capable of reaching the intestine by the systemic route, resulting from uptake of the virus in cells of the monocytic lineage in the oral cavity and subsequent extravasation of these cells in the intestinal mucosa. Subsequent shedding resulting from this alternative route of intestinal infection can theoretically occur by basolateral (cell-free or cell-associated?) infection of enterocytes (2), or by shedding from monocytic cells (3), the latter which can result in phenotypic changes of the virus by mutation or different post-translational modifications.

6.1.4 Total faecal RNA copies, infectious titres and neutralizing antibodies during FECV infections

The availability of FECV-susceptible cell cultures allowed for the first time to compare infectious titres with RT-qPCR titres, and to assess the presence of neutralizing serum antibodies. Two different RT-qPCR tests were used: 1) the generally used qPCR targeting the 3' part of the genome and all subgenomic mRNAs, and 2) a newly developed qPCR recognizing ORF1b (chapter 3) which is only present in genomic RNA. When comparing both RT-qPCRs, it was found that by using the 3' qPCR for the assessment of viral genomes as is regularly done, a titre overestimation of 3 to 4.3 $\log_{10}$ occurs, explaining why only 1/1,000,000 to 1/100,000,000 of these "particles" seemed to be infectious. When using the 5' qPCR, the difference between total genomic RNA and infectious virus was more acceptable, although this difference varied in between the time points p.i. at which the shedding was monitored. Indeed, whereas total genome copies were 3-4 $\log_{10}$ higher than infectious titres during the first week, this ratio further increased to 4.5-8 $\log_{10}$ during the remainder of the

experiment. This decreased infectivity coincided with the onset of neutralizing antibodies (Figure 6.4).

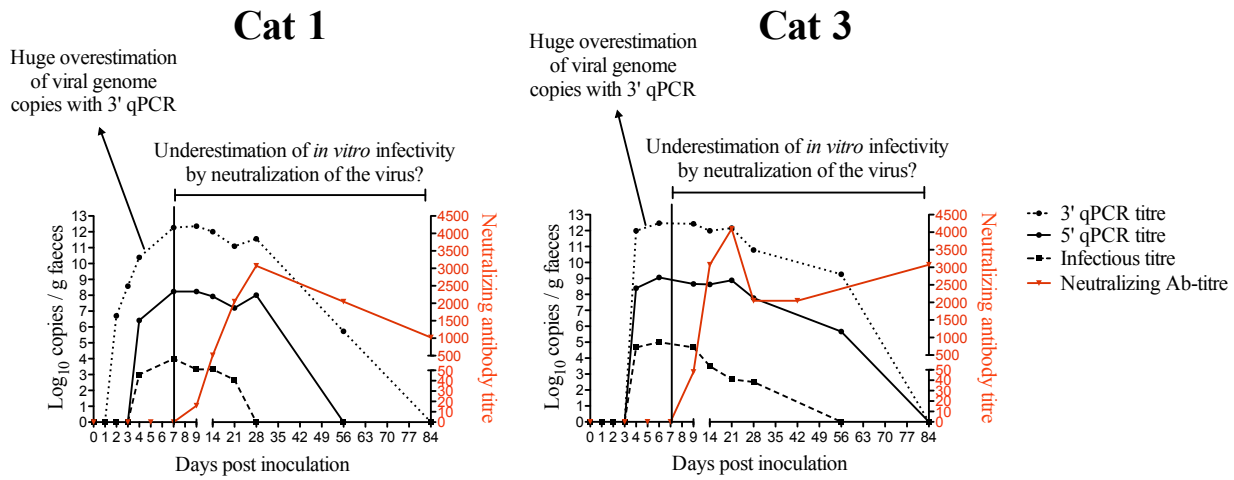

Figure 6.4. Quantification of virus and neutralizing antibodies in the two cats with the normal infection pattern.

The presence of the neutralizing IgA antibodies in faecal suspensions should be further investigated, but it seems reasonable that these started to affect the infectivity estimation in our cell culture-based assay. This could also explain the variable infectivity of faecal field samples described in chapter 3. In addition, the highly neutralizing capacity of antibodies is potentially the reason why kittens can remain fully protected by maternal antibodies during the first weeks of life, and why superinfections only rarely occur during natural infections (Addie & Jarrett, 1992; Addie *et al.*, 2003; Herrewegh *et al.*, 1997). However, it remains a mystery how FECV manages to continue infection for many months or even years, whereas other enteric viruses would already have been cleared within 1-2 weeks p.i. Extensive research on FIPV has revealed that this virus has many immune-evasion strategies (Cornelissen *et al.*, 2007, 2009; Dedeurwaerder *et al.*, 2014; Dewerchin, 2008; Dewerchin *et al.*, 2006; Vermeulen *et al.*, 2013), and it seems reasonable to assume that this is not only a hallmark of FIPV, but also determines the strengths of the "harmless" parent virus, allowing it to reside within all cat populations by establishing long-lasting infections.

6.2 Targeting FIP by managing the faecal-oral transmission of FECV (Chapter 5.2)

It has been recommended before that: "Control of FIP must be directed first at control of its parent virus, and should that fail, at FIPV itself" (Addie *et al.*, 2004). However, not only the development of curative tools to overcome FIPV, but also the prevention of FECV infections have shown to be more easily said than done, since FECV is still residing in virtually all multi-cat households worldwide. The major strength of this virus is its capability to establish

unnoticed, long-lasting infections, thereby seemingly 'co-existing' with cats. This is why most owners are not aware of the presence of this virus until one or more of their cats succumb(s) to FIP. And even then, they often do not realize that except for their FIP cat, most other cats are infected with an FCoV at that time and that the losses from FIP have their roots in these concealed infections. Shocked by the sudden death of their young and valuable cats, many owners often desperately search/ask for efficient control measures to prevent future FIP losses. Early weaning followed by complete isolation of kittens can be an effective way to prevent transmission from positive animals to kittens (Addie *et al.*, 2009; Addie & Jarrett, 1992). However, the fact that this method does not guarantee success, and notably the fact that reduction in FIP losses does not outweigh the impact on the socialisation explains why this is not regularly applied in practice. Therefore, many cat owners start to spend a lot of money on antibody testing and group their cats according to these results in order to reduce the infection pressure, but this strategy is also very often fruitless. FECV remains endemic in cat populations by continuous transmission from (long-lasting) shedders to negative animals. Consequently, it should theoretically be feasible to control infections by avoiding contact between negative animals and shedders by grouping of cats based on their shedding status. However, knowing that FECV can be easily transmitted via fomites and that most catteries are established in a normal one- or two-storey house with a lot of people movement, the practical feasibility of this strategy has been questioned. In addition, some reports mention intermittent shedding in cats (Addie & Jarrett, 2001; Pedersen *et al.*, 2008), and that a negative status can only be stated with certainty after 5 consecutive negative monthly tests (Addie & Jarrett, 2001). The enormous demand for control measures, and the lack of concrete data if and how FECV transmission can indeed be managed in normal households prompted us to evaluate whether this method of grouping would be practically feasible. Two essential conditions should be fulfilled for this method to be successful: 1) a highly sensitive PCR is needed to avoid false negative testing, and 2) the amount and housing of cats should allow for grouping of animals in at least 2 separate rooms. In Belgium, catteries are typically small to medium sized and established in normal one or two story houses, making grouping in separate rooms practically feasible. Knowing that the 5' qPCR was able to detect various field strains (chapter 3) and gave the best correlation with the infectivity (chapter 5.1), this PCR was used in 2 average-sized catteries (chapter 5.2). It was clearly shown that grouping of cats based on their shedding state is practically feasible and cost-effective if one wants to avoid FECV transmission to kittens. By monitoring both shedding and antibody titres at regular time points, present thesis confirmed that antibody titres are not good parameters to assess a cat's

infectivity, and hence that it is certainly more worthwhile to invest in tests that monitor faecal shedding. In contrast to previous reports, no extremely variable intermittent shedding that could have interfered with the grouping was noticed with the RT-qPCR test used in the present thesis. However, two features that were noticed were 1) that shedding in positive cats could vary between low and high in between the samplings, and 2) that cats that were recovering from infection and were negative at one sampling, could be very low positive the next sampling, but these cats were no longer infectious for other cats, and hence this kind of 'intermittent shedding' did not interfere with the grouping.

Despite the lack on restrictions for clothing, handling of cats, and people movement, the persistent shedder could be very effectively identified in household 1 (1 year after the start of the monitoring), and this cattery was even totally cleared from FECV shedders after the persistent shedder had been removed. In household 2, 7/14 cats were still shedding 1 year after the start of the monitoring, but by grouping of cats, reinfection of the negative cats was efficiently prevented. These observations show that direct contact between cats and presence of persistent shedders are the major reasons for endemic FECV infections within a household. Consequently, if one wants to prevent FECV transmission to kittens without the need for early weaning, it should be assured that the queen and all future in contact animals are negative (= generate a negative breeding population), or that the complete cattery is free of shedders (= eradication of FCoVs) (Figure 6.5). Theoretically, the latter strategy is the most effective in assuring that no kittens will become infected. However, eradication of FCoVs takes at least 1-2 years, and is currently, due to the widespread distribution of FCoVs, only feasible in catteries that do not import new breeding animals. Therefore, the most practically feasible approach is to make a negative breeding population, as was done in the present thesis by regular monitoring of shedding and grouping of cats. It was shown that with this strategy, a sufficient amount of negative cats could be obtained within 9-12 months. The major drawback of this approach is that there is a continuous risk for inadvertent transmission of the virus to the negative kittens, especially when positive kittens/young cats (which shed enormous amounts of virus) are imported. Indeed, an inadvertent transmission of the virus was observed in the present thesis from a newly imported kitten to the negative kittens in the adjacent room. Therefore, it is certainly recommended to consider separation of young positive cats from negative cats by grouping in non-adjacent rooms and/or to prevent import of young cats as long as the negative kittens are present. Knowing that this separation strategy is effective in reducing FECV infections, sensitizing veterinarians/cat owners and applying

this strategy in more catteries could already be an enormous step in the right direction to deal with FIP.

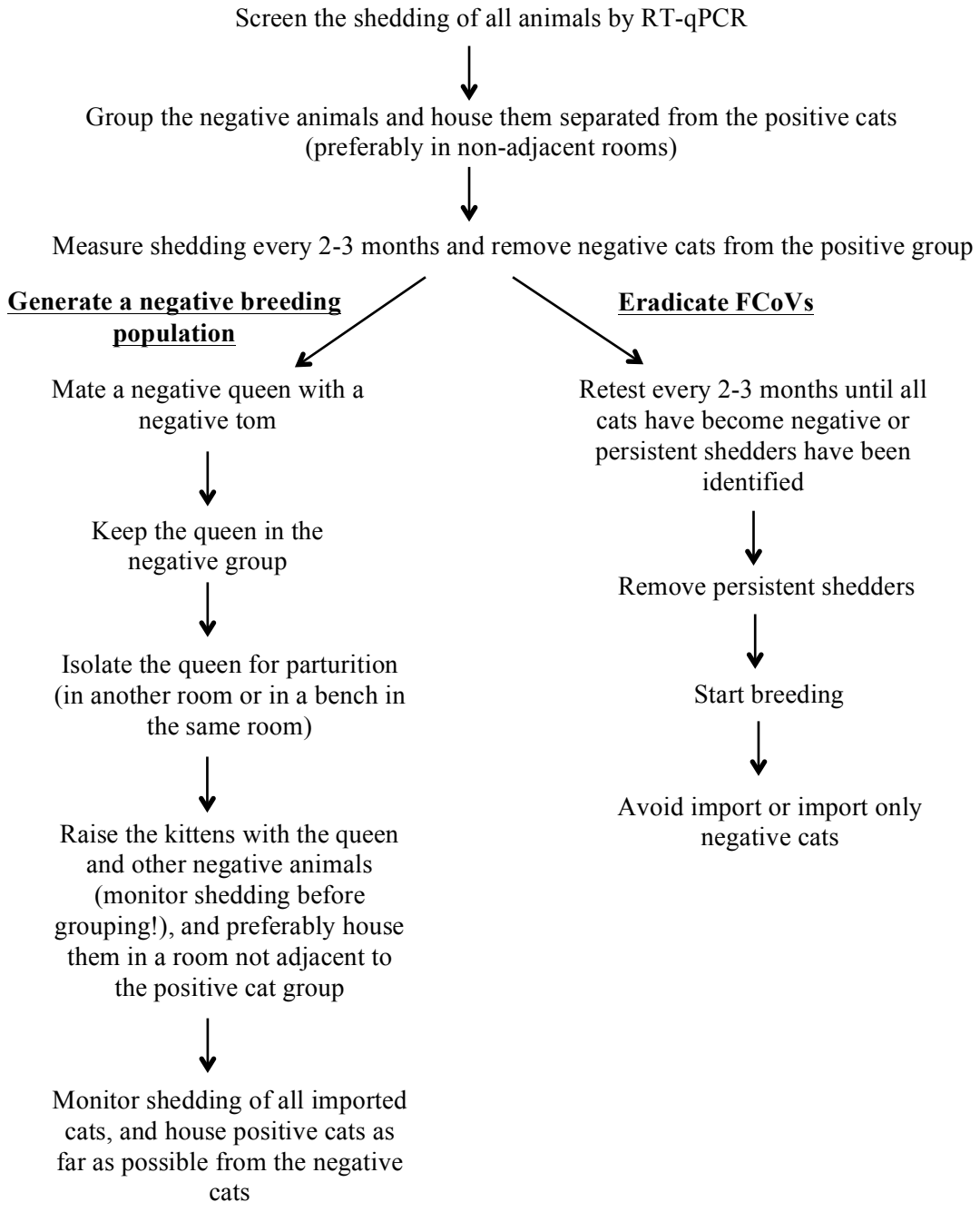

Figure 6.5. Recommendations for the prevention of feline enteric coronavirus transmission to kittens.

Notwithstanding the feasibility, the long-lasting or even persistent shedding of the virus remains the major restriction and the reason why control of FECV transmission will always remain a time- and money-consuming activity. In addition, as long as FECV remains endemic in cat populations, control of FECV infections will be unfeasible in very large catteries or in

other multi-cat households (shelters/shops) where new cats are regularly imported or grouping cannot be appropriately performed. In depth analysis of serotype I FCoV-host interactions will be of key importance if one wants to combat future FIP cases, as only these insights will allow the development of adequate preventive or curative tools. Further investments in development of cell lines for the propagation and study of serotype I FIPVs should therefore be the next crucial step to be taken.

6.3 Targeting FIP: future perspectives on FCoV curative and preventive measures

When making statements on the development of new anti-FCoV measures, the question arises which measures (preventive or curative) are most opportune to invest in, and if these should target FECV, FIPV, or both.

For many viral infections, **vaccination** has undoubtedly been very effective to reduce morbidity/mortality or even completely eradicate a virus within/from a certain population. In contrast to those successful vaccines, the efficacy of the only commercial available FIP vaccine (Primucell® FIP) is rather insufficient. In addition, all other attempts to provide better vaccines have failed so far, and many vaccines even induced accelerated disease progression. Knowing that vaccination completely relies on the host immune system, this failure is actually not surprising, as FCoVs (or at least FIPV) are masters in evading the cat's immune system on all levels (innate, humoral and cellular) (Cornelissen *et al.*, 2007, 2009; Dedeurwaerder *et al.*, 2014; Dewerchin, 2008; Dewerchin *et al.*, 2006; Vermeulen *et al.*, 2013). So far, vaccination trials have only been based on the evaluation of protection against FIPV challenge, and one can wonder if vaccine development and efficacy testing against FECV would be a more reasonable approach, as this is the only virus that circulates between cats. However, given that 1) FECV escapes from protective local immunity (as evidenced by its long-lasting shedding), 2) memory immune responses are lacking (as evidenced by the continuous reinfections), and that 3) theoretically one replication cycle is sufficient to generate FIPV mutants, this approach will probably not be the most effective way to combat FIP.

The development of adequate **curative measures** will most probably be the key to tackle FIP. Antiviral drug development can be directed against viral proteins or against host proteins that are used by the virus to complete its replication. The advantage to target viral proteins is that those antivirals have fewer side effects than if one would target host proteins involved in normal physiological processes. The counterpart of targeting viral, and especially coronaviral, proteins is that these viruses can become rapidly resistant resulting from their high mutation

rate. Hence, combatting FCoVs, as all other coronaviruses, with antivirals will possibly need a multi-drug/target approach to be successful. In addition, the development of new FCoV treatments should ideally be directed against both pathotypes, because this cannot only help in curing cats from FIP, but can maybe also help in making the restriction of FECV transmission in multi-cat households more efficient by clearing the long-lasting FECV infections in positive cats. Combining the above described management strategy with "healing" the positive cats from long-lasting/persistent infections will be a very effective approach to tackle FIP. In that way, every household would be able to rapidly clear FECV infections and avoid re-entrance of the virus by testing and/or treating incoming cats. In light of this strategy, present thesis aimed at providing insights in host and viral proteases involved in the replication of serotype I FECV. In addition, this thesis also provided additional information on proteases involved in serotype II FCoV 79-1683 and TGEV replication (Chapter 4.2). In contrast to FCoV 79-1683 and TGEV, serotype I FECV did not depend on low pH, furin cleavage and/or cathepsin B cleavage for its entry in enterocytes, but the exact fusion trigger could not be revealed. However, a yet to be identified serine protease was recognized as involved in the replication. Interestingly, this serine protease was found to be virion-associated, and not only involved in serotype I FECV, but also in FCoV 79-1683 and TGEV infections. In addition, it was shown that this viral protease fulfilled its job between 60 and 120 min p.i. Based on the kinetic study performed with E64d, which showed that the viral cysteine proteases fulfilled their function from 30 to 120 min p.i., this indicates a role of the serine protease in a post-entry step, but the identity and function of this protease remain elusive. Figure 6.6 overviews a model for serotype I FECV and serotype II FCoV 79-1683 entry and replication in enterocytes based on data obtained in the present thesis. As stated above, these data confirm that serotype I viruses follow different entry pathways compared to serotype II viruses in epithelial cells, and hence that the future search for effective antivirals in cell-based assays should be performed with the clinically relevant serotype I viruses.

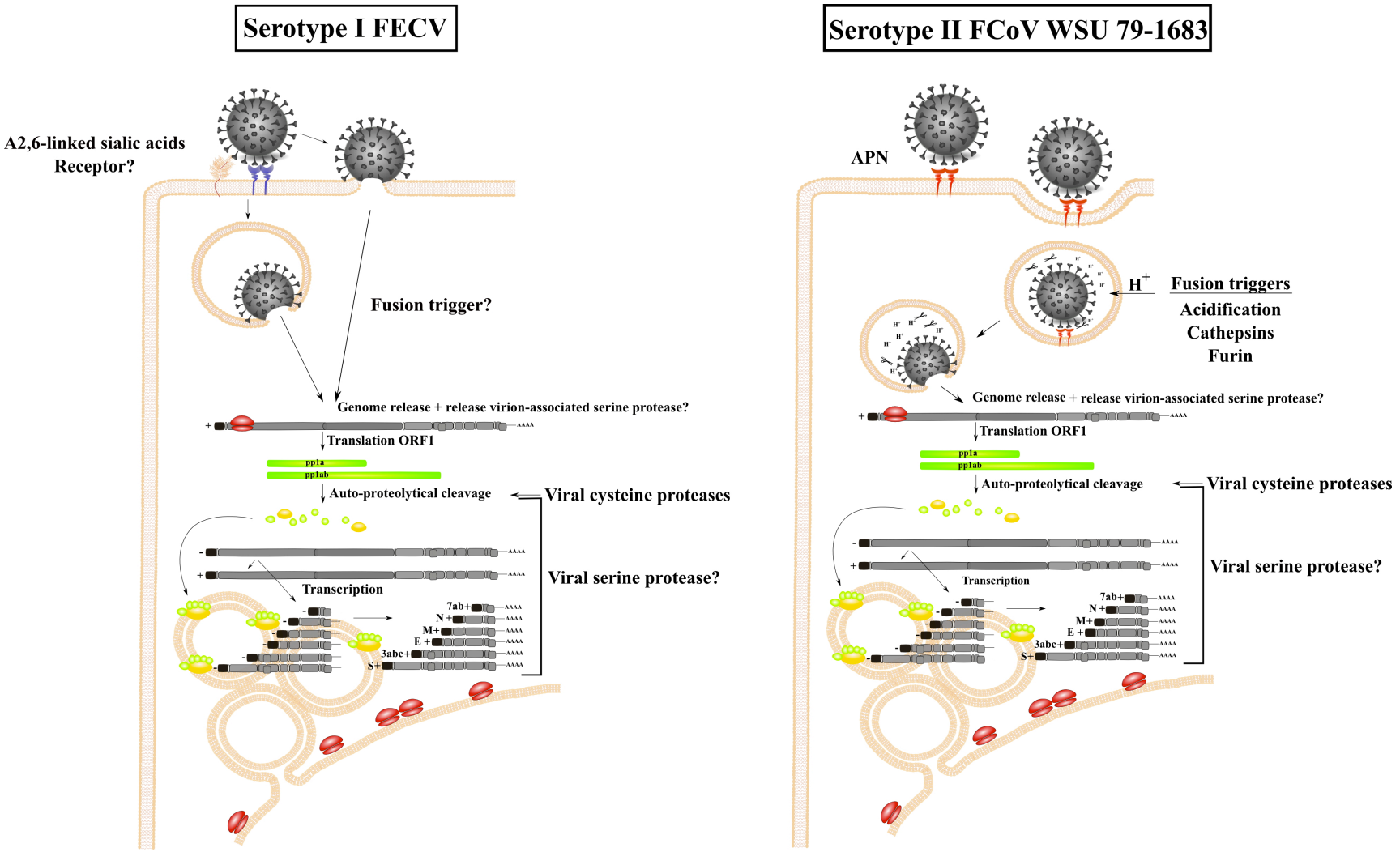

Figure 6.6. Model for serotype I and II FCoV entry and replication in epithelial cells.

In the present thesis, considerable attention was given to FECV, and it was shown that this parent virus can certainly be a future target if one wants to reduce the FIP incidence. However, FIP cases will remain to originate from insurmountable FECV infections, especially in places where large amounts of cats reside and are regularly imported. To deal with these FIP cases, investments should be made to identify as much druggable targets as possible in the FIPV-monocyte/host interaction. In contrast to FECV, it will be ethically more accepted to use drugs that have somewhat more side effects, as FIPV causes a life-threatening disease. The major problems in case of FIP, however, are that by the time a cat is diagnosed with FIP, 1) the virus has already caused extensive tissue damage for which we can wonder whether this is still reversible, and 2) that the disease is progressing in pyogranulomas, and it is unknown if optimal drug concentration/penetration occurs in these environments. Consequently, if one wants to cure FIP with any future drugs, decent discriminatory/confirmatory tests will be needed not only to enhance the survival rate by an early diagnosis of the disease, but also to avoid unnecessary treatment (or euthanasia as long as there are no treatments) of non-FIP cats. In order to establish a discriminatory test, full genome comparison has been performed on multiple enteric strains of healthy cats and tissue strains of FIP cats, although it recently became clear that one should be careful when making conclusion on these comparisons, as systemic 'FECVs' also differ from enteric 'FECVs'. Indeed, whereas one study reported to find specific 'FIPV mutations' in the spike by comparing faecal strains of healthy cats with tissue strains of FIP cats (Chang *et al.*, 2012), a following study reported that these mutations were also found in tissues of non-FIP cats and hence were not a hallmark of FIPV (Porter *et al.*, 2014). Recently, a new test was launched based on these 2 mutations to aid veterinarians in the definite diagnosis of FIP. So far, no data are available on the sensitivity, specificity and predictive values of this test based on analysis of effusions/biopsies of a large number of both FIP and non-FIP cats. However, it was stated that blood samples are not recommended for the test, since the viral load in blood is often too low to be detected. This implies that even with such tests, it will remain challenging to obtain an early diagnosis. In addition, although these 2 mutations have been found in 96% of all FIP cases, it remains unknown if and how these mutations contribute to the onset of FIP. The fact that they are also found in tissues of non-FIP cats indicates that FIP is most probably the consequence of several (consecutive) mutational events in the genome. As stated above, the search for the FIPV-determining mutations has been seriously hampered by huge inter-FECV variations and by the lack of tools to study the effect of a certain mutation on the phenotype of the virus. Consequently, 3 tools will be absolutely required to solve the crucial question on

the pathotype-switching mutations: 1) genome sequences of multiple FECV/FIPV tandem strains will be needed to search for the exact FIP-related genetic changes, and hence to decrease the amount of non-FIP related inter-FECV variations when making comparisons, 2) manipulable FCoV genomes should be available to confirm a certain mutation to be the cause of the cell tropism- and pathotype switch, and 3) FCoV-sensitive cell cultures (enterocytes and monocytes) should be available to grow these viruses and rapidly screen the effect of a certain mutation on the phenotypical behaviour of the virus. By establishing feline enterocytes cultures (chapter 3) and collecting field material, from which until now 1 FECV/FIPV tandem has been generated (chapter 5.2), this thesis has provided at least some indispensable tools to find the "Holy Grail" in feline coronavirus research.

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Summary-Samenvatting

Summary

Feline infectious peritonitis (FIP) is caused by a feline coronavirus (FCoV) and has remained one of the few insurmountable and highly feared infectious causes of death in cats to date. In **Chapter 1**, an introduction is given on the classification of FCoVs in two pathotypes (feline enteric coronavirus (FECV) and feline infectious peritonitis virus (FIPV)) and two serotypes (serotype I and II), on the general viral structure and proteins, the replication cycle, and the current understanding of the pathogenesis. It is also described that an early and certain diagnosis of FIP remains challenging and that adequate preventive and/or curative tools against FIP are still lacking. Given the fact that FECV infections precede the onset of FIP, they can be important targets in the fight against this fatal disease. Since the main part of the FECV-enterocyte interactions studied in present thesis involved the early beginning of the infection, the second part of chapter 1 overviews the current knowledge on this entry pathway for coronaviruses in general.

Due to its low-pathogenic character, FECV has not received much attention, and hence very little information is available on the FECV-enterocyte/cat interactions and the possibility/feasibility to control this virus in the fight against FIP. Therefore, this thesis focussed on this root of all troubles to add upon the knowledge on the FECV pathogenesis and to provide insights in how its transmission can be restricted. These aims are formulated in **Chapter 2**.

At the start of this project, no representative *in vitro* model nor FECV strain were available for the study of FECV-enterocyte interactions. **Chapter 3** describes the research that was performed to address this lack. Therefore, long-term feline intestinal epithelial cell cultures were established from primary ileocytes and colonocytes by simian virus 40 T-antigen- and human telomerase reverse transcriptase-induced immortalization. Next, the reliability of these cultures as *in vitro* model for enteric FCoV infections was investigated. This was done by comparing the replication capacity of the enteritis-inducing serotype II FCoV WSU 79-1683

and the FIP-inducing FCoV WSU 79-1146 with the replication capacity in primary cultures. These experiments demonstrated that the avirulent, enterotropic FCoV strain 79-1683 infected enterocytes much more efficient compared to the FIP-inducing strain 79-1146, and that both viruses infected the immortalized enterocyte cultures similarly to what was observed for the primary cells. Consequently, it was concluded that the continuous cell cultures were reliable as *in vitro* model to study enteric FCoV infections. After having established this *in vitro* model, it was investigated whether these cultures would allow the propagation and study of field strains in order to make future research more reliable. Therefore, the enterocyte cultures were inoculated with faecal suspensions from healthy cats and with faecal or tissue suspensions from FIP cats. The cultures were susceptible to infection with different serotype I enteric strains and two of these strains (FECV UCD and UG-FH8) were further propagated. No infection was seen in cultures inoculated with FIPV tissue homogenates. Based on the data obtained in chapter 3, it was concluded that a new reliable model for FCoV investigation and growth of enteric field strains was established. In contrast to FIPV strains, FECVs showed a clear tropism for intestinal epithelial cells, giving an explanation for the observation that FECV is the main pathotype circulating among cats.

The establishment of these enterocyte cultures and high titre serotype I FECV strains allowed the further unravelling of the FECV-enterocyte interactions. As nothing was known for these interactions, **Chapter 4** bundles the research that was performed to address some of the players involved in the early beginning of FECV infections. Given that sialic acids act as attachment factors/receptors in many coronavirus infections, the role of these carbohydrates in FECV infections was investigated. In addition, knowing that coronavirus spike proteins require activation by low pH and/or proteases, the effect of various pH drop- and protease inhibitors was also examined.

Chapter 4.1 describes how the FECV infectivity was affected by 1) neuraminidase (NA) treatment of enterocyte cultures, 2) NA treatment of the virus, and 3) the addition of sialylated compounds to the inoculum. NA treatment of cells greatly enhanced (up to 72 times) infection efficiency, showing that terminal sialic acid residues on the cell surface are not receptor determinants and even hamper efficient virus-receptor engagement. When studying the effect of NA treatment of the virus, it was shown that desialylated virus infected the cells up to 7 times better compared to untreated virus, but that this increased infectivity was lost by the addition of sialyllactoses (α2,6- over α2,3-linked). These results gave an indication that FECV has a sialic acid binding capacity that becomes more pronounced by NA treatment of the virus

and that, although they are not receptor determinants, attachment to sialic acids can give the virus some advantages in its interaction with the enterocyte surface. Seemingly conflicting data were obtained when the infectivity of desialylated viruses were investigated in desialylated cells, since, in contrast to untreated cells, NA treatment of the virus was detrimental in NA-treated cells. However, the fact that sialic acids could serve as attachment factors, but are not a prerequisite for the initiation of enterocyte infections, could explain this feature, as receptor engagement becomes most efficient when the virus is not delayed/distracted by non-receptor sialylated molecules. These data strongly suggested that FECV resembles transmissible gastroenteritis virus (TGEV) in its sialic acid requirement, and hence that sialic acids are not receptor determinants, but that binding to sialic acids can be a strategy for this virus to attach to highly sialylated mucins covering the enterocytes. The FECV-mucin interaction was studied by performing competitive inhibition experiments with 2 different, commercially available mucins, porcine gastric mucin (PGM) and bovine submaxillary mucin (BSM). Whereas PGM did not at all affect FECV infectivity in concentrations up to 50 mg ml^{-1}, BSM potently inhibited FECV infections from 0.5 mg ml^{-1} onwards. This effect was much less evident in desialylated cells, confirming that FECV-sialic acid/mucin interactions occur in untreated cell cultures, but that these interactions are not a prerequisite for the initiation of enterocyte infections *in vitro*. These data demonstrated that FECV is capable of interacting with mucins (rich in α2,6-linked sialic acids such as BSM), and a hypothesis was raised that this mucin binding capacity potentially not only allows FECV to escape from the intestinal flow by binding to the mucus layer, but that covering itself with mucins is probably also a strategy to survive the low pH and/or proteolysis in the upper gastro-intestinal tract.

In **Chapter 4.2**, the effect of various lysosomotropic agents and protease inhibitors on the replication of serotype I FECV strains UCD and UG-FH8, serotype II FCoV strain WSU 79-1683, and TGEV, in enterocytes was assessed. It was shown that, in contrast to FCoV 79-1683 and TGEV, serotype I FECV entry occurred independently of acidic pH or cathepsin B. In addition, the furin inhibitor decanoyl-RVKR-CMK did not affect serotype I FECV entry or infectivity of progeny virus, but did inhibit FCoV 79-1683 and TGEV entry. The serine protease inhibitor AEBSF strongly reduced replication of all FCoVs and TGEV, when it was continuously present before and during replication or added after inoculation. Interestingly, this reduction resulted from the inhibition of a virion-associated serine protease, since a similar inhibition was observed when only the virus was treated with AEBSF. By performing a kinetic study, during which AEBSF was added at different time points p.i., it was shown

that this protease fulfilled its function between 60 and 120 min p.i. As expected, since coronavirus' polyprotein processing occurs by viral cysteine proteases, the cysteine protease inhibitor E64d also inhibited replication, when it was continuously present before and during replication or added after inoculation. Neither AEBSF nor E64d inhibited replication when only the cells were pre-treated. Based on all these data, it was concluded that in contrast to serotype II FCoVs, serotype I FECVs do not rely on low pH, cathepsin B and/or furin cleavage for entry, but the exact fusion trigger could not be revealed. These observations strengthen the worries that care should be taken when generalizing data obtained with serotype II FCoVs to serotype I viruses, especially when it concerns viral entry. In addition, it was shown that serotype I FECVs (and some other alphacoronaviruses) carry a virion-associated serine protease that is potentially involved in the initiation of the replication, thereby identifying a new target for future drug development.

In **Chapter 5**, the *in vivo* FECV-host interactions were studied by either performing experimental infections (chapter 5.1) or by follow-up studies in catteries (chapter 5.2).

Chapter 5.1 describes the research results that were obtained by monitoring oral and faecal shedding, neutralizing antibodies, viraemia, and leukocyte subsets for 3 months after oral inoculation of 3 FIV-, FeLV-, and FCoV-negative cats with serotype I FECV UCD. Virus shedding was quantified by 2 different RT-qPCRs (one recognising the 3' of all genomic and subgenomic (m)RNAs (3' qPCR), and the other recognising the ORF1b of the genomic RNA (5' qPCR)), and by virus titration in enterocyte cultures. In two of the three cats, faecal shedding started within 2-4 days p.i., and viral RNA remained detectable in faeces for 2 months. Infectious virus was found from day 4 until day 28 p.i., and neutralizing antibodies were found from 9 days p.i. onwards. A cell-associated viraemia was detected in both cats at infrequent time points after the onset of faecal shedding. No abnormal leukocyte numbers were noticed, except for a granulocytopenia in cat 1. It was found that by using the 3' qPCR for the assessment of viral genomes as is regularly done, a titre overestimation of 3 to 4.3 $\log_{10}$ occurs, making it not surprising than that only 1/1,000,000 to 1/100,000,000 of these "particles" seemed to be infectious. When using the 5' qPCR, the difference between total genomic RNA and infectious virus was more reasonable, although this difference varied in between the time points p.i. at which the shedding was monitored. Indeed, whereas total genome copies were 3-4 $\log_{10}$ higher than infectious titres during the first week, this ratio further increased to 4.5-8 $\log_{10}$ during the remainder of the experiment. This decreased infectivity coincided with the onset of neutralizing antibodies, and it was suggested that

neutralizing antibodies in faeces could have led to an underestimation of infectivity *in vitro*, but this needs to be further investigated. Surprisingly however, one of the three cats showed an aberrant infection pattern, characterized by a delayed faecal shedding (from day 14 instead of day 2-4 p.i.), absence of infectivity in enterocytes cultures of the excreted virus, a delayed rise in antibody titres, and viraemia that was detected far before any intestinal replication. To link the early viraemia with the delayed faecal shedding, an alternative route of intestinal infection was hypothesized, speculating that the virus could have been taken up by permissive (monocytic) cells in the tonsils, from where subsequent viraemia occurred, finally resulting in the extravasation of these infected cells in the intestinal mucosa. The lack of *in vitro* infectivity of the excreted virus from this cat was explained by the fact that excretion occurred from monocytic cells and that adaptation to/replication in these cells could have changed the virus, but this needs to be further confirmed. No abnormalities or differences could be seen in leukocyte numbers compared to the other two cats, with the exception of $CD8^+$ regulatory T cells, but if and how these cells played a role remains elusive.

The huge demand for control measures and the lack of data on the feasibility to control FECV transmission in the prevention of FIP prompted us to monitor FECV shedding in cattery cats and control its transmission by avoiding contact between shedders and negative animals. In **Chapter 5.2**, FCoV genomes were semi-quantitatively assessed in regularly taken faecal samples with the 5' qPCR, and cats were grouped in the house based on their shedding state, generating at least one positive and one negative group. One year after the start of the monitoring, the persistent shedder could be identified in household 1, and this cattery was even totally cleared from FCoV after the removal of the persistent shedder. In addition, 2 FCoV-negative litters were raised. However, this cattery could not be kept negative due the import of FECV shedders, but separating these cats from the negative cats could prevent the virus to spread again throughout the complete cat population. Due to the success of this strategy in this household, the same method was applied in another household, where separating shedders from non-shedders was also successful to avoid reinfections. In contrast to household 1, 7 out of 14 cats remained shedding for more than 1 year, and future follow up is needed to identify the real persistent shedder(s). Grouping based on antibody titres is a strategy that is still regularly applied in practice, but by comparing faecal shedding with antibody titres, it was clearly demonstrated that antibody titres are not good parameters to reliably estimate the infectivity of the cat. From all data obtained in both households, it was concluded that FECV transmission can be successfully controlled in small to medium-sized catteries (<15 animals) by regularly monitoring of shedding and grouping of cats, without the

need for strict measures concerning people movement, clothing, or handling of cats. Cost-effectiveness of this strategy depended on the household, showing that especially in multi-cat environments with major FIP problems it is worthwhile to thoroughly monitor FECV transmission, as this will prevent enormous financial losses by kitten mortality. Although effective, monitoring is (and will always be) a time- and money-consuming approach, due to the worldwide distribution, the long-lasting shedding and the highly contagious character of FECV. This further supports the need to invest in the development of efficient tools for the prevention and/or treatment of FCoV infections.

In **Chapter 6**, all data obtained in the present thesis are reviewed and discussed. A hypothetical model is depicted for the role of sialic acids in the initiation of intestinal FECV infections, and it is discussed that both passage through the stomach and intestinal infection by the systemic route can potentially result in faecal shedding of the virus. In addition, guidelines are given to reduce the FIP incidence in small to medium-sized catteries by monitoring of the FECV transmission. Given that this management strategy is time- and money-consuming due to the long-lasting shedding of FECV and that this will be hardly feasible in very large catteries or other multi-cat households where many cats from different origin are regularly imported (shelters, shops), the final part discusses some future perspectives to tackle FIP and describes some potential targets in serotype I FECV infections in enterocytes identified in the present thesis.

In summary, the main achievements and conclusions obtained in the present thesis include:

- Two novel feline intestinal epithelial cell lines were established by immortalization of primary ileocytes and colonocytes.
- The established enterocyte cultures allowed for the first time to propagate FECVs and to study FECV-enterocyte interactions. In addition, this will be one of the necessary tools to study underlying viral factors involved in the pathotype switch.
- FECV has a sialic acid binding capacity (α2,6- over α2,3-linked) that is partially masked by virion-associated sialic acids.
- Attachment to sialic acids is not a prerequisite for the initiation of *in vitro* enterocyte infections, and even delays/distracts the virus from efficient receptor engagement. However, this feature potentially gives FECV advantages in its confrontation with the harsh digestive conditions and mucus barrier *in vivo*.
- In contrast to serotype II viruses, serotype I FECVs do not depend on acidic pH, cathepsin B and/or furin for entry in enterocytes, but the exact fusion trigger remains

to be elucidated.

- AEBSF is a potent inhibitor of FCoV and TGEV replication, and targets a yet to be identified virion-associated serine protease that fulfils its job between 60 and 120 min p.i.
- Experimental FECV infections in three cats revealed an aberrant infection pattern in one cat, characterized by a delayed faecal shedding (from day 14 instead of day 2-4 p.i.), absence of infectivity in enterocytes cultures of the excreted virus, a delayed rise in antibody titres, and a viraemia that was detected far before any intestinal replication. Based on these data, it was hypothesized that intestinal FECV infections can potentially not only be initiated by passage through the stomach, but also by the systemic route.
- The generally used 3' RT-qPCR gives an overestimation of viral genome copies of about 3-4.3 $\log_{10}$.
- Restriction of FECV infections by management of the faecal-oral transmission, and not by antibody titre determination, is a feasible strategy to reduce the number of FIP cases in small to medium-sized catteries.

Samenvatting

Feliene infectieuze peritonitis (FIP) wordt veroorzaakt door een felien coronavirus (FCoV) en blijft tot op heden één van de weinige onoverkomelijke en erg gevreesde virale infecties bij katten. **Hoofdstuk 1** geeft een literatuuroverzicht over de classificatie van feliene coronavirussen in 2 pathotypes (felien enterisch coronavirus (FECV) en feliene infectieuze peritonitis virus (FIPV)) en 2 serotypes (serotype I en II), over de algemene virusstructuur en virale eiwitten, de replicatiecyclus en de huidige kennis over de pathogenese. Hoofdstuk 1 beschrijft ook dat het stellen van een vroege en zekere FIP diagnose moeilijk is en dat adequate preventieve en curatieve middelen nog steeds ontbreken. FIPV ontstaat door mutatie tijdens een FECV infectie, en dus kan FECV een belangrijk doelwit zijn in de strijd tegen FIP. In de huidige thesis werd vooral de nadruk gelegd op de initiële FECV-enterocyt interacties, en het tweede deel van hoofdstuk 1 geeft dan ook een literatuuroverzicht van de huidige kennis omtrent opname van coronavirussen in het algemeen.

Omdat FECV op zich onschadelijk is, is er in het verleden relatief weinig aandacht besteed aan dit virus. Bijgevolg is er heel weinig geweten over de FECV-enterocyt/kat interactie en de mogelijkheid om dit virus aan te pakken in de strijd tegen FIP. Omdat FECV aan de basis ligt van elke FIPV infectie, was het doel van de huidige thesis om de kennis omtrent de FECV-cel/kat interacties te verruimen en betere inzichten te krijgen in hoe de transmissie van dit virus kan beperkt worden. Deze doestellingen worden beschreven in **hoofdstuk 2.**

Aan het begin van het huidige project was er geen enkel representatief *in vitro* model of cultiveerbare FECV stam beschikbaar om de FECV-enterocyt interactie te kunnen bestuderen. **Hoofdstuk 3** beschrijft het onderzoek dat werd uitgevoerd om aan dit tekort tegemoet te komen. Hiervoor werden, via expressie van simian virus 40 T-antigen en humaan telomerase reverse transcriptase, geïmmortaliseerde, continue cellijnen gemaakt van primaire feliene intestinale epitheelcellen afkomstig uit het ileum (ileocyten) en het colon (colonocyten). Om na te gaan of deze cellen geschikt waren als *in vitro* model voor enterocyt infecties werd de

replicatie-capaciteit van het enteritis-inducerende serotype II FCoV WSU 79-1683 en het FIP-inducerende FCoV WSU 79-1146 bestudeerd en vergeleken met hun replicatie-capaciteit in primaire cellen. Deze experimenten toonden aan dat de avirulente, enterotrope 79-1683 stam de enterocytenculturen veel efficiënter infecteerde in vergelijking met de FIP-veroorzakende 79-1146 stam. Bovendien was de replicatie-capaciteit van beide virussen vergelijkbaar met wat werd gezien in primaire cellen, en werd besloten dat de geïmmortaliseerde cellijnen dus betrouwbare modellen zijn voor het bestuderen van enterische FCoV infecties. Nadien werd onderzocht of deze cellijnen ook zouden toelaten om serotype I veldvirussen te groeien en te bestuderen om zo het toekomstig FCoV onderzoek meer relevant te kunnen maken. Daarvoor werden de cellen geïnoculeerd met mestsuspensies van gezonde katten en mest- of weefselsuspensies van FIP katten. Het werd aangetoond dat de enterocyten-cellijnen gevoelig waren voor verschillende serotype I enterische stammen en 2 stammen (FECV UCD en UG-FH8) werden verder opgegroeid om te kunnen gebruiken voor latere *in vitro* studies. Na inoculatie met FIP weefselsuspensies werd geen infectie gezien. Gebaseerd op de gegevens verkregen in hoofdstuk 3 werd geconcludeerd dat een nieuw betrouwbaar *in vitro* model werd gemaakt voor FCoV onderzoek en groei van enterische stammen. In tegenstelling tot FIPV stammen hadden FECV stammen een duidelijk tropisme voor enterocyten, wat een verklaring kan bieden voor het feit dat FECV het belangrijkste pathotype is dat tussen katten wordt overgedragen.

Het ontwikkelen van de feliene enterocyten-cellijnen en het groeien van enterische stammen tot voldoende hoge titers liet toe om de FECV-enterocyt interacties verder te ontrafelen. Aangezien niets geweten was over deze interacties werd in de huidige thesis de focus gelegd op het begin van de infectie, en de resultaten bekomen uit deze experimenten werden gebundeld in **hoofdstuk 4**. Aangezien siaalzuren vaak betrokken zijn in coronavirus infecties werd eerst onderzocht of deze suikers ook betrokken zijn in FECV infecties. Wetende dat de coronavirus spike proteïnen geactiveerd moeten worden door zure pH en/of proteasen vooraleer het genoom kan worden vrijgesteld, werd nadien ook onderzocht wat het effect was van verscheidene pH drop- of protease-inhibitoren op de FECV infectie.

Hoofdstuk 4.1 beschrijft hoe de infectiviteit van FECV beïnvloed werd door 1) neuraminidase (NA) behandeling van de enterocyten culturen, 2) NA behandeling van het virus en 3) het toevoegen van siaalzuurhoudende componenten aan het inoculum. NA behandeling van de enterocyten zorgde voor een sterke toename in het aantal geïnfecteerde cellen (tot 72 keer meer), waaruit kon opgemaakt worden dan siaalzuren geen receptor

determinanten zijn en zelfs een efficiënte FECV infectie verhinderen *in vitro*. Bij het bestuderen van het effect van de NA behandeling van het virus werd aangetoond dat NA-behandeld virus de cellen tot 7 keer beter infecteerde, maar dat deze toename in infectiviteit teniet werd gedaan door het toevoegen van sialyllactoses (α2,6- meer dan α2,3-sialyllactose). Deze resultaten gaven een indicatie dat FECV een siaalzuur-bindende capaciteit heeft die meer uitgesproken wordt door NA behandeling van het virus en dat, ondanks het feit dat het geen receptordeterminanten zijn, binding aan siaalzuren op het enterocyt oppervlak het virus voordeel kan geven in het infecteren van enterocyten. Op het eerste zicht tegenstrijdige gegevens werden verkregen bij het beoordelen van de infectiviteit van NA-behandelde virussen in NA-behandelde cellen, omdat in tegenstelling tot in onbehandelde cellen, NA behandeling van virus een negatief effect had in NA-behandelde cellen. Het feit dat siaalzuren kunnen dienst doen als bindingsfactoren maar dat binding niet nodig is om enterocyten te kunnen infecteren kon hiervoor echter een verklaring geven, aangezien receptorbinding het meest efficiënt is wanneer het virus niet langer wordt 'afgeleid' door niet receptor-geassocieerde siaalzuren. Dit suggereerde dat FECV waarschijnlijk lijkt op het transmissiebel gastro-enteritis virus (TGEV) in de afhankelijkheid van siaalzuren tijdens infectie, en dus dat siaalzuren geen receptoren zijn, maar dat binding aan siaalzuren waarschijnlijk een strategie is van het virus om te kunnen binden aan de mucines die het enterocyt oppervlak bedekken. De FECV-mucine interactie werd onderzocht door competitieve inhibitie experimenten uit te voeren met 2 verschillende, commercieel beschikbare mucines, namelijk porciene gastrische mucine (PGM) en boviene submaxillaire mucine (BSM). In tegenstelling tot PGM dat geen effect had op FECV infecties tot 50 mg ml^{-1}, kon BSM FECV infecties inhiberen vanaf 0,5 mg ml^{-1}. Dit inhiberende effect was veel minder uitgesproken in NA-behandelde cellen, wat opnieuw bevestigde dat FECV-siaalzuur/mucine interacties optreden in de celculturen maar geen voorwaarde zijn om enterocyten te infecteren *in vitro*. Deze bevindingen toonden aan dat FECV in staat is om met mucines (rijk in α2,6-gelinkte siaalzuren zoals BSM) te interageren, en een hypothese werd opgesteld dat deze interactie waarschijnlijk niet enkel een strategie is van het virus om te kunnen ontsnappen aan de intestinale flow, maar waarschijnlijk ook gebruikt wordt om zichzelf te beschermen tegen lage pH en proteasen in het begin van het gastro-intestinaal stelsel.

In **hoofdstuk 4.2** werd het effect van verscheidene lysosomotrope agentia en protease inhibitoren op de replicatie van serotype I FECV stammen UCD en UG-FH8, serotype II FCoV WU 79-1683 en TGEV in enterocyten onderzocht. In tegenstelling tot FCoV WSU 79-1683 en TGEV was de serotype I FECV replicatie onafhankelijk van zure pH of cathepsine B.

De furine inhibitor decanoyl-RVKR-CMK had geen effect op de serotype I FECV opname of infectiviteit van geproduceerde virussen, maar wel op de start van de replicatie van FCoV 79-1683 en TGEV. De serine protease inhibitor AEBSF had een sterk reducerend effect op de replicatie van alle FCoVs en TGEV wanneer het continu werd toegevoegd voor en tijdens de replicatie of wanneer het werd toegevoegd na inoculatie. Dit was het resultaat van de inhibitie van een virion-geassocieerd protease, aangezien dezelfde reductie in replicatie werd gezien wanneer enkel het virus werd behandeld met AEBSF. Aan de hand van een kinetische studie waarbij AEBSF op verschillende tijdstippen na inoculatie werd toegevoegd, werd aangetoond dat dit serine protease zijn functie vervulde tussen 60 en 120 min p.i. Zoals verwacht, gegeven dat het coronavirus' polyproteïne geknipt wordt door virale cysteïne proteasen, inhibeerde de cysteïne protease inhibitor E64d de replicatie wanneer het continu werd toegevoegd voor en tijdens de replicatie of wanneer het werd toegevoegd na inoculatie. Noch AEBSF of E64d inhibeerde de replicatie wanneer enkel de cellen werden voorbehandeld. Gebaseerd op de gegevens uit hoofdstuk 4.2 kon besloten worden dat in tegenstelling tot serotype II FCoVs, serotype I FECVs niet afhankelijk zijn van lage pH, cathepsine B en/of furin voor hun entry, maar de exacte fusie trigger kon nog niet achterhaald worden. Deze observatie bevestigt dat het extrapoleren van data gebaseerd op serotype II virussen naar infecties met serotype I virussen met de nodige voorzichtigheid moet gebeuren, zeker wat de opname van het virus betreft. Bovendien kon geconcludeerd worden dat serotype I FECVs (en sommige andere alfa-coronavirussen) een virion-geassocieerd serine protease bevatten dat waarschijnlijk betrokken is in de start van de replicatie en dus een belangrijk doelwit kan zijn in toekomstige therapieën.

In **hoofdstuk 5** werden de *in vivo* FECV-kat interacties bestudeerd door enerzijds katten experimenteel te infecteren (hoofdstuk 5.1) en anderzijds door kattenkwekerijen op te volgen (hoofdstuk 5.2).

Hoofdstuk 5.1 beschrijft de onderzoeksresultaten die verkregen werden door, na experimentele inoculatie van drie FIV-, FeLV- en FCoV-vrije katten met serotype I FECV UCD, de orale en fecale uitscheiding, de neutraliserende antistoftiters, viremie en leukocyten subsets op te volgen gedurende 3 maanden na inoculatie. Virus uitscheiding werd gekwantificeerd met behulp van 2 verschillende RT-qPCRs (één die het 3' uiteinde van alle genomische en subgenomische (m)RNAs (3'qPCR) en één die ORF1b van het genomische RNA herkent (5'qPCR)), en met behulp van virustitratie in de enterocytenculturen. Bij twee van de drie katten startte de uitscheiding vanaf 2-4 dagen p.i. en bleef viraal RNA

detecteerbaar in de feces gedurende 2 maanden. Infectieus virus werd gevonden van dag 4 tot dag 28 p.i. en neutraliserende antistoffen werden merkbaar vanaf dag 9. Een cel-geassocieerde viremie werd gezien op inconsistente tijdstippen na het beginnen van de fecale uitscheiding. Er werden geen abnormale leukocyten aantallen waargenomen, met uitzondering van een granulocytopenie bij kat 1. Er werd aangetoond dat een titer overschatting van 3-4.3 $\log_{10}$ werd gemaakt indien de 3' qPCR gebruikt werd om het aantal virale genoom kopieën in te schatten zoals vaak wordt gedaan. Het is dan ook logisch dat enkel 1/1.000.000 tot 1/100.000.000 van die 'partikels' infectieus waren. Het verschil tussen totale virale RNA kopieën en infectieuze titers was meer plausibel met het gebruik van de 5' qPCR, alhoewel dit verschil afhankelijk was van het tijdstip p.i. waarop het werd beoordeeld. De hoeveelheid totaal viraal RNA was namelijk 3-4 $\log_{10}$ hoger dan de infectieuze titer tijdens de eerste week na inoculatie, maar deze ratio steeg naar 4.5-8 $\log_{10}$ tijdens de rest van het experiment. Deze verlaagde *in vitro* infectiviteit viel samen met het opkomen van neutraliserende antistoffen in het bloed en er werd gespeculeerd dat neutraliserende antistoffen in de mest een onderschatting van de infectieuze titer konden hebben veroorzaakt, maar dit moet nog verder bevestigd worden. Verrassend genoeg was er één van de drie katten die een afwijkend infectiepatroon vertoonde. Dit was gekenmerkt door een sterk verlate fecale uitscheiding (vanaf dag 14 in plaats van dag 2-4 p.i.), gebrek aan infectiviteit van het uitgescheiden virus in the enterocyten culturen, een vertraagde opkomst van antistoffen en een viremia die reeds detecteerbaar was lang voordat enige intestinale replicatie optrad. Om deze vroege viremie te kunnen linken met de vertraagde intestinale replicatie werd een hypothese opgesteld die een alternatieve route voor intestinale infectie voorstelt. Hierbij wordt vooropgesteld dat FECV mogelijks door gevoelige (monocytaire) cellen in de tonsillen kan worden opgenomen, van waaruit viremie optreedt en waarna deze cellen finaal in de intestinale mucosa zullen uittreden. Het gebrek aan *in vitro* infectiviteit werd verklaard het gevolg te kunnen zijn van de excretie vanuit monocytaire cellen, waarbij het virus fenotypische veranderingen had ondergaan door adaptatie aan/replicatie in deze cellen, maar dit moet nog verder onderzocht worden. Er werden geen abnormaliteiten of verschillen in leukocyten aantallen waargenomen in vergelijking met de andere 2 katten, met uitzondering van de $CD8^+$ regulatorische T cellen, maar of en hoe deze cellen betrokken zijn blijft ongekend.

De enorme vraag naar controle maatregelen en het gebrek aan gegevens over de haalbaarheid om de FECV transmissie te beperken in de preventie van FIP stimuleerde ons om in kattenkwekerijen de FECV uitscheiding te monitoren en te onderzoeken of de transmissie kon

beperkt worden door contact tussen uitscheiders en negatieve dieren te vermijden. In **hoofdstuk 5.2** werden FCoV genomen semi-kwantitatief beoordeeld met de 5' qPCR in meststalen die op regelmatige tijdstippen werden genomen. Op basis van deze resultaten werden katten gegroepeerd in het huis in op z'n minst één positieve en één negatieve groep. Eén jaar na de start van de monitoring kon de persisterende uitscheider geïdentificeerd worden in huishouden 1. Deze kwekerij was zelfs volledig vrij van uitscheiders na het verwijderen van de persisterende uitscheider en twee FCoV-negatieve nesten konden worden opgegroeid. De kwekerij kon echter niet volledig negatief worden gehouden door de regelmatige import van nieuwe FECV uitscheiders, maar door deze katten gescheiden te houden van de rest kon wel voorkomen worden dat het virus zich weer over de volledige kwekerij ging spreiden. Door het succes van deze strategie werd deze ook in een tweede kwekerij toegepast, waar het scheiden van uitscheiders en negatieve dieren ook succesvol was. In tegenstelling tot huishouden 1 bleven echter 7 van de 14 katten positief na 1 jaar monitoren, en verdere opvolging is nodig om de echte persisterende uitscheider(s) te identificeren. Groepering van katten op basis van antistoftiters wordt nog steeds regelmatig toegepast in de praktijk, maar door de uitscheiding te vergelijken met antistoftiters werd duidelijk aangetoond dat antistoffen geen goede parameter zijn om de infectiviteit van een kat in te schatten. Uit hoofdstuk 5.2 werd besloten dat FECV transmissie succesvol kan beperkt worden in kleine tot matig grote kattenkwekerijen door het regelmatig monitoren van de uitscheiding en het groeperen van katten, zonder dat strikte maatregelen nodig zijn met betrekking tot het verkeer van mensen, kledij of het aanraken van katten. In kwekerijen waar regelmatig FIP gevallen optreden, is het zeker de kosten waard om de FECV transmissie regelmatig te monitoren omdat dit de enorme economische verliezen door kittensterfte ten gevolge van FIP kan voorkomen. Hoewel deze strategie efficiënt is, zal het altijd een tijd- en geld-rovende methode blijven omdat FECV zo wijdverspreid voorkomt, langdurig wordt uitgescheiden en erg besmettelijk is. Verdere investeringen in het ontwikkelen van adequate preventieve en/of curatieve middelen zullen dan ook nodig zijn in de strijd tegen FIP.

In **Hoofdstuk 6** werd een overzicht en discussie gegeven van alle resultaten die verkregen werden in de huidige thesis. Een hypothetisch model voor de rol van siaalzuren in de start van intestinale FECV infecties werd besproken. Bovendien werd bediscussieerd dat FECV waarschijnlijk zowel via passage door de maag als via het bloed een intestinale infectie kan initiëren, wat kan leiden tot uitscheiding van het virus in de mest. Bovendien werden ook richtlijnen gegeven om in kleine tot matig grote kattenkwekerijen de incidentie van FIP te

reduceren via het controleren van de FECV transmissie. Gegeven dat deze managementstrategie tijd- en geld-rovend is omwille van de langdurig uitscheiding van FECV, en dat deze strategie moeilijker haalbaar zal zijn in erg grote kwekerijen of andere multi-kat omgevingen (asielen/verkoopscentra) waar zeer regelmatig nieuwe dieren worden binnengebracht, werden in het laatste deel van hoofdstuk 6 enkele mogelijke toekomstige benaderingen om FIP aan te pakken bediscussieerd en werden mogelijke doelwitten in de serotype I FECV replicatie, die in deze thesis werden geïdentificeerd, beschreven.

Samengevat zijn de belangrijkste verwezenlijkingen en conclusies uit de huidige thesis als volgt:

- Twee nieuwe feliene intestinale epitheelcellijnen werden ontwikkeld door immortalisatie van primaire ileocyten en colonocyten.
- Deze enterocyten-cellijnen lieten voor de eerste maal toe om FECV stammen op te groeien en FECV-enterocyten interacties te bestuderen. Bovendien zal dit één van de onmisbare tools zijn om onderliggende virale factoren betrokken in de pathotype switch te bestuderen.
- FECV heeft een siaalzuurbindend vermogen (α2,6-gelinkte- meer dan α2,3-gelinkte siaalzuren) dat gedeeltelijk gemaskeerd is door virion-geassocieerde siaalzuren.
- Binding aan siaalzuren is geen noodzaak om *in vitro* enterocyt-infecties te kunnen induceren en zorgt er zelfs voor dat het virus minder efficiënt kan interageren met de receptor. Niettegenstaande het *in vitro* nadeel van siaalzuurbinding, geeft dit FECV waarschijnlijk een voordeel *in vivo* om te kunnen omgaan met de ongunstige verteringscondities en mucus barrière in het gastro-intestinaal stelsel.
- In tegenstelling tot serotype II virussen zijn serotype I FECV stammen niet afhankelijk van zure pH, cathepsine B en/of furin voor hun replicatie in enterocyten, maar de exacte fusie trigger kon nog niet achterhaald worden.
- AEBSF is een sterke inhibitor van de FCoV en TGEV replicatie dat een nog te identificeren virion-geassocieerd serine protease inhibeert. Dit protease werkt tussen 60 en 120 min p.i.
- Experimentele FECV infectie van 3 katten toonde een afwijkend infectie-patroon in één van de katten, gekenmerkt door een sterk verlate fecale uitscheiding (vanaf dag 14 in plaats van dag 2-4 p.i.), gebrek aan infectiviteit van het uitgescheiden virus in de enterocyten-culturen, een vertraagde opkomst van antistoffen en een viremia die reeds optrad lang voordat enige intestinale replicatie te zien was. Gebaseerd op deze

gegevens werd een hypothese opgesteld dat intestinale FECV infecties waarschijnlijk niet enkel via passage door de maag, maar ook via het bloed kunnen worden geïnitieerd.

- De algemeen gebruikte 3' qPCR geeft een overschatting van het aantal virale genomen met ongeveer 3-4.3 $\log_{10}$.
- Het beperken van FECV infecties door het controleren van de feco-orale transmissie, en niet via antistoftiter bepaling, is een haalbare strategie om het aantal FIP gevallen in kleine tot matig grote kattenkwekerijen te reduceren.

Curriculum Vitae

Personalia

Lowiese Desmarets werd geboren in Kortrijk op 10 januari 1986. In 2004 behaalde zij het getuigschrift van hoger secundair onderwijs aan het Sint-Amandscollege te Kortrijk in de richting wiskunde-wetenschappen. Daarna studeerde ze verder aan de Universiteit Gent en behaalde in 2010 het diploma van master in de Diergeneeskunde (afstudeerrichting onderzoek) met grootste onderscheiding. Geboeid door het wetenschappelijk onderzoek, startte zij in oktober 2010, onder begeleiding van Prof. Dr. H. Nauwynck, een doctoraatsonderzoek aan het Laboratorium voor Virologie aan de Faculteit Diergeneeskunde van de Universiteit Gent. Dit onderzoek handelde over de pathogenese van feliene coronavirussen, in het bijzonder over het felien enterisch coronavirus (FECV) en de mogelijkheid om FECV, als voorloper van het fatale feliene infectieuze peritonitis virus (FIPV), aan te pakken in de strijd tegen FIP. Het onderzoek werd gefinancierd door een doctoraatsbeurs van het Fonds voor Wetenschappelijk Onderzoek (FWO) Vlaanderen.

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- **Desmarets LM**. Diagnose en preventie van feliene coronavirus infecties. SAVAB najaarsdag, 13 december 2014, Deinze.

Dankwoord

Dit is het dan, het laatste hoofdstuk van dit vier jaren durende avontuur. En dat was het zeker! Net zoals het een goed avontuur beoogt, vond ook dit doctoraat zijn weg over talrijke bergen, dalen, woelige wateren of eindeloos lijkende vlaktes, om uiteindelijke moe maar voldaan te stranden op het eindpunt, dat door de (onvoorziene?) omstandigheden misschien wel wat afwijkt van het initiële doel. Logisch ook, want onderzoek zou namelijk niet zijn wat het is als we op voorhand zouden weten waar we moeten uitkomen. Wat mij persoonlijk het meest zal bijblijven, is dat een doctoraat niet alleen over onderzoek gaat, maar een ontdekkingsreis is op alle mogelijk vlakken. Iedere PhD lijkt van start te gaan met het streven naar dat ene wereldschokkende of baanbrekende resultaat. Maar onder het motto "De ware ontdekkingsreis is geen zoektocht naar nieuwe landschappen, maar het waarnemen met nieuwe ogen" (Marcel Proust), maakt dit streven ook snel plaats voor de fascinatie en bewondering voor de vele mysteries waarmee we als PhD student geconfronteerd worden, maar ook voor dankbaarheid tegenover alle mensen die elke dag opnieuw klaarstaan om dit avontuur tot een goed einde te brengen. In dit laatste hoofdstuk maak ik dan ook graag tijd om alle hoofdrolspelers, figuranten en (toevallige?) passanten, die mijn weg doorheen dit doctoraat mee hebben bepaald, uitvoerig te bedanken.

In tegenstelling tot de alom bekende avonturier die schreef "For the execution of the voyage to Indies, I did not make use of intelligence, mathematics or maps", (niet zo verwonderlijk dan dat onze Christoffel aan de andere kant van de wereld uitkwam, toch?) kon dit doctoraat niet tot stand komen zonder de nodige intelligentie en wetenschappelijke input van de **promotor**. Hans, ik herinner me nog de dag waarop je op de proppen kwam met het FWO-project dat dit doctoraat moest ondersteunen. Je vroeg me wat ik er zelf van dacht en vooral of ik het (met mijn ervaring die ik reeds mocht opdoen in het labo tijdens de masterproefjaren) haalbaar zag, waarop ik toen nog vol overtuiging "ja, zeker!" antwoordde. Van het originele project over darm-explanten, receptoren en discriminerende testen is echter niet meer veel terug te vinden in dit doctoraat, maar net zoals de input hebben jouw onuitputtelijke enthousiasme, (out of the box) ideeën en neiging om van iedere gelegenheid een brainstormsessie te maken ook de output van dit werk bepaald. Bedankt om jouw passie voor het onderzoek "viraal" te maken en ons te leren dat een gezonde kritische houding, een

vleugje gezond (en liefst ook wat abstract) verstand en vooral doorzettingsvermogen en zelfvertrouwen de belangrijkste sleutels zijn tot succes.

Ook zonder de **ex-FIP'ers** zou dit doctoraat niet zijn wat het is. Hannah, Evelien en Els, jullie input heeft onmiskenbaar de fundamenten gelegd voor het huidige FIP-onderzoek, waar niet alleen ik, maar ook vele andere van hebben mogen en nog van zullen profiteren. Hannah, jij was diegene die me reeds als masterproefstudente wegwijs maakte in het labo en de knepen van het vak leerde. Ook aan de start van dit doctoraat stond je klaar om ieder probleem(pje) of teken van frustratie uit te weg te ruimen door me te laten inzien dat elke 'tegenslag' ook zijn positieve kant heeft. Ook jouw onuitputtelijke bron van kennis (inclusief de nodige referenties!) was indrukwekkend. Ik ben dan ook heel blij dat je er ook in de eindfase van dit doctoraat als deel van de begeleidingscommissie weer bij was om de puntjes op de i te zetten. Bedankt voor alles! Evelien, onze samenwerking begon ergens bij de darm-explanten en even zag het naar uit dat dit ook zou verder gezet worden in de enterocyten cellijnen, maar jouw passie voor microscopie besliste daar anders over. Maar ook al was onze tijd samen kort, ook jij hebt enorm veel voor mij betekend bij zowel de eerste stapjes als bij de laatste loodjes. Bedankt voor alle kennis, protocols en levenswijsheden die je met mij hebt gedeeld en om de tijd te nemen om deze thesis in drukke tijden toch grondig na te lezen! Dominique, ook jij vervoegde (wat onverwacht?) de begeleidingscommissie, maar na jarenlange nauwe samenwerking leek dit voor mij niet meer dan normaal. Ik heb altijd enorm veel ontzag gehad voor het geduld en de nauwkeurigheid waarmee je bepaalde zaken te lijf ging, en hoe je eigenlijk zonder al te veel moeite (persoonlijke) problemen in de groep durfde te gooien. Je leek het zelf niet altijd goed te beseffen maar je bent een straffe madam, die niet bang is om de handen uit de mouwen te steken of wat overuren te kloppen indien nodig. Je was dan ook vaak op onmenselijke tijdstippen in het labo te vinden. Vooral in het begin, waar van continue cellijnen nog geen sprake was, en katten in de praktijk nu eenmaal niet binnen de 'kantooruren' moesten worden geëuthanaseerd, behoorden we samen ogenschijnlijk meer tot het meubilair dan tot het personeel van het labo. Ook al misten we daardoor wel meerdere 'social events', het was toch fijn om op die momenten bij jou een partner in crime te vinden. Maar geef toe, ik denk dat we er meer memorabele momenten aan hebben overgehouden dan we gemist hebben ☺. Bedankt voor het trainen van mijn lachspieren tijdens alle leuke, of minder leuke ('ik krijg precies kramp in mijn vingers'…. ☺) momenten en voor alle serieuze of minder serieuze babbels. Annelike, jouw luisterend oor heeft menig van ons kunnen bekoren. Het is bewonderenswaardig hoe jij op elk moment klaarstond om iedereen uit de

nood te helpen, ook op momenten waar je het zelf even niet meer zag zitten. In zal ook nooit vergeten hoe jij als een geroutineerde jager die wilde katten met je blote handen te lijf ging, terwijl Dominique en ik tot de tanden gewapend met overalls, handschoenen en borstels compleet verbouwereerd aan de grond genageld bleven toekijken ☺. Bedankt voor alles wat je voor mij hebt gedaan. Ben, ook wij hebben heel wat katjes moeten geselen de voorbije jaren, maar de resultaten mochten er zeker zijn. Bedankt voor jouw praktische, wetenschappelijke en software-gerelateerde bijdrage aan dit doctoraat. Leslie, ook jij hebt als ex-collega zeker je steentje bijgedragen aan het huidige FIP onderzoek, hoe kort jouw avontuur bij ons ook was. Ik wil je ook graag bedanken om als lid van de examencommissie de tijd te nemen om deze thesis te beoordelen.

Uiteraard zou ik ook alle andere leden van de **lees- en examencommissie**, Prof. Eric Cox, Prof. Berend Jan Bosch, Prof. Sylvie Daminet en Prof. Etienne Thiry, willen bedanken voor alle inspanningen om dit werk te beoordelen. Berend Jan, jou wil ik graag in het bijzonder nog extra bedanken voor de korte maar krachtige samenwerking en de gastvrijheid van het voorbije jaar. Als alom gekende expert op het gebied van coronavirussen en entry was het voor mij een enorme eer om met jou te kunnen samenwerken en van jou te kunnen leren. Ook Prof. Rottier wil ik graag langs deze weg bedanken. Uw niet aflatende kennis, enthousiasme en passie voor het FCoV onderzoek maakten van elke brainstormsessie een eyeopener voor mij. Bedankt om mij te willen ontvangen in het labo, elke dag was er één om te koesteren. Huihui, thanks for being my guide and partner in crime at the Utrecht University. I wish you all the best with your upcoming PhD defence and with the rest of your undoubtedly beautiful career!

Vervolgens is er het kloppende hart van de virologie, alle **administratieve en technische medewerkers** die dag na dag klaarstaan om het labo draaiende houden. Gert'je' (of Hert'je' voor de West-Vlamingen ☺), Mieke, Marijke, Dirk en Ann M., bedankt om vaak in drukke tijden klaar te staan om allerlei kleinere en grotere administratieve, en in geval van Dirk ook computer-gerelateerde, problemen op te lossen en ons op die manier heel wat werk uit handen te nemen. Ook Zeger en Loes (en in wat langer vervlogen tijden ook Geert, Fernand en Bart) wil ik graag bedanken voor het dag na dag voorzien van proper/steriel materiaal, voor hun bijdrage aan de experimentele infectiestudies, om ervoor de zorgen dat onze dieren alles krijgen wat ze nodig hebben en natuurlijk ook voor de fijne babbels tussendoor. Verder wil ik natuurlijk ook alle laboranten (Lieve, Nele, Melanie, Chantal, Carine, Tim en Ytse) bedanken voor hun onschatbare bijdrage aan alle experimenten en diagnoses. Ytse, bedankt voor het

uitvoeren van de vele extracties, PCR's, kleuringen en suspensies de voorbije 4 jaren, en om ons mini-labo'tje keer op keer van alle benodigdheden te bevoorraden. Je bent echt een toplaborante, of eigenlijk meer een pseudo-PhD student, die altijd klaarstaat om ons te helpen, niet alleen voor het uitvoeren van experimenten maar ook voor het vinden van oplossingen of om de fouten uit onze protocols te halen! Bedankt om heel wat last van onze schouders te halen en geniet met volle teugen van het prille moederschap!

Dit brengt mij vervolgens bij alle "lot"genoten, **collega (of ex-collega) PhD studenten** zonder wie dit doctoraat (en bijhorende publicaties) niet zou zijn wat het is. Zoals de meeste mensen weten, bevond de thuishaven van dit doctoraat zich op de eerste verdieping, waar tussen alle parasitologen een kleine groep virologen gedijt in het FIP/FIV/Rota-labo en bijhorende "eerste bureau". Bas, jij verscheen reeds gedurende de masterjaren op het toneel, waar ons professioneel gepruts met de darm-explanten de basis heeft gelegd voor een ongetwijfeld mooie samenwerking en vriendschap. Als vat vol kennis over de meest uiteenlopende zaken (dit moet het zijn wat jou zo aantrekt in het IWT? ☺) en fascinatie voor de meer moleculaire kant van het onderzoek ben je echt van onschatbare waarde geweest, niet alleen voor mij, maar (aan de neiging tot rij-vorming achter jouw bureau te beoordelen ☺) ook voor vele anderen. Maar daarnaast zorgden jouw goedlachse ingesteldheid (en wat onhandige vrienden ☺) voor een opperbeste sfeer en boeiende verhalen. Als rasechte Antwerpenaar was je om één of andere reden ook al snel gefascineerd door het West-Vlaamse taaltje, en ergens onderweg werd dan ook het (reeds volledig ingeburgerde) woord "Tsjoolder" geboren. En ook de vele, al dan niet door copyright beschermde, oneliners toverden telkens weer die glimlach op mijn gezicht. Dus, Tsjoolder, ik ben blij dat ik in jouw team zat, waar er vol discipline, dedication and friendship keer op keer ruimte was voor een "met mij kunde lachen hé" moment! Bedankt voor alles! Inge, ook jij kruiste niet zo snel na mijn start mijn pad, al leek jouw doorstart wat moeilijker dan verwacht. Maar jouw vechtlust (letterlijk én figuurlijk), enorm probleemoplossend vermogen en veelzijdigheid hebben niet alleen van het huidige FIV onderzoek een pareltje gemaakt, maar hebben ook velen van ons vaak terug op weg geholpen. Jouw zin voor humor en enorm aanstekelijke lach zorgden bovendien voor de nodige sfeer, terwijl jouw uit het niets opduikende drang naar opruimen en organisatorisch talent dan weer voor de nodige *dedication* en *position-switches* zorgden om het labo en bureau voor iedereen zo aangenaam mogelijk te houden. Isaura, jij kwam ons wat later vervoegen in het bureau op de eerste verdieping, maar al snel werd duidelijk dat deze nauwere samenwerking voor ons allen een enorme boost was, zowel op wetenschappelijk

niveau als qua amusementsgehalte. Jouw talenten en kennis zijn voor menig van ons al van onschatbare waarde geweest, en het was (en blijft) verbazingwekkend hoe jij vaak razendsnel een oplossing vond voor wat voor mij (en andere dierenartsen onder ons) vaak moeilijk oplosbare problemen leken. Bedankt voor je enorme bijdrage aan het FIP onderzoek! Delphine, al vanaf de eerste dag, waarop jij als volleerd FIP-onderzoeker vol overgave met ons mee discussieerde, merkten we dat jij een aanwinst zou zijn voor de FIP-groep (en onze lachspieren…). En ook de studenten mogen zich gelukkig prijzen met zo'n fantastische assistente. Ik wens je veel succes met wat nog komen zal, maar ik weet nu al zeker dat je met jouw capaciteiten (en de nodige inspanningen uiteraard ☺) het FIP-onderzoek naar een volgend niveau zal kunnen brengen. Elke, wij leerden elkaar nog niet zo lang geleden kennen, maar ik stond meteen versteld van alle werklust die je in je hebt. Ik ben er zeker van dat met deze ingesteldheid de toekomst je toelacht! En dan zijn er nog alle andere (ex-)collega's die één voor één op hun eigen manier hun steentje hebben bijgedragen ergens in de loop van dit doctoraat. Wander, Caroline, Merijn, Lennert, Sarah G., Sarah C., Annelies, Marc, Sjouke, Miet, Karen O., Karen V., Bauke, Inge H., Hanne, Mieke V., Karl, en alle andere die ik misschien nog vergeet, ik wil jullie graag bedanken voor alle steun, tips en natuurlijk ook voor de onmisbare ontspannende momenten tussendoor. Weet dat elke input van jullie kant enorm geapprecieerd werd. Ook de mensen van de immuno (Thary, Jochen, Korneel, Maria en Céline) verdienen een woordje van dank om ons uit de nood te helpen met producten of andere benodigdheden (zoals de posterkoker) indien dit nodig was. Also thanks to all past and present non-Dutch speaking people (Sabine, Sabrina, João, Dipu, Uladzimir, Amy, Angela, Hoessein, Kathlyn, Kevin, Charlie, Vishi, Garba, Jing, Yu, Wengfeng, Ivan, Ilias, José, Yewei, Fang, Jason, Tingting, Mohammed,…) for their contribution to this work and/or for the pleasant talks in between all work. I wish you all the best with your upcoming careers.

Ook alle andere medewerkers van dit doctoraat, **dierenartsen en eigenaars**, verdienen een woordje van dank voor hun bijdrage. In het bijzonder Frederik, Kathy, Kurt en Roosje zou ik willen bedanken om hun cattery voor ons onderzoek open te stellen!

Tenslotte zou dit alles niet mogelijk zijn geweest zonder de nodige steun van **familie en vrienden**. Mijn ouders (inclusief stiefouders of hoe je dit ook mag definiëren) en schoonouders wil ik uit de grond van hart bedanken voor alles wat jullie voor mij betekend hebben in de afgelopen jaren. Niemand van jullie heeft waarschijnlijk ooit echt volledig begrepen wat ik "daar in Merelbeke" zat uit te spoken ("moet iets met virussen, katten en darmen geweest zijn?") en vooral niet waarom het werk op vrijdag niet stopte zoals bij de

meeste jobs. Maar desalniettemin dit “communicatie probleem”, bleven jullie steeds vol interesse polsen naar de vorderingen en geboeid luisteren naar wat ik te vertellen had. En ook wist ik dat ik bij jullie altijd de nodige ontspanning kon vinden in drukke tijden. De mama’s wil ik graag nog extra bedanken om ons na lange werkdagen zeer regelmatig te voorzien van de nodige spijs en drank, zodat we ons geen zorgen hoefden te maken om vóór sluitingstijd de winkel nog te bereiken of het grootste deel van de meestal zeer korte avond nog te verliezen aan koken of afwassen. Bedankt voor alles! Ook Mathias en Delphine, Sofie en Arnaud en natuurlijk bijhorende schatten van kinderen wil ik graag bedanken voor alle momenten van ontspanning tussen het harde werk door. En die ontspanning was er natuurlijk ook altijd bij de vrienden! Joke, Laurent, Anne, Arne, Hannes en Kim, bedankt voor alle gezellige etentjes en uitstapjes. Joke, jij bent het organisatorisch talent van de bende, dus zonder jou zouden de voorbije jaren er misschien wel heel wat saaier hebben uitgezien. En ook van jouw neiging om regelmatig nieuwe recepten uit te proberen hebben we, als proefkonijn, altijd ten zeerste genoten! Bedankt voor al jouw inspanningen! Laurent, wij hebben voor het grootste deel hetzelfde traject afgelegd, tot in het laatste jaar diergeneeskunde, waar door mijn passie voor het onderzoek en jouw meer uitgesproken interesse in het klinische aspect van het vak onze professionele wegen gescheiden leken te worden. Maar niets was minder waar, want zonder jouw input zou dit doctoraat (en ook reeds voorbije doctoraten) maar een fractie zijn van wat het nu is. Jij was namelijk diegene die steeds onvoorwaardelijk moeite bleef doen om ons te voorzien van de nodige weefselstalen, waarmee we uiteindelijke heel wat *in vitro* tools hebben kunnen creëren. Maar naast deze professionele samenwerking, blijf je natuurlijk een vriend voor het leven en heb je altijd (bewust of eerder accidenteel ☺) voor het nodige entertainment gezorgd. Bedankt voor alles! Anne, wij leerden elkaar reeds in ver vervlogen tijden ergens in het verre Wevelgem kennen, waarna je voor ongeveer 10 jaren van de radar verdween, tot je plots opdook in het auditorium tussen alle eerstejaarsstudenten Diergeneeskunde. Sindsdien werd onze band steeds hechter. Uiteindelijk volgden we ook hetzelfde traject en konden we al onze (onderzoeks)avonturen met elkaar blijven delen tijdens de gezellige etentjes of uitstapjes. Bedankt voor alle mooie momenten en ik wens je veel succes met het finaliseren van jouw doctoraat. Hannes, ook jou wil ik graag nog bedanken voor alle mooie momenten die we samen hebben gedeeld, niet alleen tijdens onze studies maar ook erna. Ook alle mensen van de volleybal zou ik willen bedanken voor fantastische tijd die we samen hebben gehad. Mijn “carrière” heeft zich uiteindelijk noodgedwongen moeten stoppen maar ik blijf jullie overwinningen (en uitzonderlijke nederlagen ☺) op de voet volgen. Bedankt voor alle ontspannende momenten. Bie, wij leerden elkaar ook in ver

vervlogen tijden (moet nu ongeveer 19 jaren geleden zijn) kennen in het volleybalmilieu, maar sindsdien ben je altijd een belangrijk deel van mijn leven geweest. De laatste jaren was je er wat minder door je bewonderenswaardige keuze om de liefde van je leven te volgen over de hele wereld. Toch bedankt voor alle fijne babbels op momenten dat je toch even in het land was en ik hoop jullie snel hier in ons Belgenlandje te zien settelen, zodat er weer meer tijd is om bij te praten (en vooral te lachen ☺) zoals in de goede oude tijd.

En dan, last but not least, de persoon die alles vanop de eerste rij heeft mogen/moeten meemaken. Schat, wij leerden elkaar ergens tijdens de middelbare studies kennen, toen nog jong en volledig onbezonnen en nog geen idee wat de toekomst ons zou brengen. Nu, ongeveer 12 jaren later, heb ik nog steeds geen moment spijt gehad van mijn keuze om mijn leven met jou te delen. En ik geef toe, het leven ging er misschien heel wat makkelijker uitgezien hebben moest ik, met mijn voorkeur om in de vroege uurtjes het bed uit te springen, postbode geworden zijn, zoals je wel eens lachend durft aanhalen, maar ik ben blij dat je ondanks de vaak eenzame momenten mij onvoorwaardelijk bent blijven steunen in mijn keuze. Bedankt om altijd je rustige zelf te blijven en ten alle tijden voor mij klaar te staan. Ik weet zeker dat we samen een mooie toekomst tegemoet gaan!

Lowiese