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Anne-Laure Pham-Hung d'Alexandry d'Orengiani

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par

Anne-Laure Pham-Hung d'Alexandry d'Orengiani

The accessory glycoprotein gp3 of canine Coronavirus type 1 :
investigations of sequence variability in feline host and of the
basic features of the different variants

Directeur de thèse : Mme Nicole Pavio Directeur de recherche (ANSES- Maisons-Alfort)
Co-directeur de thèse : Mme Sophie Le Poder Maître de conférence (ENVA- Maisons-Alfort)

Composition du jury :

Président du jury : Mme Anne Collignon Professeur (Faculté de Pharmacie-Paris 11)
Rapporteurs : Mr Peter Rottier Professeur (Utrecht University)
Mr Etienne Thiry Professeur (Université de Liège)
Examineurs : Mme Astrid Vabret Professeur (CHU de Caen)

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ABBREVIATIONS

ACE2	Angiotensin Converting Enzyme 2
AIDS	Acquired Immunodeficiency Syndrome
APN	Aminopeptidase N
ATCC	American Type Culture Collection
ATF6	Activating Transcription Factor 6
BCoV	Bovine Coronavirus
BHK	Baby Hamster Kidney
CCoV	Canine Coronavirus
CEA	Carcinoembryonic Antigen
CM	Convuluted Membranes
CoV	Coronavirus
CPV	Canine Parvovirus
CRCoV	Canine Respiratory Coronavirus
CTD	C Terminal Domain
DBT	Delayed Brain Tumour Cells
DDP4	Dipeptidyl Peptidase 4
DMV	Double membranes vesicles
EqCoV	Equine Coronavirus
ER	Endoplasmic reticulum
ERGIC	Endoplasmic reticulum-Golgi intermediate compartments
FCoV	Feline Coronavirus
FECV	Feline Enteric Coronavirus
FIP	Feline Infectious Peritonitis
FIPV	Feline Infectious Peritonitis Virus
FPV	Feline Panleukopaenia Virus
HAE	Human Airway Epithelial
HEF	Hemagglutinin Esterase Fusion
HIV	Human Immunodeficiency Virus
IBV	Infectious Bronchitis Virus
ICTV	International Committee on Taxonomy of Viruses
IDR	Intrinsically Disordered Regions
IFN	Interferon
IRF	IFN Regulatory Factor
kB	Kilobases
kDa	kiloDalton
MERS-CoV	Middle East Respiratory Syndrome Coronavirus
MeV	Measles Virus
MHV	Mouse Hepatitis Virus
MOI	Multiplicity of Infection
NiV	Nipah Virus
nm	Nanometres
nt	nucleotides
NTD	N Terminal Domain
ORF	Open Reading Frame
PEC	Poult Enteritis Complex
PEDV	Porcine Epidemic Diarrhoea Virus

PhCoV	Pheasant Coronavirus
PHEV	Porcine Hemagglutinating Encephalomyelitis Virus
PRCoV	Porcine Respiratory Coronavirus
RBD	Receptor Binding Domain
RdRp	RNA-dependant RNA polymerase
RNA	Ribonucleic Acid
RNP	Ribonucleoprotein
RTC	Replicase transcriptase complex
RT-PCR	Reverse transcriptase – Polymerase Chain Reaction
SARS	Severe Acute Respiratory Syndrome
TCoV	Turkey Coronavirus
TGEV	Transmissible Gastroenteritis Virus
TRS	Transcriptional Regulatory Sequence
UPR	Unfolded Protein Response
UTR	Untranslated Regions
VSV	Vesicular Stomatitis Virus
WHO	World Health Organisation

INTRODUCTION

CHAPTER ONE: CORONAVIRUSES

I. History and classification

I.1. History

The first member of Coronaviruses (CoV) was isolated in 1936 as the aetiological agent of infectious bronchitis virus in chicken (Beaudette et al., 1937). Ten years after, in 1946, the transmissible gastroenteritis virus in pigs was isolated (DOYLE and HUTCHINGS, 1946), followed by the mouse hepatitis virus in 1949 (Bailey et al., 1949). In 1963, Feline Infectious Peritonitis (FIP) was diagnosed for the first time by Holzworth in Boston (HOLZWORTH, 1963), and its aetiological agent, the feline Coronavirus (FCoV), was discovered by Ward a few years later (Ward, 1970). The first isolation of canine Coronavirus (CCoV) was reported in 1971, by Binn and colleagues, in a military canine facility in Germany (Binn et al., 1974). The first human Coronaviruses (HCoV-OC43 and HCoV-229E) were characterised in 1967 as agents of human respiratory diseases (Almeida and Tyrrell, 1967) and were therefore regrouped together to form the *Coronaviridae* family. Until 2002, Coronaviruses were mostly of veterinary interest for two reasons: first, they cause important economic losses in different live animal farms (bovine, porcine) and second, human Coronaviruses caused only minor respiratory infections.

Scientific interest in Coronaviruses was restored in 2002, when a new disease named SARS (for Severe Acute Respiratory Syndrome) caused by a previously unknown Coronavirus led to a massive human epidemic worldwide (Drosten et al., 2003). Many Coronaviruses have been

identified following the SARS epidemic, including two more human Coronaviruses (HCoV-NL63 and HCoV-HKU1) (van der Hoek et al., 2004) (Woo et al., 2005), thus multiplying by three the number of Coronavirus strains. Many of them have been identified among the bat species, as they are thought to be the main reservoir (Wang et al., 2006). Exactly 10 years after the SARS-CoV emergence, another pathogen emerged in the Middle East, also causing severe respiratory distress but this time with a less efficient human-to-human transmission. Still, this new emerging Coronavirus named MERS-CoV has an unusually high mortality rate of approximately 40% and appears to have adapted from camels (Meyer et al., 2014). By the end of September 2014, more than 837 people were infected with the MERS-CoV (Middle East respiratory syndrome Coronavirus) with 319 fatal cases.

I.2. Classification

The *Nidovirales* order regroups four families: *Coronaviridae*, *Arteriviridae*, *Roniviridae* and the recently added *Mesoniviridae* (Figure 1)(Cavanagh, 1997). Nidoviruses are enveloped viruses with a non-segmented positive strand of RNA. They share some significant properties such as: the same general genomic organisation with structural proteins located downstream of a very large replicase-transcriptase gene, expression of the replicase-transcriptase polyprotein through a ribosomal frameshift and a high number of enzymes dedicated to the genome transcription and replication. Downstream genes are expressed through nested subgenomic messenger RNAs. This last property provided the name of the order, as Nido stands for “nest” in Latin.

The *Roniviridae* family contains viruses that infect shrimps, crabs and represent an economical threat to the crustacean industry (Bonami and Zhang, 2011) . The *Arteriviridae*

family was established in 1996 and contains viral pathogens of domesticated animals (Snijder and Meulenber, 1998).

The *Mesoniviridae* was proposed and accepted as an additional family in 2012, when viruses isolated from mosquitoes displaying features of the Nidoviruses, but with a genome length intermediate between the *Arteriviridae* and *Coronaviridae* families, were discovered (Zirkel et al., 2011).

The *Coronaviridae* family is the largest one of the four families, by its genomic size of 30 kB (Gorbalenya et al., 2006), the number of representatives it contains and is subdivided into two separate subfamilies, the *Coronavirinae* and the *Torovirinae*. The *Torovirinae* subfamily is divided into two genera: Toroviruses and Bafiniviruses. Toroviruses are pathogens of veterinary importance as they cause enteric diseases and consequent economic losses in the swine, cattle and horse industries (Woode, 1987) and Bafiniviruses are the only viruses of the *Nidovirales* order that infect fishes (Schütze et al., 2006). The *Coronavirinae* was originally subdivided into groups, based on serological approach. With the ICTV (International Committee on Taxonomy of Viruses) revision, it is now divided into four genera, based on phylogenetic clustering: Alphacoronavirus, Betacoronavirus, Gammacoronavirus and Deltacoronavirus (Figure1).

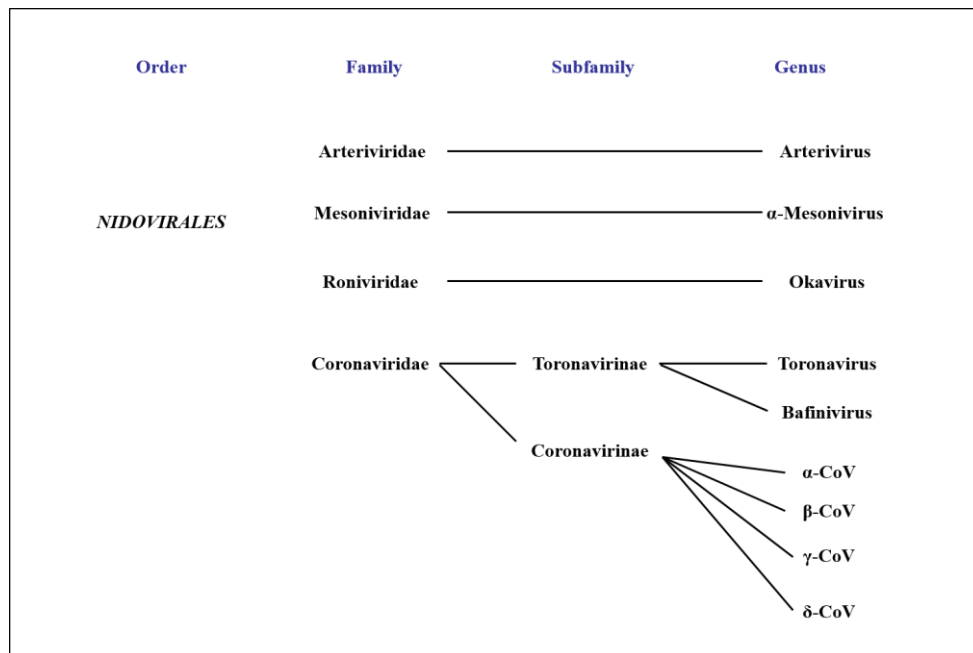


Figure 1 : Classification of the *Nidovirales* order.

Alphacoronaviruses type 1 species regroup feline FCoV, FECV (Feline enteric Coronavirus) and FIPV (Feline infectious peritonitis virus), the porcine TGEV (Transmissible gastroenteritis virus) and PRCoV (Porcine Respiratory Coronavirus) and the canine CCoV. Alphacoronaviruses also comprises human CoVs such as HCoV-229E and HCoV-NL63, but also various bat Coronaviruses. Betacoronaviruses also infect a wide range of mammals, with species such as mice, human with SARS-CoV, HCoV-OC43, HCoV-HKU1 and MERS-CoV (Table 1). Gammacoronaviruses are specific of birds, with one exception of a beluga whale Coronavirus. The deltacoronavirus genus was created in 2012 and regroups various Coronavirus from mammals to birds.

Genus	Species	Host	Acronym
Alphacoronavirus	Alphacoronavirus 1	Dogs/Pigs/ Cats	CCoV/ TGEV and PRCoV/ FECV and FIPV
	Alphacoronavirus 2**	Ferrets/ Mink	FRCV/ MCoV
	Human Coronavirus 229E	Human	HCoV-229E
	Human Coronavirus NL63	Human	HCoV-NL63
	Porcine epidemic diarrhoea virus	Pigs	PEDV
	Rhinolophus bat Coronavirus HKU10	Bats	Rh-BatCoV HKU10
	Scotophilus bat Coronavirus 512/05	Bats	Sc-BatCoV 512
	Miniopterus bat Coronavirus 1A/B	Bats	Mi-bat CoV 1A/B
	Miniopterus bat Coronavirus HKU8	Bats	Mi-batCoV HKU8
Betacoronavirus	Betacoronavirus 1	Human/Bovine/ Dogs/ Pigs/Horses	HCoV-OC43 / BCoV/ CRCoV/ PHEV/ EqCoV
	<i>Murine Coronavirus</i>	Mice/Rats	MHV/RtCoV
	Human Coronavirus HKU1	Human	HCoV-HKU1
	Middle East respiratory syndrome virus*	Human	MERS-CoV
	Tylonycteris bat Coronavirus HKU4	Bats	Ty-BatCoV HKU4
	Pipistrellus bat Coronavirus HKU5	Bats	Pi-BatCoV HKU5
	Severe acute respiratory syndrome Coronavirus	Human	SARS-CoV
	SARS related Rhinolophus bat Coronavirus HKU3	Bats	SARSr-Rh- BatCoV HKU3

	Roussettus bat Coronavirus HKU9	Bats	Ro-BatCoV HKU9
Gammacoronavirus	Avian Coronaviruses	Turkeys/Partridges/ Chicken/Peafowls	TCoV/ IBV- partridge/IBV/ IBV-peafowl
	Beluga whale Coronavirus SW1	Beluga whales	BWCoV-SW1
Deltacoronavirus	Bulbul Coronavirus HKU11	Bulbul	BuCoV HKU11
	Thrush Coronavirus HKU12	Thrush	THCoV HKU12
	Munia Coronavirus HKU13	Munia	MuCoV HKU13
	Porcine Coronavirus HKU15	Pigs	PorCoV HKU15

Table 1 : Classification of the Coronavirus family.

Representative members of the different genera of Coronaviruses with the respective host they infect and usual acronyms. Adapted from the ICTV classification.*Novel virus which has not been formally classified.

*Proposed by (Woo et al., 2012) but not formerly in ICTV classification.

II. Biology of Coronaviruses

II.1. Morphology of the virion

The Coronavirus virion is a spherical enveloped pleiomorphic particle of 118 to 136 nm (nanometres) in diameter with distinctive petal-shaped spikes on its surface, giving rise to its family name “corona” as for “crown” in Latin (Figure 2A). At least three structural proteins are located in the viral envelope: the S glycoprotein that forms the long spikes on the surface, the E protein and the M glycoprotein (Figure 2B). A fourth structural protein can be found embedded within the lipid bilayer: HE glycoprotein forms small spikes on the surface of the virion but is only present in some Betacoronaviruses, such as mouse hepatitis virus (MHV) and human Coronavirus HCoV-HKU1(de Groot, 2006). The N phosphoprotein interacts with the viral genomic RNA and forms the nucleocapsid enclosed within the viral envelope. The

nucleocapsid displays a helical symmetry, which is uncommon for positive-strand RNA viruses (Macneughton and Davies, 1978). Some accessory proteins can also be found in the viral particle.

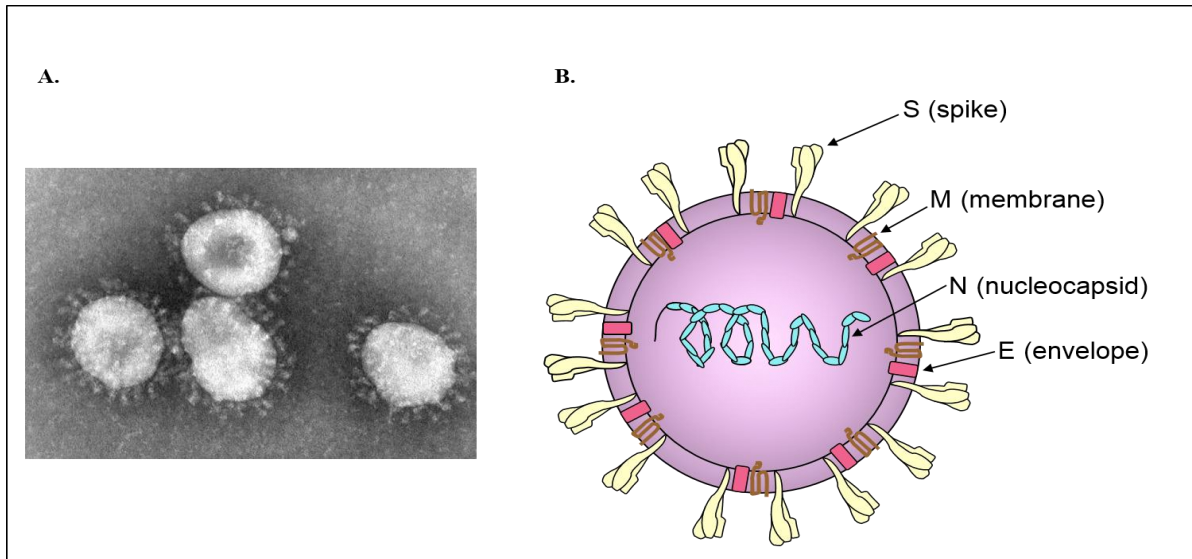


Figure 2: Morphology of the virion

A. Coronavirus particles with their distinctive crown-like appearance in electronic microscopy (Gronvall et al., 2006). B. Schematic representation of the viral particle with the structural proteins.

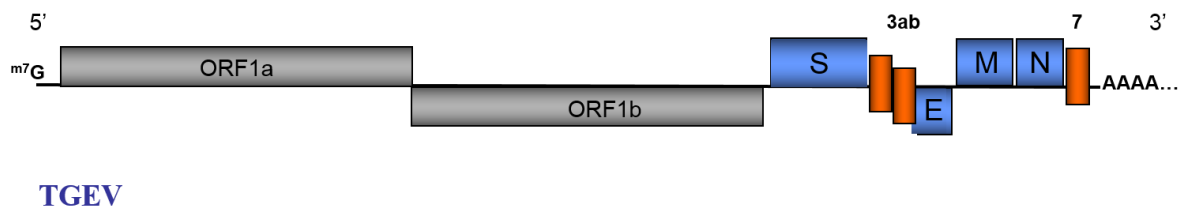
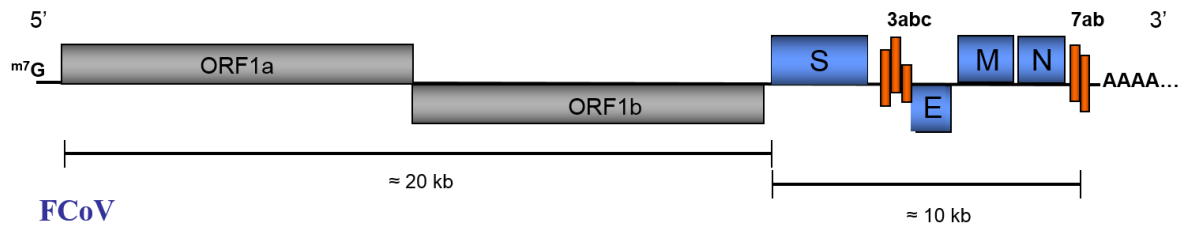
II.2. Genome organisation

Coronaviruses display the largest genome among RNA viruses which ranges from 26 to 32 kB (kilobases). It is a single strand positive-sense RNA, with a capped 5' end and a polyA tail on the 3' end. The different ORFs (Open-Reading Frames) are separated by untranslated regions (UTR) of 275 to 500 nucleotides that comprises a leader sequence of 65 bases approximately and a transcriptional regulatory sequence (TRS). The genome contains a basic set of genes always organized in the same following order: ORF1a/1b-(HE, for some Beta-CoV)-S-E-M-N (de Groot, 2006). At the 3' end, downstream of the UTR region is found the polyA tail. The genomic organisation is quite similar for all the *Coronaviridae*: ORF1a/1b occupies the first 2/3 of the genome and encodes for the 16 necessary non-structural proteins

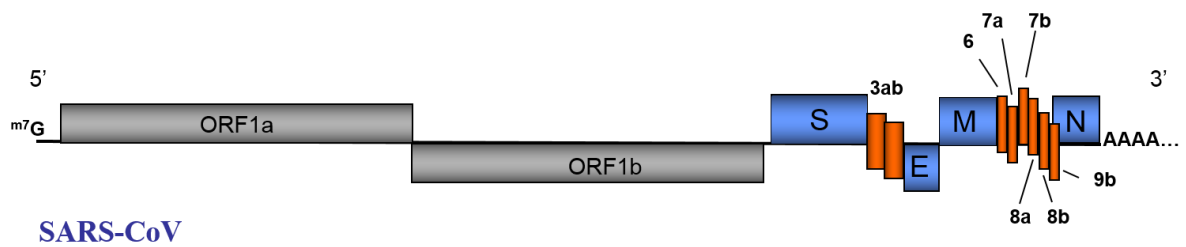
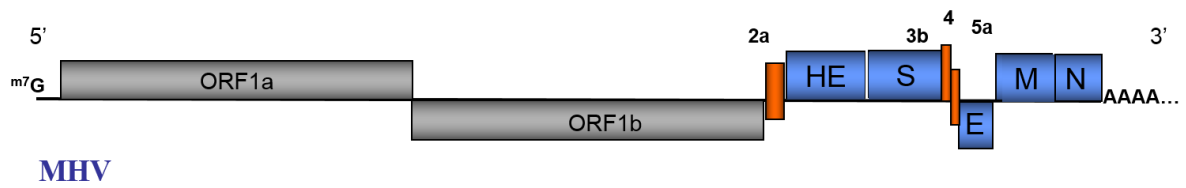
(nsp1-nsp16) required for both the replication and the transcription of the viral genome. This large replicase complex gene characterises the *Nidovirales* order and is composed of two ORFs that share a small portion of overlap. Interspaced between the structural genes are found intergenic sequences where additional ORFs encoding for accessory proteins can be found.

Those accessory proteins differ in number, size and position on the genome for each host species of *Coronaviridae* and are thus called genus (or species)-specific proteins. For example, feline Coronavirus and canine Coronavirus display two subsets of accessory proteins. The first group is the ORF3abc group that is located inside the intergenic sequence between the S and the E genes. The second group, named ORF7ab, is located downstream of the N gene. With eight different accessory proteins, SARS-CoV possesses the highest number of them (Figure 3)(Narayanan et al., 2008).

Alphacoronavirus



Betacoronavirus



Gammacoronavirus

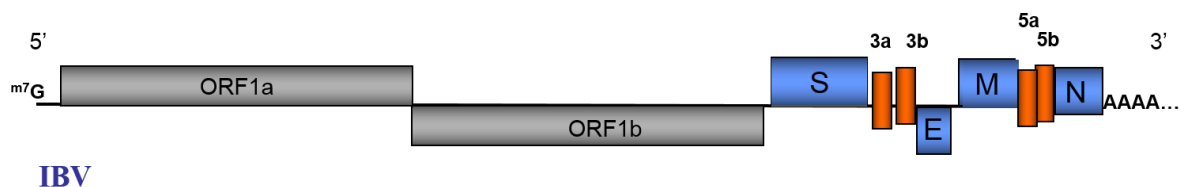


Figure 3 : Schematic representation of genomes from different Coronaviruses.

Genomic organisation of Coronaviruses with numerous accessory proteins, which are species-specific. In grey, genes of the replication/transcription. In blue, genes of the structural proteins. In orange, genes encoding accessory proteins.

II.3. Structural proteins

II.3.1. The spike S glycoprotein

The S protein ranges from 128 to 160 kDa (kiloDaltons) and is N-glycosylated during its maturation process. It assembles into trimers in order to form the distinctive spikes on the surface of the virion. S is a class I viral fusion protein that mediates membrane fusion (Bosch et al., 2003). It is responsible for the attachment to the receptor and the fusion between the cell and the viral membrane for all Coronaviruses. The large N-terminal ectodomain of S is subdivided into S1 and S2 subunits that contain the receptor binding domain and the fusion domain, respectively. Similarly to other class I fusion proteins, the S protein harbours a cleavage site between S1 and S2 domains (Figure 4). This cleavage is necessary either for an efficient virus entry into the host cell or for the cell fusion (Collins et al., 1982). PEDV (Porcine Epidemic Diarrhoea Virus) requires trypsin for efficient entry into the cell (Wicht et al., 2014). A furin cleavage motif has been recently identified in canine Coronavirus strain Elmo/02 and feline Coronavirus strains UCD and UCD8 (de Haan et al., 2008).

The S1 subunit forms the globular part of the spike and contains recognisable epitopes for host-receptor binding. The receptor binding-domain (RBD) is located on distinct parts of the S1 subunit for different Coronaviruses. For instance, MHV RBD is located at the 5' end of the S1 subunit, between a.a 1 and 330; SARS-CoV, HCoV-NL63 and HCoV-229E RBDs are located in the middle of the S1 subunit. The RBD of TGEV is located at the 3' end (Table 2).

S2 subunit is highly conserved and forms the stalk of the S protein. This S2 subunit is itself divided into different parts, in the following order: the fusion peptide, two heptad repeat domains, named HR1 and HR2, a transmembrane domain immediately downstream of HR2 and a short endodomain. The C-terminus region of S2 interacts with the M protein.

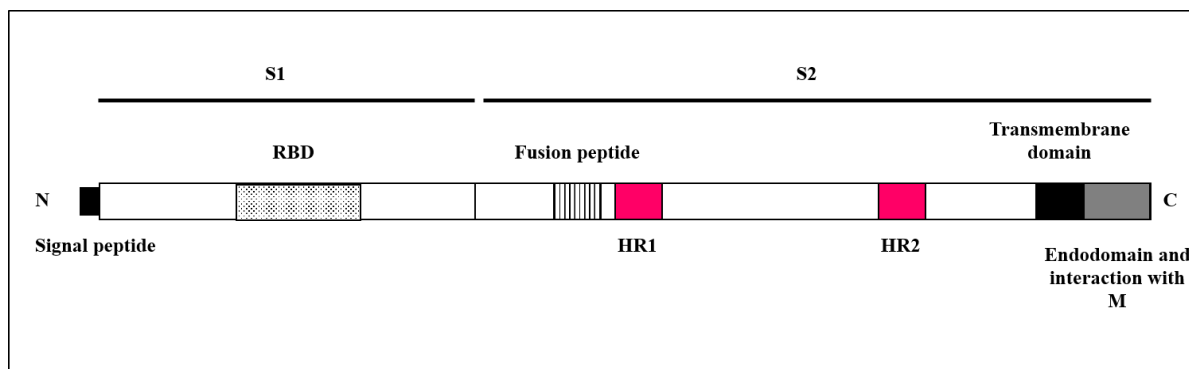


Figure 4 : Schematic linear representation of the S protein.

S1 and S2 represent the two subdomains. S1 is located on the N-terminus of the protein and contains the receptor-binding domain (RBD). S2 is located on the C-terminus of the protein and contains the fusion peptide, two heptad repeat domains (HR1 and HR2), a transmembrane domain and an interaction site with the M protein.

CoV strains	Localisation of identified RBD (amino acids positions)
MHV	a.a 1 to a.a 330 (Kubo et al., 1994)
SARS-CoV	a.a 318 to a.a 510 (Babcock et al., 2004)
HCoV-NL63	a.a 232 to a.a 684 (Hofmann et al., 2006)
HCoV-229E	a.a 407 to a.a 547 (Hofmann et al., 2006)
TGEV	a.a 579 to a.a 655 (Godet et al., 1994)

Table 2 : Localisation of the identified RBDs of Coronaviruses.

II.3.2. The envelope E protein

The E protein is the smallest structural protein, ranging from 8 to 12 kDa. It contains a short hydrophilic ectodomain, a transmembrane domain and a larger hydrophilic endodomain. E is not absolutely essential for virus replication (DeDiego et al., 2007). It is an integral membrane protein, but its topology is not yet fully resolved, as it could have a hairpin conformation, spanning the lipid bilayer twice with both termini inside the virion lumen (Arbely et al., 2004) or it could transverse the lipid bilayer once (Nieto-Torres et al., 2011). Its oligomeric state is also unresolved. The E protein deforms the lipid bilayer which could explain its role in virus

budding (Raamsman et al., 2000). For different Coronaviruses, protein E has been shown to display an ion conductivity (Wilson et al., 2004). Deletion of the E protein in TGEV or in MERS-CoV prevents the virus to properly propagate (Almazán et al., 2013). Still, it does not abolish the viral replication (DeDiego et al., 2007). Interestingly, SARS-CoV strain with a deleted E protein confers protection against parental virus and is thus considered a good candidate for vaccine development (Dediego et al., 2008). Also, deletion of the E protein in SARS-CoV significantly decreases inflammation in mice through the inhibition of NF- κ B pathway and increases their survival (Fett et al., 2013).

II.3.3. The M protein

The M protein (M for membrane) is the main structural component found in the viral envelope of Coronaviruses. It is a glycoprotein which monomer ranges from 25 to 30 kDa, with a small N-terminal exposed on the exterior of the envelope, followed by three transmembrane segments, an α -helical domain and a large carboxyterminus endodomain located either inside the virion lumen or on the cytoplasmic side of intracellular membranes. In the cell, the M protein localises to the ER (endoplasmic reticulum)-Golgi area, excepted for TGEV, SARS-CoV and FIPV, where the M protein can reach the plasma membrane (Voss et al., 2006)(Laviada et al., 1990)(Jacobse-Geels and Horzinek, 1983). Glycosylation is mainly N-linked, as it is the case for the SARS-CoV (Nal et al., 2005) (Oostra et al., 2006) and IBV (Stern and Sefton, 1982), but exceptions are found with O-glycosylation for MHV (de Haan et al., 1998) and HCoV-OC43 . When co-expressed with the N protein, M is able to form virus like particles (Tseng et al., 2013). Previous reports have also demonstrated that co-expressions of both M and E proteins from TGEV could form pseudo-particles and induce α -Interferon synthesis (Baudoux et al., 1998). As for the SARS-CoV, expressions of E, N and M

are required for the formation of virus-like particles (Siu et al., 2008). The M protein forms dimers that give the shape to the virion envelope and allows interaction with both the S and the N proteins.

II.3.4. The N protein

The N protein is a phosphoprotein of 46 kDa that packages the genomic RNA into a helical ribonucleoprotein (RNP). The N proteins of Coronaviruses share between 20 to 30% of sequence identity. It assembles into dimers and binds to viral RNA at multiple sites. The N protein is organised into two distinct domains: the N-terminus domain (NTD) and the C-terminus domain (CTD) that are interspaced with intrinsically disordered regions (IDRs) that regulate the RNA binding activities of the NTD and CTD. The IDR includes a serine-arginine rich region with putative phosphorylation sites that may regulate the protein function and its interaction with the M protein through the C-terminus domain of N (Narayanan et al., 2000). These phosphorylation sites vary in number according to the Coronavirus species. As such, for MHV, six putative phosphorylation sites exist (White et al., 2007), as well as four sites for TGEV (Calvo et al., 2005) and IBV (Infectious bronchitis virus) (Chen et al., 2005). The heavy phosphorylation is thought to trigger conformational changes in the N protein and enhances its affinity to the viral RNA (Ma et al., 2010). The way the N protein interacts with the viral RNA to form the RNP is still under studies.

II.3.5. The hemagglutinin-esterase (HE) protein

The HE protein is found on the virion of some Coronaviruses of the Beta-genus, such as MHV, bovine Coronavirus (BCoV), human Coronavirus HCoV-OC43, equine Coronavirus

(EqCoV), porcine hemagglutinating encephalomyelitis virus (PHEV) and human Coronavirus HCoV-HKU1. The monomer contains a large ectodomain, a transmembrane domain and a small endodomain. It forms short spikes composed of disulphide-bonded dimers, ranging from 65 to 70 kDa. It shares a 30% amino-acid sequence homology with the hemagglutinin protein of influenza C virus, HEF (Hemagglutinin-Esterase Fusion), therefore suggesting the evolution from a common ancestor through recombination (de Groot, 2006). The HE protein contains two specific activities: first, it is a hemagglutinin binding to sialic acids. Second, the HE protein displays an acetylerase activity that cleaves acetyl groups from 9-O or 4-O-acetylated neuraminic acids. These properties suggest that the HE protein could be involved in the attachment of the virion to the host cells. For MHV, HE enhances the neurovirulence (Kazi et al., 2005) in the mouse host, but is generally not essential for the virus replication (Masters, 2006) and is actually counter-selected during virus passages in tissue cultured cells (Lissenberg et al., 2005).

II.4. Non-structural proteins

The first event that follows the release of the genomic RNA is the translation of the ORF1a and 1b. ORF1a is translated into a polyprotein named pp1a and codes for 11 non-structural proteins (nsp1-nsp11). During this translation process, with a fixed ratio of one third, a ribosomal frameshift extends pp1a into a longer polyprotein, named pp1ab, therefore allowing the production of additional nsps (nsp12-nsp16) (Bredenbeek et al., 1990). This frameshift phenomenon is due to a “slippery” sequence of eight nucleotides and a pseudo-knot structure, both located upstream the ORF1a stop codon (Brierley et al., 1989). The primary translated products are processed by two proteases embedded within the polyproteins into 16 nsps.

Nsp3 carries the first protease activity and contains one or two papain-like proteases (for MHV), named PL1^{pro} and PL2^{pro} (Baker et al., 1993). Nsp3 allows the release of nsp1, nsp2 and nsp3. The second protease activity is carried by nsp5, which is responsible for the release of the remaining 13 nsps. Due to its similarities with the 3C proteinase of the Picornavirus, nsp5 is often designated the 3C-like proteinase (3CL^{pro}).

Functions of the processed nsps have been largely investigated, as can be seen in table 3. Apart from the protease activities of nsp3 and nsp5, some have an impact on host-virus interaction or are directly involved in RNA synthesis. Nsp12 carries the RNA-dependant polymerase activity, while nsp13 the helicase activity. Nsp8 is also an RNA polymerase by a *de novo* mechanism (Imbert et al., 2006). Two other nsps display functions which are unique to the *Nidovirales* order. The first is the 3'-5' exonuclease activity of nsp14. Recent studies with Exo-N defective mutants of MHV and SARS-CoV demonstrated that nsp14 acts as a proof-reading enzyme (Minskaia et al., 2006). Such activity is probably crucial for the maintenance of the large Coronavirus genome. The second is the endonuclease activity carried by nsp15 (NendoU). NendoU cleaves downstream and upstream of uridylate sequences to produce molecules with 2'-3' cyclic phosphate ends (Ivanov et al., 2004).

Together with N and possibly with cellular proteins, the nsps proceed to form the replicase-transcriptase complex (RTC). The RTC is localised in specific structures: double-membranes vesicles (DMV) and convoluted membranes (CM). Nsps 3, 4 and 6 altogether seem to play a major role in membranes rearrangement that leads to their formation. This structure is tightly connected to the endoplasmic reticulum, as the RTC is made from modified endoplasmic reticulum membranes, induced during Coronavirus infection (Knoops et al., 2008).

Such organisation, along with viral proteins, confers a suitable environment for the viral RNA synthesis, as it offers protection from defence mechanisms of the infected cell.

The RTC has thus two distinguishable activities: first, as a replicase, it will recognize the genome as a template for copies of genomic strands and second, as a transcriptase, it will generate copies into subgenomic mRNAs for viral translation.

Non-structural proteins	Activities
Nsp1	Induces RNA degradation process
Nsp2	Unknown
Nsp3	Protease activity and membranes rearrangements
Nsp4	Membrane rearrangements
Nsp5	Protease activity
Nsp6	Membrane rearrangements
Nsp7	RdRp activity
Nsp8	RdRp activity by <i>de novo</i> mechanism
Nsp9	RNA linking protein
Nsp10	RNA linking protein, co-factor for nsp16
Nsp11	Unknown
Nsp12	RdRp activity
Nsp13	Helicase activity, RNA 5' triphosphatase
Nsp14	Exonuclease activity/ 7-methyltransferase
Nsp15	Endonuclease activity
Nsp16	2' O-Methyl transferase

Table 3: Functions of the non-structural proteins.

II.5. Accessory proteins

In addition to the non-structural proteins and the different structural proteins, the Coronavirus genome displays supplementary ORFs that encode accessory proteins. These accessory proteins are specific to each CoV species and their genes can be found anywhere in the intergenic regions between ORFs of the structural proteins, except between E and M. Most accessory genes are preceded by a specific transcriptional regulatory sequence (TRS), for further translation into functional proteins. Some, like the ORF3b of the SARS-CoV, are synthesized through internal ribosome entry site (Rota et al., 2003). They are designated according to their position in the viral genome and thus, identical names may be attributed to proteins, which differ in both sequences and functions. For instance, ORF3a of CCoV is not homologous to the ORF3a of the SARS-CoV, but both genes share the same localisation behind the S gene. FCoVs and CCoVs display two groups of accessory proteins, known as group 3abc and 7ab (see Figure 3). One particularity of genotype I CCoV is that it contains an additional accessory ORF, compared to other CCoV genotypes, directly downstream of the S gene and preceding ORF3a. That ORF, named ORF3, bears no homology with any other viral protein (Lorusso et al., 2008).

Studies on accessory proteins have exhibited their dispensability for viral growth *in vitro* (Haijema et al., 2004)(Yount et al., 2005)(Shen et al., 2003). However, maintenance of their sequences in viral genome suggest that they are relevant *in vivo*. Numerous studies demonstrated a wide variety of functions with implication in viral pathogenesis (See Chapter one, part IV.3.3), in host or tissue tropism (See Chapter one, part IV.3.1). No function has been attributed to CCoV accessory proteins. Among the ORF3abc of FCoV, the most studied protein is the ORF3c, which plays a role in the tissue tropism of the virus (Bálint et al., 2012). Some accessory proteins are also structural components of the viral particle such as 3a, 6, 7a,

7b and 9b of the SARS-CoV (Ito et al., 2005)(Huang et al., 2007)(Huang et al., 2006)(Schaecher et al., 2007)(Xu et al., 2009).

III. Virus life cycle

III.1. Virus attachment and entry

The attachment of the virion to its host cellular receptor constitutes the first step of the infection. The interaction between the RBD of the S1 subunit of the S protein and its receptor on the cell surface is determinant for the Coronavirus host species range and tissue tropism. As can be seen in table 4, receptors for a few Coronaviruses have been identified. Coronaviruses bind either to a variety of proteins or to sialic acids for entering into the cell. Among Alphacoronaviruses, TGEV, PEDV, type II CCoV (CCoV-II), type II FCoV (FCoV-II) and HCoV-229E use the cell membrane-bound metalloprotease, aminopeptidase N (APN) of their respective host-species as receptor (Delmas et al., 1992)(Yeager et al., 1992)(Tresnan et al., 1996)(Tusell et al., 2007). APN, also known as CD13 for mammalian aminopeptidase N, is a zinc-binding glycoprotein with an endopeptidase activity that is expressed on the apical surfaces of respiratory and intestinal epithelium as well as on synaptic junctions. FCoV-I and CCoV-I do not use the feline APN as a receptor (Hohdatsu et al., 1998). To this day, no receptor for these Coronaviruses has been described.

The receptor for HCoV-NL63, another human Coronavirus belonging to the Alpha-genus, is the angiotensin-converting enzyme 2 (ACE2). ACE2 is a carboxypeptidase expressed in epithelial cells of the lungs, intestine, as well as in heart, kidney and other tissues. It is the same receptor that is shared by the SARS-CoV, which belongs to the Betacoronavirus (Kuhn et al., 2004). Some other receptors for the Betacoronaviruses have been described such as

MHV, which belongs to the carcinoembryonic antigen (CEA) family of the Ig superfamily (CEACAM1) (Williams et al., 1991). The MERS-CoV receptor, DDP4 for dipeptidyl peptidase 4, also known as CD26, is a serine exopeptidase that is expressed on many cell types (Raj et al., 2013). As opposite to these Betacoronavirus receptors of protein nature, BCoV and human HCoV-OC43 Coronavirus bind to sialic acids and more precisely recognise N-acetyl-9-O-acetylneuraminic acids on the cell surface. For gammacoronaviruses, α 2,3 linked sialic acids are the only known receptor for IBV (Winter et al., 2006). No receptors for any deltacoronaviruses have been identified yet.

Coronavirus	Identified receptor
FCoV-I	Unknown
FCoV-II	Feline Aminopeptidase N
CCoV-II	Canine Aminopeptidase N
TGEV/PRCoV/PEDV	Porcine Aminopeptidase N
HCoV-229E	Human Aminopeptidase N
HCoV-NL63	Angiotensin-converting enzyme 2
SARS-CoV	Angiotensin-converting enzyme 2
MHV	Murine carcinoembryonic antigen-related adhesion molecule (mCEACAM)
MERS-CoV	Dipeptidyl peptidase 4
BCoV	N-acetyl-9-O-acetylneuraminic acids as co-receptor
HCoV-OC43	N-acetyl-9-O-acetylneuraminic acids as co-receptor

Table 4: Known receptors of different Coronavirus species.

After the S protein binds to its specific receptor, the virus enters the cell. This particular step involves conformational changes of the S protein, which are initiated by receptor-binding but may also require additional changes, such as pH-acidification or proteolytic activation.

Mechanisms differ between Coronaviruses species and strains. Some Coronaviruses such as most MHV strains fuse at neutral pH with plasma membranes, whereas others, such as TGEV (Hansen et al., 1998), FCoV (Regan et al., 2008), SARS-CoV (Simmons et al., 2004) and HCoV-229E (Nomura et al., 2004) are pH-dependant and fuse with the endosomes.

To this day, it is still unclear how these rearrangements are triggered, but they are thought to allow the exposition of the fusion peptide (located upstream of HR1) through the cleavage between subunits S1 and S2. Once the viral nucleocapsid is delivered into the cytoplasm, the positive RNA strand is uncoated for translation and transcription, but the mechanism of this process is not yet well described.

III.2. Viral RNA synthesis

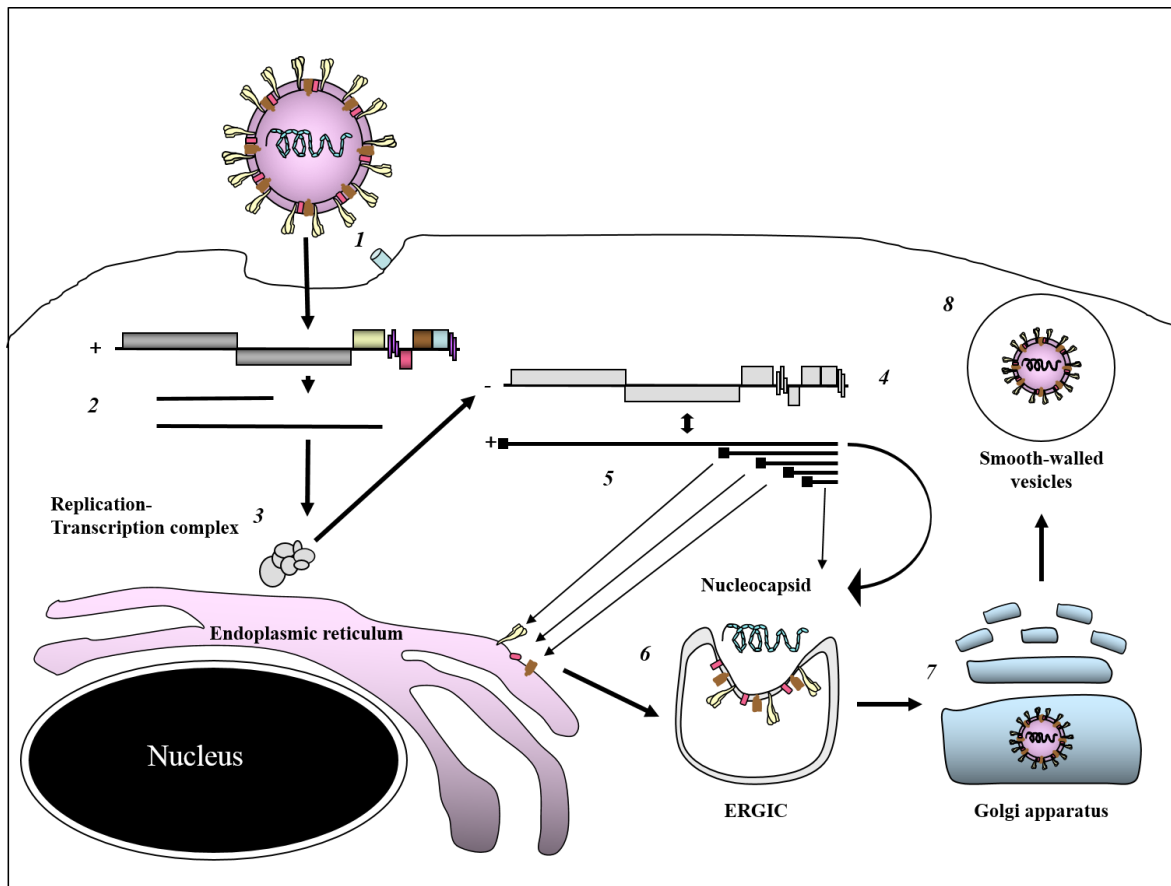


Figure 5 : Schematic representation of the viral replication cycle

1- Attachment and entry of the virus into the cell. 2- Once the nucleocapsid is delivered into the cytoplasm, viral RNA is uncoated for translation of ORF1a/1b. 3- Synthesized proteins will assemble and form the RTC. 4- The RTC will initiate the transcription of a full length (-) RNA and (-) sgRNAs. 5- In turn, they will serve as template for synthesis of (+) full-length RNA and (+) sgRNAs. 6- Translated proteins will undergo maturation in the ER and ERGIC where they will assemble to form the viral particle. 7- The viral particle undergoes a maturation process while transiting in the Golgi apparatus. 8- The viral particle will be secreted out of the cell through smooth-walled vesicles, by exocytosis.

Both the replication and transcription of the viral genome is processed by the RTC. The initiation of replication starts at the UTR (untranslated) 3' end of the viral genome, which is recognized by the RTC. It then synthesises both genome-length strand and multiple subgenomic RNAs (sgRNAs). The latter serve as templates for the translation of both structural and accessory proteins. In all cases, genome-length and sgRNAs result from negative strand intermediates, all displaying a complementary “body”-TRS at their 3' end (Figure 5).

Transcription is initiated at the 3' end of the genome and RdRp (RNA-dependant RNA polymerase) either synthesises a full-length negative strand, either pauses at one of the different TRSs, located in-between the different ORFs, all displaying a complementary "body"-TRS at their 3'end (Sawicki and Sawicki, 1986). All produced RNAs possessing the 3' complementary sequence of the body-TRS will be redirected to the 5' end of the genomic RNA, where fusion of body-TRS and leader-TRS will occur, leading to a discontinuous transcription mechanism. Negative sgRNAs acquire a complementary leader sequence that serves as template for the production of positive-sense sg mRNAs. The leader sequence initiates the synthesis of the positive-sense strand through the complementary sequence located at the 3' end of all the negative-sense RNA strands. All negative strands/subgenomic strands therefore share the same 3' and 5' ends. Full length and subgenomic strands serve as templates for the synthesis of multiple copies of the corresponding positive strands (Figure 6). This peculiar discontinuous transcription mechanism may explain the enhanced potential of Coronaviruses to recombine (Nagy and Simon, 1997). As a discontinuous transcription mechanism implies a dissociation of the nascent RNA from the RNA template in order to fuse with the leader sequence, fusion with another RNA leader sequence could occur, therefore leading to recombination (Sawicki et al., 2007). Coronavirus recombination is thought to occur by a copy-choice mechanism, like those described for Poliovirus(Pasternak et al., 2006). Recombination can take place between different CoVs infecting the same cell, leading to the emergence of a new strain, like the emergence of serotype II FCoV (See Chapter two, part III.2). Possible recombination events with cellular genes or heterologous viral genes have also been hypothesised. For instance, presence of the HE protein for some Betacoronaviruses may have been acquired through a recombination with the Influenza C virus (Luytjes et al., 1988)(Zeng et al., 2008). The frequency rate of recombination event is estimated at 25% for the genome of MHV, which represents the highest rate for RNA viruses (Fu and Baric, 1994).

Recombination sites analyses demonstrated the presence of hot spots across the viral genome. For instance, in IBV, hot spots have been characterised in nsp2, the envelope E gene, the M gene and downstream of the N gene (Jackwood et al., 2012). This phenomenon may have different consequences according to the recombination site. Changes within S would lead to a new serotype of virus, or to the recognition of a different RBD, that would generate a possible host-jump. Changes in non-structural proteins may alter the replication/transcription mechanism, and in turn, modify the pathogenicity of the new recombinant virus.

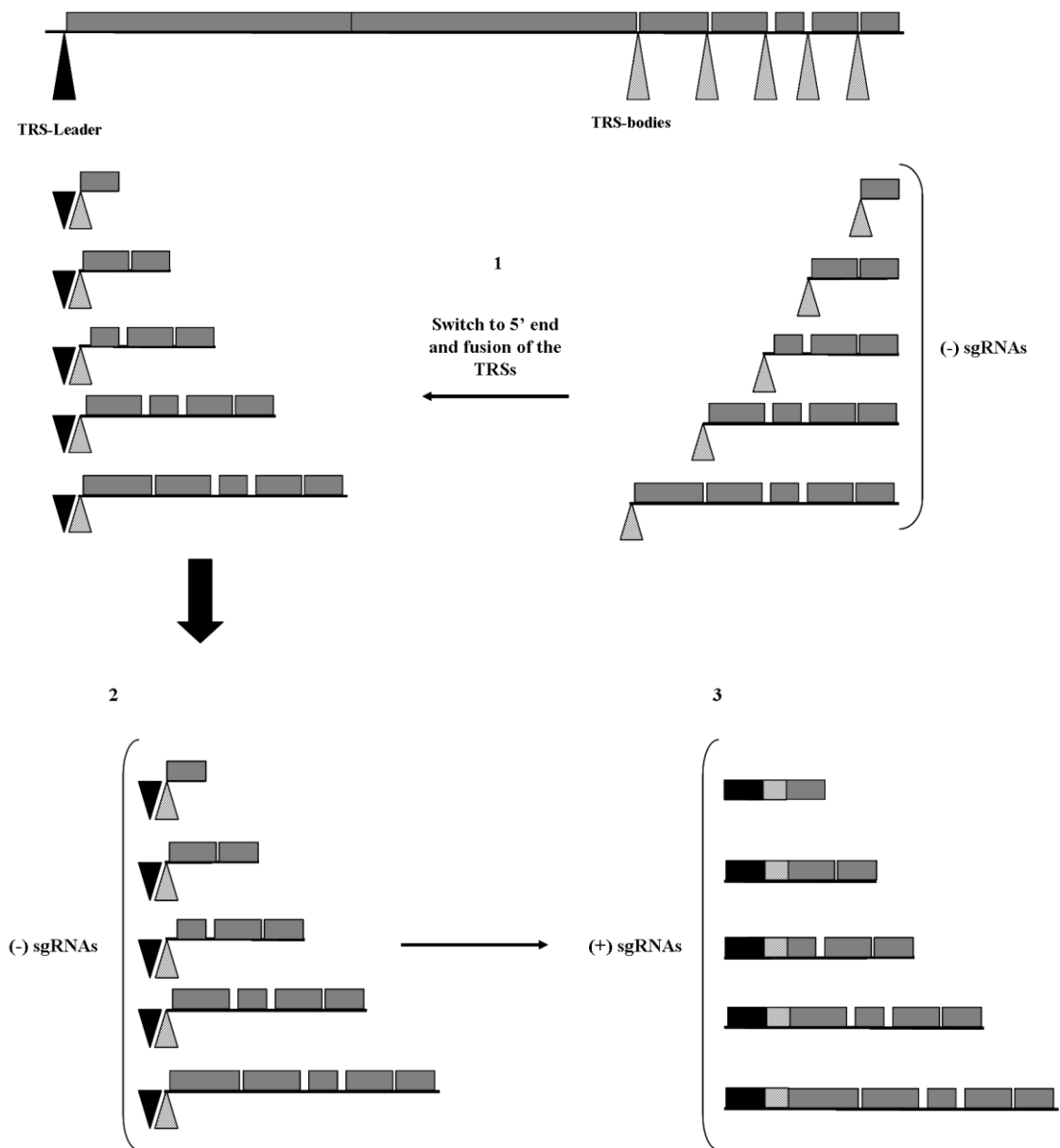


Figure 6 : Representation of the discontinuous transcription of Coronaviruses.

1- Synthesis of negative strand RNAs can be continuous and leads to the negative genome strand or be discontinuous and leads to the different (-) sgRNAs. TRS-body sequences allow a switch and fusion to the TRS-leader sequence at the 5' end. 2- (-) sgRNAs will possess the same 5' end and will then serve as templates for 3- synthesis of (+)sgRNAs.

III.3. Assembly and release of the virions

Translation follows synthesis of the positive RNA strands. Only the 5' gene of the nascent positive strand will serve for the production of the different proteins. Once structural and accessory proteins are translated, they will follow a maturation process in the ER.

M, S and E proteins are inserted into the ER for translation maturation and will transit to the ERGIC (Endoplasmic reticulum-Golgi intermediate compartments) for the particle formation (Klumperman et al., 1994)(Tooze et al., 1984)(Krijnse-Locker et al., 1994). Interactions of the N protein with both the viral RNA and M will complete the particle formation (Kuo and Masters, 2002). To this day, it is still unclear how the nucleocapsid traffics from DMVs to the budding compartment. After budding, progeny virions undergo condensation during their transport in smooth-walled vesicles to the plasma membrane, before release by exocytosis into the extracellular environment.

IV. Human and veterinary diseases

IV.1. Human diseases

IV.1.1. Human Coronaviruses associated with mild respiratory diseases

The first isolated human Coronaviruses were HCoV-229E and OC43 in 1967, from patients with upper respiratory tract infections (Almeida and Tyrrell, 1967). These two human Coronaviruses cause mild upper and lower respiratory tract infections, have a low mortality rate and are often associated with the common cold. Both Coronaviruses have seasonal infectious cycles, with a peak of infectivity usually situated during winter and early spring, in

countries with a temperate climate. First infections usually occur during early childhood and re-infections are frequent throughout life and are symptomatic in 45% of the cases.

Shortly after the SARS epidemic, two more human Coronaviruses were identified: HCoV-NL63, first described in 2004 (van der Hoek et al., 2004) and HCoV-HKU1, which was discovered in 2006 in Hong-Kong (Woo et al., 2005). Both are also associated with mild respiratory diseases. HCoV-NL63 belongs to the Alpha-genus and diverged from HCoV-229E in the 1800s. It was first isolated from a 7 months old child in The Netherlands, suffering from bronchiolitis. This virus was independently reported by another Dutch group, from a respiratory sample, dating from 1988, of a young child suffering from pneumonopathy (Fouchier et al., 2004), therefore suggesting that this strain was already circulating among the human population without being identified before. In children infections, HCoV-NL63 has been associated with acute bronchiolitis (Ebihara et al., 2005).

HCoV-HKU1 was first isolated from a 71 years old patient, suffering from pneumonia, but remains to this day an agent of mild and severe respiratory tract infection. The seriousness of the respiratory infection depends in majority of the underlying condition of the patient, whether he is immunocompromised or displays a chronic disease.

IV.1.2. Severe acute respiratory syndrome virus-SARS-CoV

The SARS epidemic started in the Guangdong Province in Southern China in November 2002, with an outbreak of “acute respiratory syndrome” resulting in 300 human cases and 5 deaths. By the end of February 2003, it spread out of the Chinese frontiers to Hong Kong, where an infected physician contaminated 16 individuals, who travelled to at least 6 different countries and therefore triggered the second wave of the outbreak (Centers for Disease Control and Prevention (CDC), 2003). The epidemic resulted in more than 8270 cases and

775 deaths, making approximately a 10% death rate (Poon et al., 2004). The epidemic had a huge economic impact with a total loss of approximately 40 billion dollars and spread over to 30 different countries (Caulford, 2003). International cooperation of scientists and medical healthcare combined with drastic hygiene measures helped to control the outbreak. Quarantine was the only intervening strategy as no effective therapeutic exists.

After an incubation period of two to six days, patients develop general symptoms of flu, associated with fever, asthenia, shivering, anorexia and stiffness (Cheng et al., 2013). Severe respiratory symptoms occur several days after general symptoms. SARS-CoV has a strong tropism for the low respiratory tract and preferentially infects type 1 pneumocytes, where the infection is productive. It also infects macrophages and dendritic cells but infection is abortive and leads to the production of pro-inflammatory cytokines, such as CXCL10, CCL2... This immune dysregulation leads to pneumopathy. Patients can exhibit either localised or diffused alveolar damages. Pathologies of the lungs are associated with localised/diffused alveolar damages, increase of macrophages and epithelial cell proliferation. Over time, alveolar damages progress and lead to acute lung injury. In the most severe cases, damages develop into an acute respiratory distress syndrome, necessitating mechanical ventilation. For these patients, CD4 and CD8 lymphocytes drastically drop, whereas neutrophils and pro-inflammatory cytokines are elevated. SARS patients can also develop gastrointestinal symptoms (about 30%), as SARS-CoV has been shown to replicate in enterocytes (To et al., 2004). Enteric symptoms are associated with vomiting, diarrhoea and abdominal aches. For 10% of the patients, evolution of the disease lead to a fatal issue.

IV.1.3. Middle East respiratory syndrome virus-MERS-CoV

MERS-CoV was first identified in Saudi Arabia in 2012 and has since been circulating widely in the Arabian Peninsula (Zaki et al., 2012). As a new emerging pathogen with a symptomatology close to the SARS-CoV infection, it is under scrutiny of the WHO. Its infection differs among patients and while it can be asymptomatic for some, it can also cause fever, cough, respiratory distress, pneumonia, leading to death in 30% of the cases. Most severe and fatal cases have been found in immunocompromised patients, people with chronic diseases, such as diabetes or cancer and elderly patients. Some gastrointestinal symptoms have been reported as well as kidney failure. To this date, MERS-CoV displays a limited human-to-human transmission. All cases are linked to the Middle East region (Arabi et al., 2014).

IV.2. Domesticated animal diseases

IV.2.1. Infectious bronchitis virus

Infectious bronchitis virus was the first Coronavirus infection described in the late 1930s. Its infection causes a highly contagious disease with high morbidity and low mortality and results in great economic losses in the poultry industry. Many strains and antigenic forms of the virus exist, with some strains restricted to geographical areas, whereas others extend over the world. The virus is spread via aerosol and infects the respiratory tract. IBV causes respiratory distress, asphyxia, mucus accumulation and tracheal rale among chicken (Chen et al., 1996). Post-mortem examination reveals tracheitis, infiltration of lungs with lymphocytes and frequent cloudy airsacs.

The female reproductive tract is also affected and causes a decrease in egg production. Still, the physiopathological mechanisms of the viral replication in the oviduct epithelium leading the fertility decrease have not been investigated.

Some IBVs have a peculiar kidney tropism. During the 1960s, circulating IBV strains in Australia had a prominent kidney tropism, causing nephritis with urate crystals inside the collecting tubules. These strains had a high mortality rate, especially in young animals. In the 1990s, an IBV strains named QX emerged in China. This strain has now extended to Europe and South America and induces severe nephritis in younglings. It is also strongly associated with proventriculitis (Yu et al., 2001).

Other avian diseases have now been recognised as consequences of Coronavirus infection. Turkey Coronavirus (TCoV) is another avian Coronavirus infection of economic importance. It was recognised in the 1970s as the causative agent of an enteric disease known as Bluecomb, or transmissible enteritis of turkeys (Panigrahy et al., 1973). Combined with other agents (virus, bacteria, protozoa and fungi), TCoV has been regrouped in the poult enteritis complex (PEC) (Pakpinyo et al., 2003). PEC is a general term that regroups multifactorial and transmissible intestinal diseases of young turkeys of up to 7 weeks old, with signs including enteritis, slow development, impaired feed utilisation and frequent immune dysfunction. TCoV is found predominantly in the digestive content (41% in jejunum, 39% in caecum).

In January 2014, the aetiological agent responsible for fulminating disease in Guinea fowl has been identified as a novel avian CoV (Liais et al., 2014). Fulminating disease, or X disease causes acute enteritis with a high mortality rate, often leading to the entire destruction of the flock. Some birds exhibit pancreatic degeneration but lesions are commonly restricted to the intestinal tract. A virus was long suspected as the aetiological agent of this rare disease, but as propagation was unsuccessful, only electronic microscope analyses could be considered.

Here, Liais and al. used unbiased high-throughput sequencing onto both field cases and experimentally infected animals.

IV.2.2. Porcine Coronaviruses

Several Coronaviruses affect the swine industry but the first discovered is the transmissible gastroenteritis virus, which has been known since the 1940s. TGEV is highly contagious and causes severe enteritis and diarrhoea. Its mortality rate approaches 100% for young piglets, therefore causing huge economic losses. TGEV infects epithelial cells of the intestinal and respiratory tract. The evolution of the virus gave rise to PRCoV (Porcine Respiratory Coronavirus), infecting only epithelial cells of the lungs. PRCoV presents an attenuated virulence and a cross protection against TGEV (Bernard et al., 1989) and since the emergence of PRCoV in the 1980s, TGEV is no more a threat to the swine industry. PEDV was first identified in Europe. It is now a source of concern in Asia with severe outbreaks and has recently emerged in North America in 2013 (Chen et al., 2014). PEDV induces lesions of the small intestine and causes severe diarrhoea, with a high mortality rate in younglings.

PHEV, a Betacoronavirus, was first isolated in 1962 in Canada from suckling piglets with encephalomyelitis (Greig et al., 1962) and replicates in epithelial cells of the respiratory tract as well as the central nervous system. This virus causes encephalomyelitis, vomiting and wasting disease in suckling piglets. Nervous symptoms include convulsions and nystagmus. It invades the central nervous system via the peripheral nervous and infect neuronal cells (Gao et al., 2011).

The last discovered porcine Coronavirus, HKU15, belongs to the Deltacoronaviruses. It was first identified in Hong-Kong during epidemiologic surveillance and was recently reported in the USA in pigs with clinical diarrhoea (Woo et al., 2012)(Wang et al., 2014). Further

investigations are needed to determine the distribution of this virus among the swine industry and whether it is responsible for clinical diseases.

IV.2.3. Feline enteric Coronavirus and feline infectious peritonitis virus

Feline Coronavirus infections are widely spread among this population. Ten to 50% of household cats are infected and this number can reach 80% of seropositive animals in cat-breeding facilities. Avirulent FCoV, which cause usually mild enteric symptoms are referred as FECV strains, for feline enteric Coronavirus. Virulent strains, named FIPV, induce the feline infectious peritonitis, a lethal disease. In 2% to 5% of the cases, FECV infected cats develop FIP, after mutation of FECV into FIPV (Pedersen et al., 1981)(See chapter IV.3.1).

Unlike FECV, which replicates in enterocytes, FIPV has a major tropism for monocytes/macrophages (Kipar et al., 2005). Infection of these cells leads to the secretion of pro-inflammatory cytokines like $\text{TNF}\alpha$, $\text{IL-1}\beta$ and GM-CSF. These cytokines attract neutrophil cells at the site of infection and in turn, provoke pyogranulomatous lesions, which are pathognomonic of FIP infections (Berg et al., 2005). Secretion of $\text{TNF}\alpha$ triggers the apoptotic death of T cells and CD8^+ in particular, leading to lymphopenia, frequently observed during the course of infection (de Groot-Mijnes et al., 2005). Evolution of the FIPV infection can have two different symptomatic forms: an effusive or a non-effusive one (wet or dry forms). The wet form is characterised by accumulation of protein-rich liquid in the abdomen or pleural cavity of the infected animal. For the dry form, symptoms vary according to the affected organs. In almost 10% of the cases, neurological symptoms are linked with ataxia, vestibular disorders or seizures. Uveitis is also frequently associated with neurological disorders. Abdominal organs may in turn be affected, leading to renal or hepatic failure.

IV.2.4. Canine Coronaviruses

Different canine CoVs (CCoVs) belong to the Alpha and Betacoronaviruses. CCoV from the Alpha-genus were described since the 1970s and are important entero-pathogens of canine population. They are widely spread, especially in kennels and animal shelters. Canine CoV infections present a high morbidity and a low mortality, except for younglings. Infection is mainly restricted to the intestinal tract. Symptoms include loss of appetite, diarrhoea, vomiting and eventually death. Death of the animal is often a consequence of co-infection with canine parvovirus, canine distemper virus or other pathogens.

In 2007, an Italian group reported fatal cases of infected pups by a new virulent strain of Alphacoronavirus, named canine pantropic CoV. The infection caused a systemic disease and death within two days after pups developed symptoms. Those symptoms include fever, haemorrhagic diarrhoea, vomiting, leukopenia, ataxia, anorexia, depression and seizure. Viral RNA was detected in intestinal contents of pups, as well as lungs, spleen, liver, kidney and brain. A difference in mortality rate exists between young pups and older pups, as the latter of 6 months old recover from the disease (Decaro et al., 2007). Epidemiological studies conducted in different European countries indicate that this new strain is widely distributed in dog populations (Decaro et al., 2013).

In 2003, a new canine Coronavirus with pulmonary tropism was detected by RT-PCR, in a kennel in United Kingdom. This new virus, CRCoV (Canine Respiratory Coronavirus), presents a close relationship with BCoV for both the spike and the replicase genes and belongs to the beta-genus (Erles et al., 2003). It is responsible for mild respiratory infections, and its implication in the kennel cough syndrome is under investigations.

IV.3. Molecular determinants of pathogenesis

IV.3.1. Tropism switch and pathogenesis

Degree of pathogenesis correlates for some CoVs with their cell type tropism. The tropism switch of peculiar strains results in an increase, like FCoV, or decrease, like TGEV, of pathogenesis.

In all examples, mutations in the S protein are determinant and sometimes in the accessory proteins. Feline infection peritonitis probably arises from cats already infected by FECV, which mutates into highly virulent FIPV (Poland et al., 1996). FIPV gains a tropism for monocytes/macrophages and loses the ability to infect enterocytes (Pedersen, 2009). Different mutations have been suggested to explain that specific phenomenon. In 2012, Chang et al. demonstrated that more than 95% of the sequenced strains in their study, shared the same two mutant codons in the S protein sequence of FIPV (Chang et al., 2012). These two identified mutations are M1058L and S1060A and occur in the S2 fusion-peptide subdomain of the S protein. Substitutions within this peptide could alter the tropism of the virus and facilitate the infection of monocytes/macrophages, which would in turn, lead to FIP. However, another recent study conducted in United Kingdom led to the conclusion that these substitutions are effectively linked to the systemic spread of FCoV in the organism, but not necessarily with the occurrence of FIP (Porter et al., 2014). At last, another study identified the molecular determinants of virulence in the S protein, but within the furin cleavage motif between the S1 and S2 domains (Licitra et al., 2013). The cleavage sequence is conserved in FECV strains but varies in strains recovered from tissues of FIP cats. Again, whether these mutations are related to diffusion of FCoV in cat tissues or FIP pathogenesis is unclear. Moreover, mutations in the accessory protein 3c are also involved in the tropism switch (see below).

Another well described example of tropism switch in correlation with a change of pathogenesis, is the transmissible gastroenteritis virus that underwent mutations to become the avirulent porcine respiratory Coronavirus. TGEV is an enteropathogen, but also exhibits replication in the respiratory tract (Underdahl et al., 1975). In 1986, Pensaert et al. demonstrated for the first time the relationship between TGEV and PRCoV, this last virus being a mutant of TGEV (Pensaert et al., 1986). In contrast to TGEV, PRCoV replicates solely in the respiratory tract and displays a reduced pathogenicity. Both viral strains present a high nucleotide sequence identity, with exception of the S gene and ORF3 sequence of PRCoV which present deletion (Ballesteros et al., 1997). Both TGEV and PRCoV use the APN as a receptor but deletion in the 5' end of S prevents its binding to sialic acids and thus prevents the virus to replicate in the gastrointestinal tract. Therefore, PRCoV has lost the enteric tropism and only keeps the ability to replicate in the respiratory tract (Rasschaert et al., 1990).

At last, in 2008, Mardani et al. published results on sequence analyses of the circulating IBV strains in Australia that did not induce renal lesions of the infected flocks, in contrast to the usual IBV strains (Mardani et al., 2008). Sequencing of these novel circulating strains of IBV revealed mutations in the S1 subdomain of the S protein and the partial and complete loss of accessory proteins ORF3 and ORF5, respectively, in parallel to the gain of a new ORF, named X1, located in place of the ORF3. Its sequence revealed no homology with any known Coronavirus sequence. As S1 interacts with the host cell receptor, mutations might have altered its binding to receptors, resulting in the loss of the kidney tropism. Still, as displayed by previous examples, tropism switch is attributed to the observed phenomena of mutations in the S protein and modifications in the accessory proteins.

IV.3.2. Interaction with the host innate immune response

Type I Interferon (IFN) has been discovered in 1987 by Isaacs and Lindemaan (Isaacs and Lindenmann, 1987) and constitutes the first defence line by a host against a viral pathogen. It influences protein synthesis, growth and processes of cell survival, besides regulating innate and adaptive immune system. Still, viruses have developed many different strategies to circumvent host innate antiviral response. Viral proteins may interfere with multiple steps of the innate response, to establish a sustainable infection. Coronaviruses encode several proteins affecting type I IFN pathway and have been extensively studied for the SARS-CoV.

Some structural proteins function as interferon antagonists. M and N proteins of the SARS-CoV inhibit the synthesis of type I IFN (Siu et al., 2009)(Kopecky-Bromberg et al., 2007). Non-structural proteins also modulate the innate immune response through diverse mechanisms. Nsp1 antagonises type I IFN by three different mechanisms: inactivation of host protein translation, degradation of host mRNA whereas viral RNA is not affected by this process and third, inhibition of STAT1 phosphorylation, a critical transcription factor of IFN signalling (Kamitani et al., 2006). Nsp3 inhibits the IFN synthesis by antagonising IRF3 (IFN-regulatory factor 3), a key element in the IFN inducing pathway (Devaraj et al., 2007). Finally, the IFN antagonist effect of nsp16 is due to its 2' O-methylation activity of viral RNA that protects the genome from recognition by MDA5, an inducer of the IFN pathway (Daffis et al., 2010). Some accessory proteins may also counteract the IFN pathway (see below).

IV.3.3. Roles of the accessory proteins in pathogenesis

The accessory proteins are virus-specific and are dispensable for the viral replication when studied *in vitro* (Haijema et al., 2004)(Yount et al., 2005)(Shen et al., 2003)(Hodgson et al.,

2006)(Casais et al., 2005). Still, as they are conserved under selective pressure, they somehow confer a selective advantage to the virus, in *in vivo* infections. During the intense studies that followed the SARS outbreak, one noticeable aspect of the SARS-CoV was the uncommonly high number of these accessory proteins it displayed. SARS-CoV contains eight ORFs, namely ORF3a, 3b, 6, 7a, 7b, 8a, 8b and 9b (McBride and Fielding, 2012). They, as well as accessory proteins of some other Coronaviruses, have been extensively studied and many modulate the viral replication or virulence of the virus or even help evade the innate immune system. They are therefore involved in viral pathogenesis. Properties, localisation and functions of studied accessory proteins of Coronaviruses widely differ from each other and are summarised in table 5.

Despite their high diversity in sequences and subcellular localisation, they share some common functions and properties.

Three of them have been identified as viroporins: ORF4a of the HCoV-229E, ORF3 of PEDV and ORF3a of the SARS-CoV. They share common membrane topology with three transmembrane domains and all seem to localise in the assembling budding site of CoVs. They regulate membrane permeability and regulate viral production, probably by influencing the release step of viral particles.

Accessory proteins may also regulate other steps of viral replication. ORF7a of SARS-CoV is also localised where CoV generally assemble and is thought to favour viral assembly (Fielding et al., 2004). ORF6 of the SARS-CoV enhances viral production at low MOI (multiplicity of infection). Moreover, in an adapted mouse model of SARS infection, recombinant SARS-CoV deleted of ORF6, induces lower morbidity and mortality, with decreased virus titres compared to the wild-type virus. ORF6 is thought to induce ER membrane rearrangements and favours the formation of DMVs (Zhou et al., 2010).

Many accessory proteins counteract the antiviral effect of IFN-induced pathway. Mechanisms of inhibition pathways have been investigated for some of them. ORF3b of the SARS-CoV is an antagonist of type I IFN, capable of inhibiting RIG-I and MAVS pathways (Freundt et al., 2009). ORF6 of the SARS-CoV inhibits by a different way the IFN induction. This protein blocks nuclear translocation of the transcription factor STAT1 and therefore impairs the JAK-STAT pathway and the transcription of type I IFNs (Kopecky-Bromberg et al., 2007). Among MERS-CoV accessory proteins, ORF4a, ORF4b and ORF5 are also type I IFN antagonists (Yang et al., 2013). ORF4b, but also ORF4b of the closely related Bt-CoV-HKU4 and HKU5 localise in the nucleus but their precise mechanisms of inhibition remain unknown (Matthews et al., 2014). On the opposite, ORF4a has been demonstrated to block the MDA5-dependant IFN activation (Niemeyer et al., 2013).

Among Alphacoronaviruses, FCoV ORF7a is also a type I IFN antagonist. Recombinant FCoV virus, deleted of the ORF7a and 7b, is susceptible to IFN treatment and trans-complementation with ORF7a restores the resistance to IFN. However, ORF7a is not completely efficient to confer resistance to IFN for the twice deleted ORF3abc and ORF7ab virus, suggesting a synergic effect of ORF3abc and 7a. Detail mechanisms of the action mode of these accessory proteins remain to be investigated (Dedeurwaerder et al., 2014).

In some cases, presence of the accessory proteins reduces pathogenesis and virulence. Recombinant TGEV, deleted of ORF7, has a higher cytopathic effect and extensive injuries in infected new-born piglets in comparison with the parental virus. The ORF7 encoded protein of TGEV interacts with a protein phosphatase (PP1). This association leads to the inactivation of eIF2 α by its dephosphorylation and therefore prevents the activation of RNase and RNA degradation (Cruz et al., 2011). Another example is the ORF3c from FCoV, which is intact in healthy infected cats but is frequently mutated in FIP cats (Pedersen et al., 2012). With a deleted ORF3abc recombinant virus, Balint et al. demonstrated that this ORF is necessary to

maintain the replication of FCoV in the enteric tract (Bálint et al., 2014). Hsieh et al. also suggest that viruses with the intact ORF3c have a diminished replication efficiency, which could be correlated with a lower virulence (Hsieh et al., 2013). Controversial studies pointed the eventual role of FCoV ORF7b that leads to a secreted protein. In the field, phylogenetic studies are discrepant. Where some authors suggest the maintenance of ORF7b only in asymptomatic infections, others observe deletion of ORF7b in both FIP and healthy cats (Kennedy et al., 1998)(Lin et al., 2009a). In IBV, deletion of ORF3b has been linked with an increased virulence pattern in chicken embryos, but the mechanistic pathway remains unclear. Many functions of other accessory proteins of CoVs remain unknown and are still under investigation.

<u>Strain</u>	<u>Name</u>	<u>Localisation</u>	<u>Characteristics and functions</u>
FCoV	ORF3abc	Perinuclear region	Sustains the viral replication in enterocytes (Bálint et al., 2014)
FCoV	ORF7a	Unknown	Type I IFN antagonist synergistically with ORF3abc proteins (Dedeurwaerder et al., 2014) Deletions associated with FIP cases (Kennedy et al., 2001)
FCoV	ORF7b	Secreted	Controversial studies about the importance of deletions in FIP cases (Herrewegh et al., 1995)
HCoV-229E	ORF4a	ERGIC	Forms oligomers through disulphide bonds. Ion channel activity Enhances the viral production (Zhang et al., 2014)
TGEV	ORF7	ER and plasma membranes	Attenuates virulence Counteracts cell antiviral response (Cruz et al., 2011)
PEDV	ORF3	Unknown	Ion channel activity Enhances the viral production (Wang et al., 2012)

SARS-CoV	ORF3a	Cytoplasm, Golgi, plasma membrane and viral particle	Interacts with S, M, E and 7a. Form oligomers through disulphide bonds. Enhances viral production. Induces apoptosis (Shen et al., 2005)(Freundt et al., 2010)
SARS-CoV	ORF3b	Nucleolus and mitochondria	Inhibits type I IFN through inhibition of IRF3 and mitochondrial antiviral response (Kopecky-Bromberg et al., 2007)(Yuan et al., 2005)(Freundt et al., 2009)(Khan et al., 2006)
SARS-CoV	ORF6	ER Golgi apparatus Viral particle	Enhances viral replication Induces ER stress Modulates host protein nuclear import Stimulates DNA synthesis (Kopecky-Bromberg et al., 2007)(Hussain and Gallagher, 2010)
SARS-CoV	ORF7a	ER ERGIC Viral particle	Interacts with S and ORF3a Role in the assembly of the viral particle Inhibits cellular translation (Vasilenko et al., 2010)
MERS-CoV	ORF4a	Cytoplasmic puncta	Type I IFN antagonist Binds double-stranded RNA (Niemeyer et al., 2013)(Yang et al., 2013)
MERS-CoV BtCoV-HKU4 BtCoV-HKU5	ORF4b	Nucleus	Type I IFN antagonist Inhibit NF-KB pathway (Matthews et al., 2014)
IBV	ORF3b	Unknown	Deletions correlate with virulence in chicken embryos (Shen et al., 2003)

Table 5 : Localisation and roles in pathogenesis of the most extensively studied accessory proteins.

CHAPTER TWO: CORONAVIRUS INTERSPECIES TRANSMISSIONS AND ADAPTATION TO A NEW HOST

I. Understanding the interspecies transmission

Emerging viruses in the human population are a major threat to public health. A zoonosis is defined as the process in which an animal disease can be transmitted to another species (usually the human species). It has been estimated that more than 70% of zoonosis affecting humans find their origins from wildlife reservoirs. Many examples illustrate viral zoonosis, such as AIDS (Acquired Immunodeficiency Syndrome), SARS, Ebola, Hantaviruses and Influenza, among others. Examples such as measles and rabies demonstrate that this phenomenon is not new and has occurred long before human awareness of virus existence. Different stages allow an interspecies transmission of a disease. They are described hereafter.

I.1. Exposure

The first stage of an interspecies transmission is the exposure of the new host species to the viral pathogen. Contact needs to be made between the “donor” host and the “recipient” host, being the human species for zoonosis. Small changes in both the donor and the recipient host can have big incidences on the exposure to viral pathogen. Both hosts are dynamic and their nature as well as their behaviour needs to be considered. Some circumstances are particularly important to take into account, in order to explain the dynamics of diseases exposures (Woolhouse, 2002).

Living with companion animals may expose the owner to new pathogen. For instance, cowpox virus can be transmitted to humans by cat contacts, a widely spread pet animal (Schulze et al., 2007). Dogs are a source of common history with rabies and contamination.

Animal production and farming conditions expose human population to animal pathogens. It is believed that domestication of small ruminants for farming about 7000 or 8000 years ago favoured the exposure of humans to the Rinderpest and allowed adaptation of measles to human beings (Diamond, 1997). The latter is a highly contagious respiratory disease, caused by MeV (Measles virus). As witnessed by wet markets in China, mingling of different species that have separate ecological niches under stressful conditions, can alter animal immune responses and allow contact of these animals with foreign pathogens, such as avian Influenza (Kilpatrick et al., 2006) and SARS-CoV.

Feeding habits can become a source of contamination, with consumption of uncooked pork meat in industrialized countries, exposed to Hepatitis E infections (Ruggeri et al., 2013). Hunting bush meat is also considered a risk factor as hunters are in close contact with animals' body fluids, organs and tissues. It could lead to the successful transmission of Ebola virus and Simian foamy viruses from primates (Wolfe, 2005).

Urbanisation and deforestation disrupts ecological habitats and brings wildlife animals closer to livestock and human living areas. It was suggested that the Nipah virus outbreak in pigs and human population in Malaysia and Singapore in 1999, is a consequence of the environmental mismanagement (Looi and Chua, 2007). Pig farms installed at the forest's border were contaminated through bat excretions. Forest fruit bats, such as *Pteropus hypomelanus* and *Pteropus vampyrus* are natural hosts for the Nipah virus (Chua et al., 2002).

Climate changes can also have radical effects on animal populations. Periodic outbreaks of the Rift Valley Fever virus are associated in Africa with modifications of the river flow due to some heavy flooding and rainfalls (LaBeaud et al., 2008). El Nino phenomenon also affects

the distribution of rodent population and increases the human exposures to Hantaviruses. Camping in wilderness caused some Hantavirus infections as campers have inhaled aerosolized urine excretions of rodents (Flood et al., 1995).

Extensive tourism, commercial exchanges with animal transportations favours novel contacts between donor species and humans and benefits to the importation of new diseases in naive human populations. This example has been highlighted with outbreak of monkeypox infection among pet owners of prairie dogs in the United States (Maskalyk, 2003). Prairie dogs were imported from Ghana and transmitted monkeypox virus by biting their owners.

Behaviour and features of the reservoir host also influences exposure (Wong et al., 2007). Bats are notorious for being present on all continents of the globe (excluding Polar Regions), for flying long distances, for living up to 35 years and for displaying natural habitats particularly close to human populations and livestock. All these ecological features allow them to disseminate viruses quite easily. Also, mingling of different bat species and herds, which is quite a unique feature for mammals, enhances viral genetic material exchange.

I.2. Infection

The next step for an effective inter-species transmission is the infection of the new host by the viral pathogen. As such, the virus must be able to infect appropriate cells of the recipient host. Multiple restrictions exist to prevent that process. First of all are the physical host barriers, such as skin, mucosal surfaces... For example, neuraminidases of Influenza viruses prevent them from being bound to cell surface glycans and therefore be inactivated by the cell (Matrosovich et al., 1998). Second, is the ability for the virus to enter the cell through receptor binding of the new host cell. At this stage, two main processes may contribute to the

transmission in new host. First, the mutation of the viral receptor-binding domain extends the host range capacity.

This first process is portrayed by the transmission of Feline Panleukopaenia Virus (FPV) to dogs. Multiple mutations in the viral capsid allowed FPV to bind to the orthologous receptor of the new canine cell, leading to the emergence of Canine Parvovirus (CPV) (Hueffer et al., 2003). Indeed, as seen in Chapter I, the key determinant protein for CoV cell entry is the spike protein S and more precisely, the RBD. A very well-studied example is the S protein of the SARS-CoV during the human epidemic. Point mutations within the RBD drastically affect the affinity of the S protein to its receptor and extend the host susceptibility. Sheahan et al. demonstrated that single substitutions K479N and S487T allowed the civet SARS-CoV to replicate in Vero cells and in human airway epithelial cells (HAE) and DBT cells (delayed brain tumour cells) expressing human ACE2 receptor (Sheahan et al., 2008). The gained host range was directly linked to a single substitution.

A second process that contributes to the transmission in a new host is the ability of the viral pathogen to bind to the new host receptor without any required modification. As an example of receptors allowing entry of viral strains, feline APN can bind to FCoV but also TGEV, CCoV-II and HCoV-229E (Tresnan et al., 1996). Viruses using sialic acids as receptors can also jump to new hosts. For instance, the ability of BCV and HCoV-OC43 to bind sialic acids has been proposed to be directly related to their broader host ranges (Schwegmann-Wessels and Herrler, 2006). Another example would be the swine that is susceptible to both avian and human Influenza viruses, without prior mutations from the virus or the host receptor (Kahn et al., 2014). Pigs can therefore generate many novel reassortant Influenza viruses which could represent a new threat to human health (Ma et al., 2008).

I.3. Host adaptation

The last condition for a virus to sustain into its new host, is its ability to establish an effective individual-to individual transmission and fully adapt itself to its new environment. This last phase can take months or years for a virus to be fully adapted to its new host. As described in 2007 by Wolfe et al, five different stages exist for adaptation of a virus to a new host and are illustrated in Figure 6.

In the first stage, the virus stays specific to its host reservoir in natural environmental conditions.

In a second stage, in favourable conditions described earlier, the virus will infect a new host species, without any viral cycle among the new infected host population. Rabies virus is a good example of human dead-end host, as spill over of the disease is only done in one way, from animal to human. Rabies cannot sustain into the human population without repeated re-introductions from the non-human reservoir.

Along the third stage, the virus establishes a continuous infectious chain between its natural host and the new host species, but in this stage, the transmission among the new species is not well established. Ebola and MERS-CoV infections highlight this stage.

It is during the fourth stage that the virus is able to trigger an epidemic among the recipient species, as the transmission is well established. SARS-CoV is a notorious example of this stage as the virus was widely spread around the globe.

Finally, it is during the last stage that the virus has adapted itself to its new host in such a manner that it is only able to circulate among the new species. It is the case of HIV (human immunodeficiency virus), which originates from the Simian retrovirus, but is now able to infect only humans (Hillis, 2000). Still, despite its full adaptation to the human species, viruses continue to evolve, as HIV-1 (human immunodeficiency virus type 1) uses the CCR5

co-receptor in the early stages of infection, but later switches to the CXCR4 co-receptor. The co-receptor change is often accompanied with a drop of CD4+ and hastens progression of the disease (Philpott, 2003).

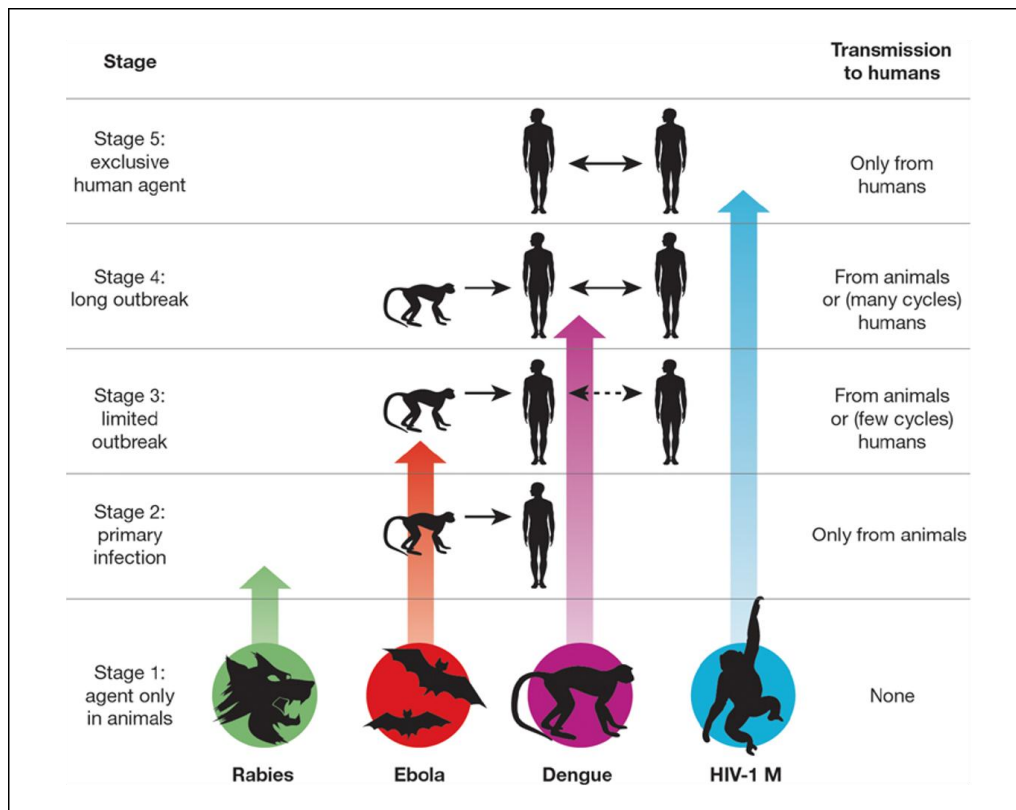


Figure 7 : Five different stages of zoonotic virus adaptation.

Description by (Wolfe et al., 2007).

The first stage represents a virus contained in its natural reservoir. Second stage represents infection of humans, without any human-to-human transmissions, opposite to the third stage where the transmission is rare and limited. The fourth stage allows an established transmission among human beings. The fifth and last stage represents an exclusive pathogen of the human species, which results from the previous adaptive stages.

II. From animal Coronaviruses to zoonotic diseases

II.1. HCoV-OC43 strain: a strictly human respiratory virus with bovine origins

Until 2005, only partial sequences of HCoV-OC43 strain were available, thus challenging studies of this human virus. Publication of the full genomic characterization of HCoV-OC43 by Vijgen et al. lifted the veil on the animal origins of this virus (Vijgen et al., 2006). Acquisition of the full sequence of the lab strain (named VR759) showed the close relationship between the human strain HCoV-OC43 and the porcine strain PHEV with the E sequence, sharing 99.6% of sequence identity. Concerning other sequences such as HE, S, M and N, a strong relationship was also established with the bovine Coronavirus, BCoV. Molecular clock calculations permitted the authors to date the zoonotic transmission event back to 1890, where a common ancestor for both bovine and human virus is most plausible. The most likely circumstance for the emergence of this human strain would be a host-jump phenomenon and an adaptation of the bovine strain to the human species. The authors observed the presence in the human strain of a 290 nucleotides deletion at the N-terminus of the N protein sequence, leading to two small ORFs. In the bovine strain, this ORF located inside the N sequence is intact and encodes a 207 a.a length protein, of unknown function (Vijgen et al., 2006). Significance of this deletion is unknown and unfortunately, no further studies on this observation were carried out. However, it is plausible that this deletion occurred during the host switch and may have played a role in the adaptation of the virus to the human strain.

II.2. From bat SARS-CoV to SARS-CoV

Shortly after identification of the SARS-CoV, animal reservoir and potential sources of contamination were sought. Wet markets were investigated and masked palm civets as well as raccoon dogs displayed SARS-CoV RNA. Nevertheless, samples from these animals in the wild or at least, not in contact with a wet market, were negative. Still, studies from other wild animals in the Hong-Kong area revealed that Chinese horseshoe bats displayed antibodies to SARS-CoV proteins and hosted a phylogenetically close Coronavirus (Li et al., 2005). It was then assumed that Chinese horseshoe bats were the natural reservoir for the bat SARS-CoV related strains named SARS-like CoV (SL-CoV). Until 2013, all SL-CoV discovered in bats were proven incapable of using ACE2 as a receptor in contrast to the civet-SARS-CoV and human SARS-CoV, therefore leading to the hypothesis that civets and raccoon dogs served as intermediate hosts between bats and humans. In 2013, Ge et al isolated the first SL-CoV from bat which uses ACE2 from bats, civets and humans for cell entry (Ge et al., 2013). This finding strongly suggests that direct bat-to-human infection is a plausible scenario for some bat-SL-CoVs. In this hypothesis, civets and raccoon dogs are not intermediate hosts but are infected either through humans or bats. Two hypotheses are maintained in the chronology of this outbreak to explain the emergence of SARS-CoV in the human population (Wang and Eaton, 2007). The first one consists of a direct bat to human transmission followed by a human to small mammals' transmission. The second possibility is that civets could represent an intermediate between humans and bats.

Accumulation of full-length genome of SARS during the early, middle and late phase of the epidemic provided unique data to study the molecular determinants of human adaptation of the virus. Figure 8 illustrates the major genetic differences between strains from bats, civets and the different epidemic stages of the human strains. Major differences have been observed

in ORF1a, in the RBD of S, in ORF3 and ORF8. During the early and the middle phase of the epidemic, substitutions such as A₃₀₄₇V, A₃₀₇₂V were observed in the ORF1a. During the middle phase to the late phase of the outbreak, observations included A₂₅₅₂V in the ORF1a gene and E₁₃₈₉D in the ORF1b gene (Chinese SARS Molecular Epidemiology Consortium, 2004). Functional significance of those substitutions remain unclear, still these changes have been strongly hypothesized as being key events in the expanding human epidemic. Mutations were identified as the K479N and S487T in the RBD of the S1 domain from civets' strains and subsequently increased the binding affinity to the human receptor ACE2 (Qu et al., 2005). These mutations were therefore key determinants for the affinity of S with the human ACE2 receptor. Interestingly, comparative analyses of both humans and civets SARS-CoV strains also detected an intriguing 29 nucleotides deletion in the ORF8ab sequence of human isolates. The 29-nt deletion of ORF8ab is a unique characteristic of the human isolates (Oostra et al., 2007). This deletion leads to the disruption of the ORF8ab into two smaller products, ORF8a and ORF8b.

With a retrospective aspect, this SARS epidemic enlightened its zoonotic potential and triggered massive research on this virus and its natural host, the bat.

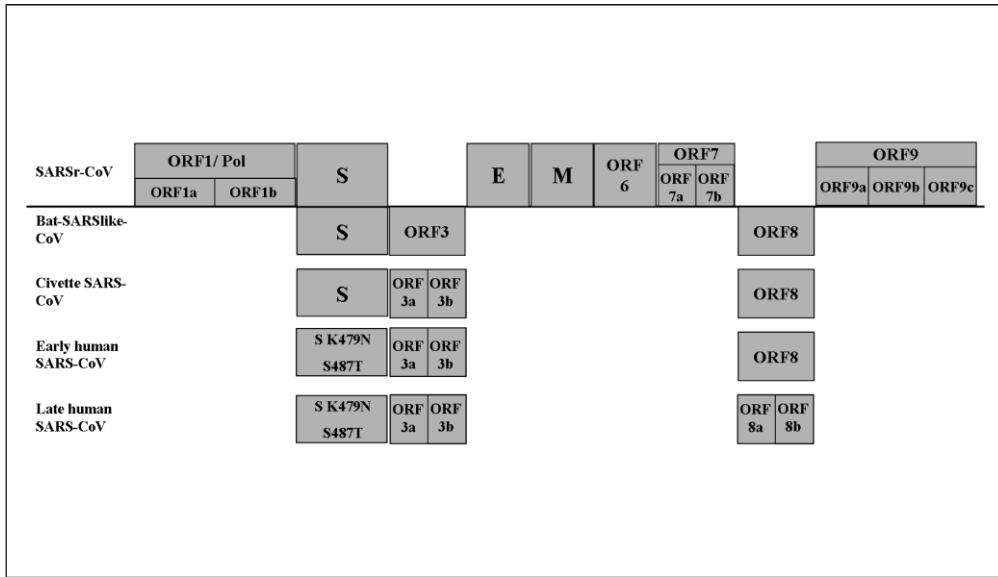


Figure 8 : Representation of the SARS-CoV genome during different stages of the epidemic. Illustration of the major genetic differences between bat strains, civet strains and human strains at different stages of the epidemic. Adapted from (Balboni et al., 2012).

II.3. Emergence of the MERS-CoV: role of the dromedary camel

Soon after the discovery of MERS-COV, it was hypothesized that the virus emerged from an animal reservoir. Bat CoV-HKU4 and HKU5 are phylogenetically close to the MERS-CoV, suggesting a common ancestor in bats. This hypothesis was reinforced by the detection of small genomic fragments of MERS-like-CoV in bats from various countries (Lelli et al., 2013). However, to date, no complete sequence of MERS-like-CoV was recovered from bats. It has been recently demonstrated that MERS-CoV arises from an ancestor bat CoV named Neoromicia capensis bat CoV (NeoCoV) that infects this particular bat species in Africa (Corman et al., 2014).

Furthermore, bats do not have direct contact with human or at least rare and for a very short time and potential for an intermediate host intervention was sought. As such, samples were collected in different domesticated and wild animals from the Middle East region and neutralizing antibodies to the MERS-CoV were found in camels, leading to the speculation

that they represent the intermediate host (Meyer et al., 2014). Camels and dromedaries are companion animals that live close to their owners in that region, as they are raised in farms for both races and leisure.

Further investigations revealed the presence of MERS-CoV RNA in camels in close contact with infected humans (Nowotny and Kolodziejek, 2014). Recently, Azhar et al isolated and sequenced MERS-CoV from a patient and one of his dromedary camels. Isolates from both the patient and the camel were found identical and serological investigations conducted in parallel indicated that MERS-CoV infected the camel in a first place and later, the patient (Azhar et al., 2014). These findings strongly suggest that camels can transmit MERS-CoV to humans in close contact.

In contrast to the SARS-CoV, person-to-person transmission of MERS-CoV occurs in peculiar situations of a prolonged contact or the presence of risk factors such as immunodeficiency.

III. Interspecies transmissions among animal Coronavirus

III.1. Avian Coronaviruses

IBV still causes economic losses, despite efforts on vaccine development. As a matter of fact, many genotypes and serotypes exist, impeding any cross-protection among them. Also, new types of this virus arise from mutations and recombination events, making infections particularly difficult to control. Recently, publications of Korean survey have identified recombination events between indigenous Korean IBV strains and a vaccine strain (Massachusetts serotype) used, therefore questioning the use of live-attenuated vaccines (Song et al., 2013).

Moreover, IBV has been demonstrated to infect a wider host range of birds than previously thought, like peafowls and teals (Cavanagh, 2005). Recombination events among different strains, coupled with mutations, deletions and insertions, play a critical role in the emergence of genetic variants of IBV strains.

Sequence analysis of TCoV (Turkey Coronavirus) has been shown that TCoV results from the recombination event between IBV and an unidentified Coronavirus (Jackwood et al., 2010). TCoV bears the structural backbone of IBV but a different S gene of unknown origin. Pheasant Coronavirus (PhCoV) is also closely related to IBV and its S gene also presents heterogeneity. Overall, IBV, TCoV and PhCoV demonstrate high degrees of genetic similarities (Cavanagh et al., 2002), except for the S gene, suggesting cross-species transmission of IBV to chicken to at least turkeys, pheasants, Guinea fowl, etc...

III.2. Feline, canine and porcine Coronaviruses

TGEV, FCoV and CCoV are regrouped in the same Alphacoronaviruses 1 species due to their very close relationship. Molecular studies demonstrate that they evolved from a common ancestor, probably from bats and then from different cross-species transmissions, from pigs to dogs and dogs to cats, leading to the different genotypes of FCoVs and CCoVs.

It is thought that CCoV genotype II (CCoV-II) results from the ancestral adaptation of TGEV to dogs, accompanied with the loss of accessory gene 7a/b. Among CCoV-II strains, some named CCoV-IIb are characterised by an N-terminal portion of S related to TGEV. Double recombination between CCoV-II and TGEV occurred in order to obtain this strain, thus suggesting a cross-species transmission of TGEV to dogs (Wang and Lu, 2009). Another recombination event between CCoV-IIa and CCoV-I has been recently described, leading to

CCoV-A76 isolate, harbouring an S1 gene originating from CCoV-I (Figure 9)(Regan et al., 2012).

Cross-species transmissions between cats and dogs have also given rise to different genotypes of FCoV and CCoV (Figure 9).

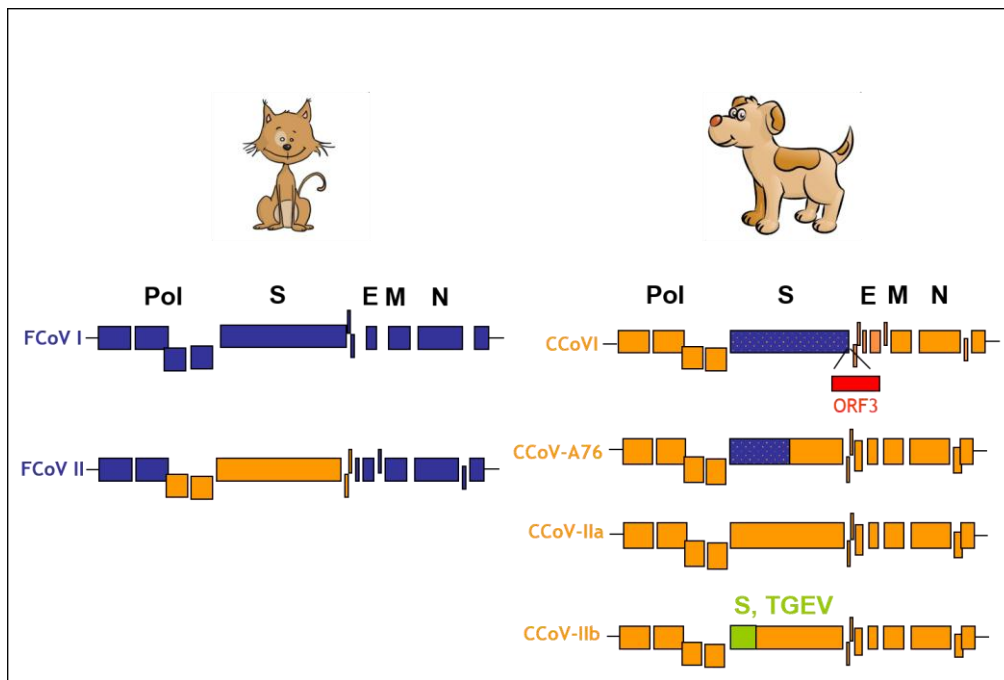


Figure 9: Recombination events between feline, canine and porcine Coronaviruses.

FCoV-II results from the double recombination event between CCoV-IIa and FCoV-I. Genes represented in blue are of feline origins. Genes in orange are of canine origins. Genes in green represent those of porcine origins. In red is represented ORF3, discovered in 2008.

Feline and Canine Coronaviruses are distinguished into two main genotypes for each species (types I and II). Historically, FCoV genotypes were discriminated *in vitro* by use of neutralization assays. Cross neutralisation assays indicated that type II FCoV was closely related to CCoV-II. In 1998, Herrewegh and al. produced evidence of the relationship between FCoV-II and CCoV-II (Herrewegh et al., 1998). Indeed, comparative sequence analyses of different part of the genome allowed identification of FCoV-II as the product of a double recombination event between FCoV-I and CCoV-II. The S gene of the two different studied strains of FCoV-II (79-1146 and 79-1683) exhibited a very close amino acid

homology with the S gene of CCoV-II (91% identity). On the opposite, downstream genes such as M, N and ORF7a/7b and upstream of the ORF1a/b are closely related to FCoV type I. The compiled data strongly suggest a homologous recombination event between FCoV-I and CCoV-II, giving rise to FCoV-II, with the backbone genome of FCoV-I and the S gene originating from CCoV-II (Herrewegh et al., 1998). Recombination sites have been investigated and the different locations have demonstrated that FCoV-II variants originated from independent recombination events. Thus, these findings have put on display an important frequency of host-jump between feline and canine CoVs and co-infections of their hosts. Advantages conferred to the FCoV-II strains are still unclear to this day. Double recombination necessitates a co-infection step with both FCoV-I and CCoV-II strains. Origins of the species that hosted this recombination is still under debate. As the feline APN allows CCoV-II infections on one side whereas canine APN is strictly host-dependant on the other side, it is tempting though to believe that feline cells could have hosted this double-recombination event.

Sequence analysis of CCoV-I demonstrated that its S gene shares an uncommonly high sequence homology with FCoV-I (more than 80%) (Pratelli et al., 2003). In 2008, Lorusso et al. provided evidence of a new uncharacterised accessory protein, unique to the CCoV-I strain, downstream of the S gene (Lorusso et al., 2008). This new gene named ORF3 encodes for an N-glycosylated glycoprotein gp3 of unknown function. As comparative sequences analyses of CCoV-II and FCoV-II resulted in identification of ORF3 remnants, new evolutionary hypotheses for these strains have been suggested (Figure 10). CCoV-I and FCoV-I share the same common ancestor. During RNA recombination events that led to the emergence of CCoV-II, the ORF3 sequence has been lost. To this day, no advantageous properties linked to the presence of ORF3 for CCoV-I strains have been suggested.

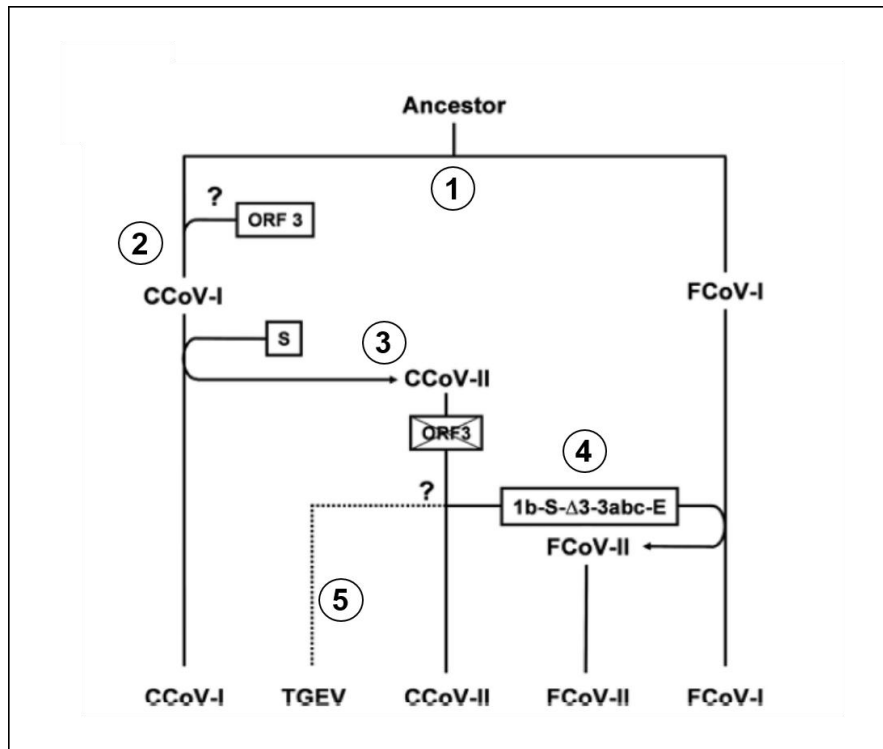


Figure 10: Evolutionary hypothesis of canine and feline strains.

From (Lorusso et al., 2008).

1. CCoV-I and FCoV-I diverged from a common ancestor. 2. CCoV-I acquired a supplemental accessory gene through an unknown process. 3. CCoV-II is the recombinant product of CCoV-I and an unknown strain. It subsequently lost the ORF3 gene while acquiring a novel S gene in this event. 4. FCoV-II is the result of a double recombination event between CCoV-II and FCoV-I. 5. TGEV results from the adaptation of CCoV-II to the porcine species, through an unknown mechanism accompanied with the loss of ORF7b and the inactivation of ORF3b.

III.3. Available models for the study of Coronavirus inter-species transmissions

To assess the molecular determinants of host specificity, *in vitro* and *in vivo* models have been developed and highlight viral residues or regions that play a role in transmission or host specificity. As an example, MERS-CoV does not replicate in small laboratory mammals, such as the Syrian hamster, mouse or ferret, even if they all display orthologous DPP4, the identified receptor. It seems that the virus is host restricted to the human and non-human primates, Rhesus macaques in this case. A recent study has determined the host restriction of the MERS-CoV animal models to be regulated at the receptor binding level. Expression of human DPP4 is necessary to make mice susceptible to MERS-CoV infections. In a recent

developed model, mice were transduced with adenoviral vectors expressing the human DPP4, prior to infection with the MERS-CoV. Thus, mice developed pneumonia but with lesions less severe than in human infections. *In vitro* studies also pointed the critical importance of human DPP4. BHK cells (Baby Hamster Kidney) and ferret cells became susceptible to MERS-CoV when transduced with human DPP4. Moreover, by modelling the interactions between the RBD of the S protein of the MERS-CoV with DPP4, five amino acids were identified to be particularly important, in positions 291, 295, 336, 341 and 346 (van Doremalen et al., 2014).

An exchange of these amino acids in hamster DPP4 allowed the MERS-CoV to infect BHK cells. Still, its replication is significantly lower than in human cell line, demonstrating at the same time the need for further adaptation of the virus to the host.

MERS-CoV can also use the DPP4 of camels, goats, cows and sheep, as a receptor. BHK cells are susceptible to MERS-CoV infection after transfection by one of these species-specific DPP4. Those results correlate with previous studies demonstrating that among goats, alpaca, camel, sheep, bat, bovine or rodent cells, only camels and goats can support MERS-CoV replication (Eckerle et al., 2014). Discrepancies between studies focusing on DPP4 receptor and cell lines for sheep and cows may suggest that other factors than the receptor are essential for MERS-CoV replication in a different host.

As seen before, for the SARS-CoV infection, only two substitutions in the RBD of the S1 domain from civets' strains significantly increase the binding affinity to the human receptor ACE2. In contrast to MERS-CoV, many animals are sensitive to SARS-CoV infections and allow replication of the virus. Among those animals are found: non-human primates, cats, ferrets, pigs, chickens and small laboratory mammals, such as mice, rats, Chinese hamsters and guinea pigs (van den Brand et al., 2008). Despite all those available models for a specific aspect of the SARS-CoV infection, no animal has been found adequate to display all clinical

aspects of the disease, except for non-human primates. A recent study of SARS-CoV infection on aged and young African green monkeys, has demonstrated the similarities of the infectious pattern of the aged monkey model compared to the clinical aspects in aging humans during the epidemics (Clay et al., 2014). In mice and rats, adaptation of SARS-CoV strains is necessary for the development of clinical signs in these animals. These adaptation studies gave information about the viral determinants necessary for the adaptation in the new host. In young BALB/c mice, adaptation needs 15 to 25 passages, leading from six to nine mutations in the spike gene, nsp9, nsp13 and M. Specific mutations at residue 436 in S has been shown to allow stronger affinity to the murine ACE2 receptor. In aged mice, adaptation needs only five passages and less mutations (Frieman et al., 2012). SARS-CoV has also been adapted to young F344 rats (Nagata et al., 2007). Adapted mutations also display substitutions in the spike protein.

Among Alphacoronaviruses, studies on the different species specific APN demonstrate the exceptional property of the feline APN to serve as a receptor for FCoV, HCoV-229E, TGEV and CCoV-II. Moreover, using chimeric APN receptor, the critical residues in feline APN, necessary to recognise the S of HCoV-229E, TGEV, CCoV and FCoV, were identified (Tusell et al., 2007). Whereas amino acids 288 to 290 are crucial for interactions with HCoV-229E, TGEV requires the region from the 732nd to the 746th residues, and both FCoV and CCoV necessitate this latter region, plus residues 764 to 788. *In vivo* experimental infections correlate with the *in vitro* data, as cats can be infected with CCoV-I and HCoV-229E, suggesting a possible role of cats as a reservoir or an intermediate host for different Alphacoronaviruses.

OBJECTIVES OF THESIS PROJECT

Coronaviruses are prone to recombine and cross the barrier species, as it is the case for FCoV-II, TCoV and human epidemics, such as SARS-CoV and the MERS-CoV. The S protein of Coronavirus is a crucial determinant of host switching and mutations in S are frequently involved in the adaptation of the binding process to the receptor of the newly infected species. However, infection of a new host also requires other adaptations from the CoV strains. In a host jump context, frequent mutations of CoV accessory proteins are observed: deletion of ORF8ab during the adaptation of SARS-CoV to humans, deletion of the ORF located inside the N sequence of HCoV-OC43. The precise roles of these accessory proteins and their mutations during the host switch remain unclear.

FCoV and CCoV are common pathogen and inter-species transmissions through recombination between viral strains have already been documented. Indeed, FCoV-II is the product of a double recombination between two parental strains FCoV-I and CCoV-IIa. Moreover, as the feline APN is able to serve as a receptor for CCoV-II, it is likely that the S infection and recombination events took place in the feline host. CCoV-I displays an uncommon S gene that shares more than 80% homology with the S gene of FCoV-I. As S is a key determinant of host-species, the possible infection of cats by CCoV-I seems plausible.

In a context of high prevalence of FCoV and CCoV in cat and dog populations, we aimed at investigating the possible factors involved in inter-species transmission of CoV between those two animal species.

We conducted phylogenetic studies in cat samples recovered from animals living or not in the same household as dogs. With the discovery of atypical FCoV harbouring truncated forms of the ORF3 gene, we then attempted to seek the implication of the accessory gp3 protein in the adaptation of atypical FCoV in feline host.

INFECTIONS OF CATS WITH ATYPICAL FELINE CORONAVIRUSES
HARBOURING A TRUNCATED FORM OF THE CANINE TYPE I NON-
STRUCTURAL ORF3 GENE

In this first study, our aim was to investigate the diversity of CoV strains in infected cats, with a particular attention to possible cross transmissions of CCoV strains. Information about the presence of dogs in the same household of studied cats was inquired. When possible, samples of dogs in close contact were also collected and analysed for the presence of CoV.

Samples included both healthy (88 samples) and FIP cats (11 ascitic fluids) over a period of six years. Out of these hundred samples, 11 of the cats were living in the presence of a dog. Faecal samples from three dogs were analysed. RNA extractions and amplification of N and part of S genes were performed and the resulting sequences analysed. Results have demonstrated that:

- Statistics of infected cats in close contact with dogs versus non-infected cats showed that the presence of dogs in the same households had no impact on the Coronavirus infection prevalence in cat population.
- The conserved N gene recovered from both asymptotically infected and sick cats displayed no differences and segregated with the FCoV-I/FCoV-II clade when constructing the phylogenetic tree, except for six sequences.
- The remaining six sequences of the N gene clustered with the CCoV-I clade. Five of them were recovered from healthy cats while one was recovered from a FIP cat.
- Partial sequence analysis of the S gene revealed that all strains recovered from cats clustered within the FCoV-I strains, even those harbouring an N gene phylogenetically related to CCoV-I.

- In order to further investigate the six strains with an N gene related to CCoV-I, amplification of ORF3 was performed. Indeed, ORF3 was discovered in 2008 and is unique to the CCoV-I genotype (Lorusso et al., 2008). This gene is completely absent from any other Coronaviruses and displays no homology whether with viral or cellular genes. It is therefore a good genetic marker of the CCoV-I genotype. Amplification of the ORF3 was successful for five of the six sequences. Still, sequence analyses identified two identical deletions, never described before, in the ORF3 sequences. The systematic deletion of 29 nucleotides leads to a frameshift and a stop codon in the C-terminus part of the protein, leading to a predicted shorter gp3 protein, gp3- Δ 1. The small internal deletion of 27 nucleotides does not change the reading frame of the sequence and introduces a deletion of nine amino acids, leading to gp3- Δ 2.
- For one of the cats, infected with the atypical strain, we analysed the CoV strain recovered from the dog living under the same rooftop. This dog was infected with a CCoV-I strain and the displayed ORF3 sequence shared the same 29-nucleotides deletion found in the cat, strongly suggesting an inter-species transmission of CoV between the two animals.

In this study, circulation of atypical feline Coronaviruses was demonstrated. Those strains harboured truncated forms of ORF3, which have never been described before. Many questions remain regarding those accessory proteins, especially when it is now acknowledged that they are of importance in the different processes of host-jump and adaptation of Coronaviruses to novel species.



Infection of cats with atypical feline coronaviruses harbouring a truncated form of the canine type I non-structural ORF3 gene[†]

Sophie Le Poder^{a,†}, Anne-Laure Pham-Hung d'Alexandry d'Orangiani^a, Lidia Duarte^a, Annie Fournier^a, Cristina Horhogea^b, Carine Pinhas^a, Astrid Vabret^c, Marc Eloit^{a,1}

^a Université Paris-Est, Ecole Nationale Vétérinaire d'Alfort, UMR 1161 virologie INRA ENVA ANSES, Maisons-Alfort F-94704, France

^b Université des Sciences Agricoles et Médecine Vétérinaire Ion Ionescu de la Brad Iasi, Faculté de Médecine Vétérinaire, Aleea Mihail Sadoveanu, Nr. 8, cod 700489 Iasi, Romania

^c Laboratoire de Virologie, EA 2128, CHU de Caen. Avenue Georges Clemenceau, 14033 Caen Cedex, France

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Feline and canine coronaviruses (FCoV and CCoV, respectively) are common pathogens of cats and dogs sometimes leading to lethal infections named feline infectious peritonitis (FIP) and canine pantropic coronavirus infection. FCoV and CCoV are each subdivided into two serotypes, FCoV-I/II and CCoV-I/II. A phylogenetic relationship is evident between, on one hand, CCoV-I/FCoV-I, and on the other hand, CCoV-II/FCoV-II, suggesting that interspecies transmission can occur. The aim of the present study was to evaluate the prevalence of coronavirus (CoV)-infected cats according to their contact with dogs and to genetically analyse the CoV strains infecting cats. From 2003 to 2009, we collected 88 faecal samples from healthy cats and 11 ascitic fluids from FIP cats. We investigated the possible contact with dog in the household and collected dogs samples if appropriate. Out of 99 cat samples, 26 were coronavirus positive, with six cats living with at least one dog, thus showing that contact with dogs does not appear as a pre-disposing factor for cats CoV infections.

Molecular and phylogenetic analyses of FCoV strains were conducted using partial N and S sequences. Six divergent strains were identified with the N gene clustering with CCoV-I whereas the 3' end of S was related to FCoV-I. Further analysis on those six samples was attempted by researching the presence of the ORF3 gene, the latter being peculiar to CCoV-I to date. We succeeded to amplify the ORF3 gene in five samples out of six. Thus, our data strongly suggest the circulation of atypical FCoV strains harbouring the CCoV-I ORF3 gene among cats. Moreover, the ORF3 genes recovered from the feline strains exhibited shared deletions, never described before, suggesting that these deletions could be critical in the adaptation of these strains to the feline host.

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1. Introduction

Coronaviruses (CoVs) are enveloped viruses that possess the largest (27–32 kb) single-stranded, positive-sense RNA molecule of all such viruses. According to the novel classification approved by the International Committee of Taxonomy of Viruses, coronaviruses have been divided into four new genera *Alpha*, *Beta*, *Gamma* and *Deltacoronavirus* and each genus is subdivided into different species. Due to their close sequence identity, porcine transmissible gastroenteritis virus (TGEV), canine coronavirus (CCoV) and feline

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[†] Corresponding author. Tel.: +33 1 43 96 73 25; fax: +33 1 43 96 73 96.

E-mail address: slepoder@vet-alfort.fr (S. Le Poder).

¹ Present Address: Institut Pasteur, Laboratory for Pathogen discovery, 28 rue du Dr Roux, 75015 Paris.

coronavirus (FCoV) have been regrouped together, henceforth forming the *Alphacoronavirus* 1 species, within the *Alphacoronavirus* genus (Carstens, 2010).

The *Alphacoronavirus* 1 species genome includes 7 open reading frames (ORFs) flanked by 5' and 3' untranslated regions (UTRs). The 5' two thirds of the genome encode proteins involved in RNA replication and transcription (Bredenbeek et al., 1990). Genes encoding the structural proteins are located downstream; these include the spike (S) glycoprotein responsible for virus entry, the small envelope (E) protein, the transmembrane (M) protein and the nucleocapsid (N) protein, which is associated with the viral RNA. The rest of the ORFs encode unknown or less-characterised non-structural proteins. The genome of FCoVs and CCoVs includes two gene clusters encoding non-structural proteins, the ORFs 3a, 3b, 3c (located between the S and E genes) and the ORFs 7a, 7b (downstream of the N gene). These genes are not essential for virus propagation in cell culture but are important in virulence or viral tropism (Chang et al., 2010; Vaughn et al., 1995).

FCoV infections are common in domestic cats and usually remain subclinical. In some cases, FCoV causes a fatal disease called feline infectious peritonitis (FIP). Two FCoV serotypes exist and can be distinguished by an *in vitro* neutralisation assay (Hohdatsu et al., 1991). In the field, type I FCoVs are predominant (Addie et al., 2003). Type II FCoVs originate from a double recombination between type I FCoV and CCoV, resulting in an FCoV genome with the S gene and the 5' end of the M gene originating from CCoV (Herrewegh et al., 1998).

CCoV are also divided into 2 genotypes, CCoV type I (CCoV-I) and CCoV type II (CCoV-II). CCoV-II was recently subdivided into two subtypes, CCoV-IIa comprising CCoV reference strains and CCoV-IIb, which results from the recombination between CCoV-IIa and TGEV (Decaro et al., 2009). CCoV-I strains were identified in the beginning of the 2000s and were initially designated as FCoV-like strains due to their high sequence homology within the S gene of FCoV-I (Pratelli et al., 2003a,b). Lately, an additional ORF, named ORF3, that has not been detected in CCoV type II and other *alphacoronaviruses*, has been identified in CCoV-I. ORF3 is placed downstream of the S gene and encodes a non-structural glycoprotein of unknown function (Lorusso et al., 2008).

Considering the close genetic relationship between CCoV-I/FCoV-I on one hand, and CCoV-II/FCoV-II on the other hand, interspecific circulation of CoVs between both species is plausible. In 2006, a study performed in an Austrian shelter where cats and dogs lived together, did indeed suggest that some cats were infected with atypical strains related to CCoV-I (Benetka et al., 2006). The present study was designed to provide a greater knowledge of FCoV strains infecting cats from private owners, living or not in close contact with dogs.

2. Materials and methods

2.1. Clinical specimens

Rectal swabs from 88 healthy cats and 11 ascitic fluid samples from cats having clinical signs of a wet form of FIP were collected in France and Romania. The owners were questioned about the eventual presence of dogs in the household. 3 faecal samples from dogs living with cats were thus recovered. Samples were kept frozen at -80 °C until tested.

2.2. RNA extraction and RT-PCR assays

RNA from ascitic fluid and frozen faeces was extracted by using the QIAamp viral RNA Mini Kit (QIAGEN). The screening of coronavirus positive samples was performed by using the previously described primers and protocol targeting the highly conserved 3' UTR of the coronavirus genome (Herrewegh et al., 1995). Primers amplifying nucleocapsid N gene (forward, 5'-AACAAACACACCTGGAAGA-3' reverse, 5'-GTGTCATCAAACACATCTGT-3') and ORF3 (forward, 5'-CACTAAACTCAAAATGTTGATTC-3' reverse, 5'-TTAAGGATTAACATATTTCTA-3') were designated on the basis of sequences available in GenBank. RT-PCR assays were carried out using the *OneStep RT-PCR Kit* (Qiagen). Briefly, the RNA was reverse transcribed at 50 °C for 30 min and then amplified by 40 cycles of 1 min of denaturation at 94 °C, 1 min of primer annealing at 50 °C and 1 min of primer extension at 72 °C. The N PCR products were 415 bp in length located between nucleotides position 27466 and 27881 in reference to the sequence of strain FCoV 79-1146. The ORF3 PCR products size were expected around 628 bp, located between nucleotides 16 and 638 in reference to the Elmo/02 CCoV-I strain.

Amplification of the 3' end of the S gene was attempted by using specific FCoV-I and CCoV-I primers and protocols previously described (Addie et al., 2003; Pratelli et al., 2003b; Chang et al., 2012).

2.3. Bank accession numbers

The PCR products were subjected to sequence analysis (Eurofins Company). The partial sequences of the N gene of strains cats-1, -9, -21, -22, -24, -29, -30, -33, -41, -57, -61, -74, -81, -118, -122, -125, -127, -130, -139, -149, -163, -179, -180, -181, -196, -198 and dog-1 have been registered in GenBank under accession numbers JN687591-JN687617. The ORF3 sequences of strains cats-9, -22, -29, -30, -163 and dog-1 have been registered in GenBank under accession numbers JN714195-JN714200. The partial sequences of the S gene of cats-22, -29, -30, -163 and dog-1 have been registered in the European Nucleotide Archive under accession numbers HG325837-HG325840.

2.4. Sequence analysis

The ClustalW2 program (www.ebi.ac.uk/Tools/clustalw2) was used for sequence alignment. Phylogenetic tree was constructed by the Neighbour-Joining method using MEGA version 4, supplying a statistical support with bootstrapping over 1000 replicates (Tamura et al., 2007). Potential signal peptide and N-glycosylation site were determined respectively with Sigcleave (emboss.bioinformatics.nl/cgi-bin/emboss/sigcleave) and NetNGlyc (www.cbs.dtu.dk/services/NetNGlyc/) programs, respectively.

3. Results

3.1. Prevalence of CoV infection in cats living or not with dogs

Over the period 2003–2009, 88 rectal swabs from healthy cats and 11 samples of ascitic fluid from cats that developed a wet form of FIP were collected in France and Romania. The owners were questioned about the presence of dogs in the households. 19/99 (19.2%) of the cats enrolled in the study had been in contact with at least one dog (Table 1).

The presence of coronavirus was assessed by a previously described RT-PCR assay that amplifies the highly conserved 3' end of the viral genome (Herrewegh et al., 1995). All diseased cats were tested positive (Cats-1, -21, -24, -41, -74, -118, -163, -180, -181, -196 and -198) and out of the 88 healthy cats, 15 animals were shedding coronaviruses in their faeces (see Supplementary Table 1). Among those infected animals, 6/26 (23%) were living with at least one dog, which is not statistically different ($p < 0.05$) from the proportion of non CoV-infected cats in contact also with dogs (13/73) (Table 1).

Within our range of action, we sampled rectal swabs from 3 of the dogs living with CoV infected cats and only one (Dog-1), which was in close contact with Cat-22, was shedding coronavirus at the time of sampling.

3.2. Sequence analysis of N gene

Sequence analysis of a fragment of the N gene was performed on the assumption that this ordinarily conserved gene would allow

Table 1
Distribution of coronavirus-infected cats and non coronavirus-infected cats according to the presence or absence of dogs in the household.

	Contact with dogs		Total
	Yes	No	
CoV-infected cats	6	20	26
Non CoV-infected cats	13	60	73
Total	19	80	99

significant discrimination of the phylogenetic relationships between strains. The sequences obtained were compared by phylogenetic analyses with representative FCoV-I/II, CCoV-I/II and TGEV strains retrieved from GenBank. The N sequences clustered into two main clades (Fig. 1). The first clade included FCoV-I/II whereas the second clade is divided into two separate clusters comprising CCoV-I and CCoV-II/TGEV. 20/26 of the feline samples tested fell into the typical FCoV genotype. Half of them were recovered from asymptomatic infections, the others from sick cats but no genetic distinction was evident between N sequences from healthy or diseased cats. With exception given to the sequence from Cat-57, the nucleotide identity of the sequenced N genes to the reference FCoV strains was comprised between 90% and 94% (Fig. 1). Interestingly, 5 sequences recovered from healthy cats (Cats-9, -22, -29, -30 and -179) and one from a FIP cat (Cat-163) segregated within the CCoV-I cluster. The N gene of these strains displayed high sequence identity to CCoV-I reference strains ranging from 91 to 96% but less than 79% with any FCoVs. The N sequence from Dog-1 was also grouped within the CCoV-I cluster (Fig. 1).

3.3. Sequence analysis of the 3' end of the S gene

As the S gene sequences have been widely used to genotype FCoVs and CCoVs and more recently to discriminate virulent FCoV strains leading to FIP, we investigated the 3' end sequence of S with primers specific to CCoV-I and to FCoV-I strains (Addie et al., 2003; Pratelli et al., 2003b; Chang et al., 2012). By using CCoV-I primers, only the S fragment from Dog-1 was amplified and sequenced, confirming its highest sequence homology with CCoV-I over 95% (Fig. 2).

Amplification of the 3' end of S was successful with specific FCoV-I primers for 14/20 cats samples with FCoV-I N genes and for 4/6 samples with N gene clustering with CCoV-I (Fig. 2). Assays with specific FCoV-II primers failed. Sequence analyses confirmed that the amplified S fragments from Cats -22, -29, -30 and -163, whose N genes clustered with CCoV-I strains, shared the highest sequence identity with prototype or field FCoV-I strains, ranging from 89 to 97.9% and less than 86.2% with prototype CCoV-I strains (Fig. 2). In addition, the sequence from Cat-163, which died from FIP, exhibited a Leucine residue at position 1058, recently described as virulence

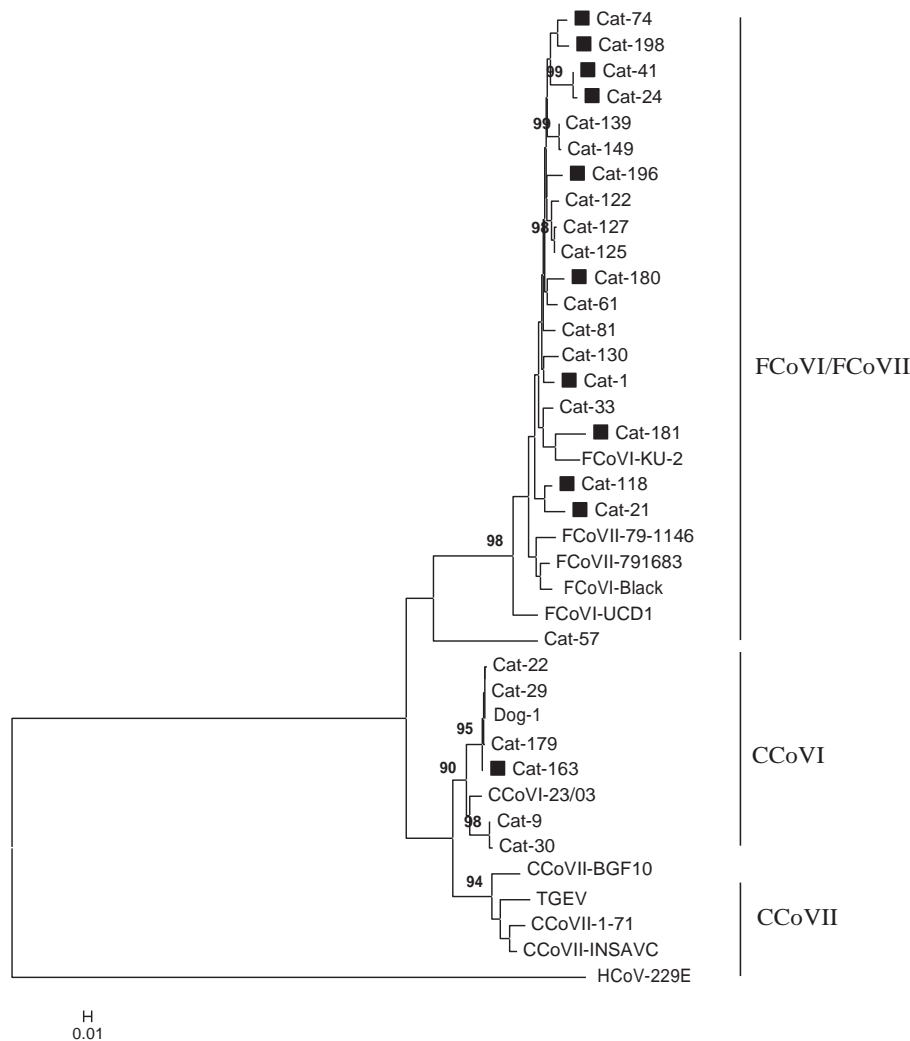


Fig. 1. Phylogenetic tree based on partial nucleotide sequences of the N gene. A neighbour-joining tree was constructed using the MEGA 4.1 software (Tamura et al., 2007). The N gene of human coronavirus 229E (HCoV-229E) serves as an outgroup. A bootstrap analysis of 1000 replicates was performed and values above 90% are indicated on the branches. Horizontal branches are drawn to scale; the scale bar represents 0.1 nucleotide substitutions per site. The following reference strains were used for phylogenetic tree construction (GenBank accession numbers are reported in parentheses): FCoV-II-79-1683 (AB086904), FCoV-II-79-1146 (DQ010921), FCoV-I-UCD1 (AB086902), FCoV-I-Black (EU186072), FCoV-I-KU-2 (AB086881), CCoV-II-1-71 (JQ404409), CCoV-II-BGF10 (AY342160), CCoV-II-INSAVC (D13096), CCoV-I-23/03 (AY548235), TGEV (AJ271965), HCoV-229E (DQ2439391). Sequences marked with j were amplified from FIP animals.

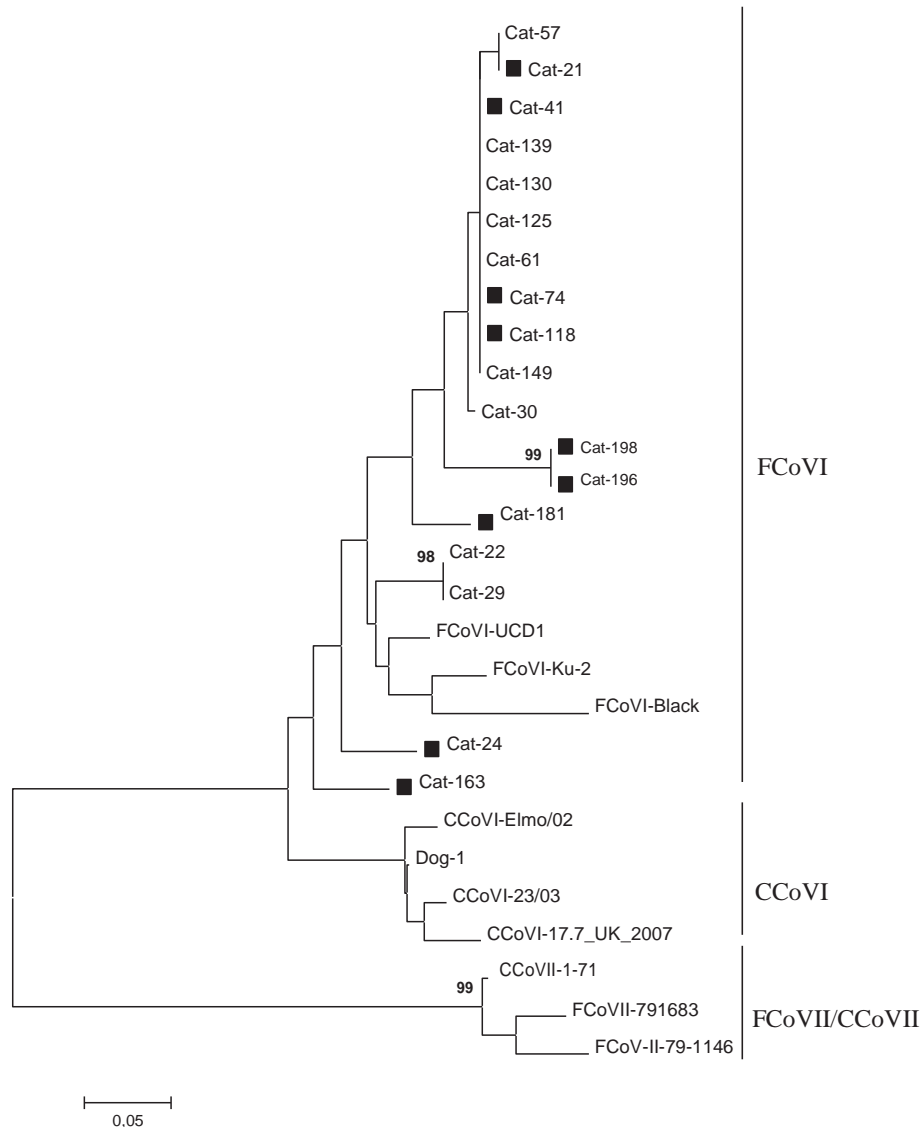


Fig. 2. Phylogenetic tree based on partial nucleotide sequences of the 3' end of the S gene. A neighbour-joining tree was constructed using the MEGA 4.1 software (Tamura et al., 2007). A bootstrap analysis of 1000 replicates was performed and values above 90% are indicated on the branches. Horizontal branches are drawn to scale; the scale bar represents 0.05 nucleotide substitutions per site. The following reference strains were used for phylogenetic tree construction (GenBank accession numbers are reported in parentheses): FCoV-Black (EU186072), FCoV-UCD1 (AB088222), FCoV-KU-2 (D32044), CCoV-23/03 (AY307021), CCoV-Elmo/02 (AY307020), CCoV-17.7_UK_2007 (JX035860), FCoV-II-79-1146 (DQ010921), FCoV-II-791683 (AB086904), CCoV-II-1-71 (JQ404409). Sequences marked with *j* were amplified from FIP animals.

marker of FCoV-I strains (Chang et al., 2012). The other S sequences retrieved from FIP infections also harbour the same mutation.

3.4. Amplification of the ORF3 gene and sequence analysis

Recently, a novel accessory gene named ORF3, located between the end of the S gene and the ORF3a gene, was discovered in CCoV-I strains. This gene is absent in all other *alphacoronaviruses* studied so far. To further characterise feline coronaviruses harbouring an N gene related to CCoV-I, we attempted to amplify the ORF3 gene by RT-PCR. Thus, ORF3 was detected in the Dog-1 sample, and in all but one cat (Cat-179) harbouring an N gene related to CCoV-I. Conversely, the attempt to detect ORF3 in a panel of strains with a feline N gene was unsuccessful.

The sequence comparison of the ORF3 amplicons revealed 85–95% identity with ORF3 nucleotide sequences from the CCoV-I reference strains. The sizes of the amplicons were shorter than the reference sequences. ORF3 sequences from Dog-1 and Cat-22 were

595 bp in length, sequences from Cats-9, -29 and -30 were 568 bp in length and the sequence from Cat-163 was only 541 bp in length, instead of 623 bp for the reference ORF3 sequences deposited in GenBank. By nucleotide alignment with sequences available from GenBank, two deletions, never described before, were identified within the ORF3 gene isolated from cats and Dog-1 (Fig. 3A). The first deletion, located between nucleotides 262 and 289, preserved the reading frame of ORF3 and resulted in the loss of 9 amino acid (aa) starting at the aa 88 (Fig. 3B). This deletion was only observed in Cats-9, -29, -30 and -163. The second deletion was common to all analysed ORF3 sequences, even in the ORF3 gene from Dog-1, which lived with Cat-22. It comprised nucleotides 461–482 and introduced an early stop codon at the aa 159 (Fig. 3B). The ORF3 retrieved from Cat-163 is shortened at the 30' end of the gene.

Computer analysis predicted that the deleted ORF3 would encode a truncated gp3 protein of 149 or 158 aa, while the gp3 protein comprises 207 aa in CCoV-I isolates studied so far (Fig. 3B). All of the truncated gp3 proteins retained the 14-aa signal peptide and

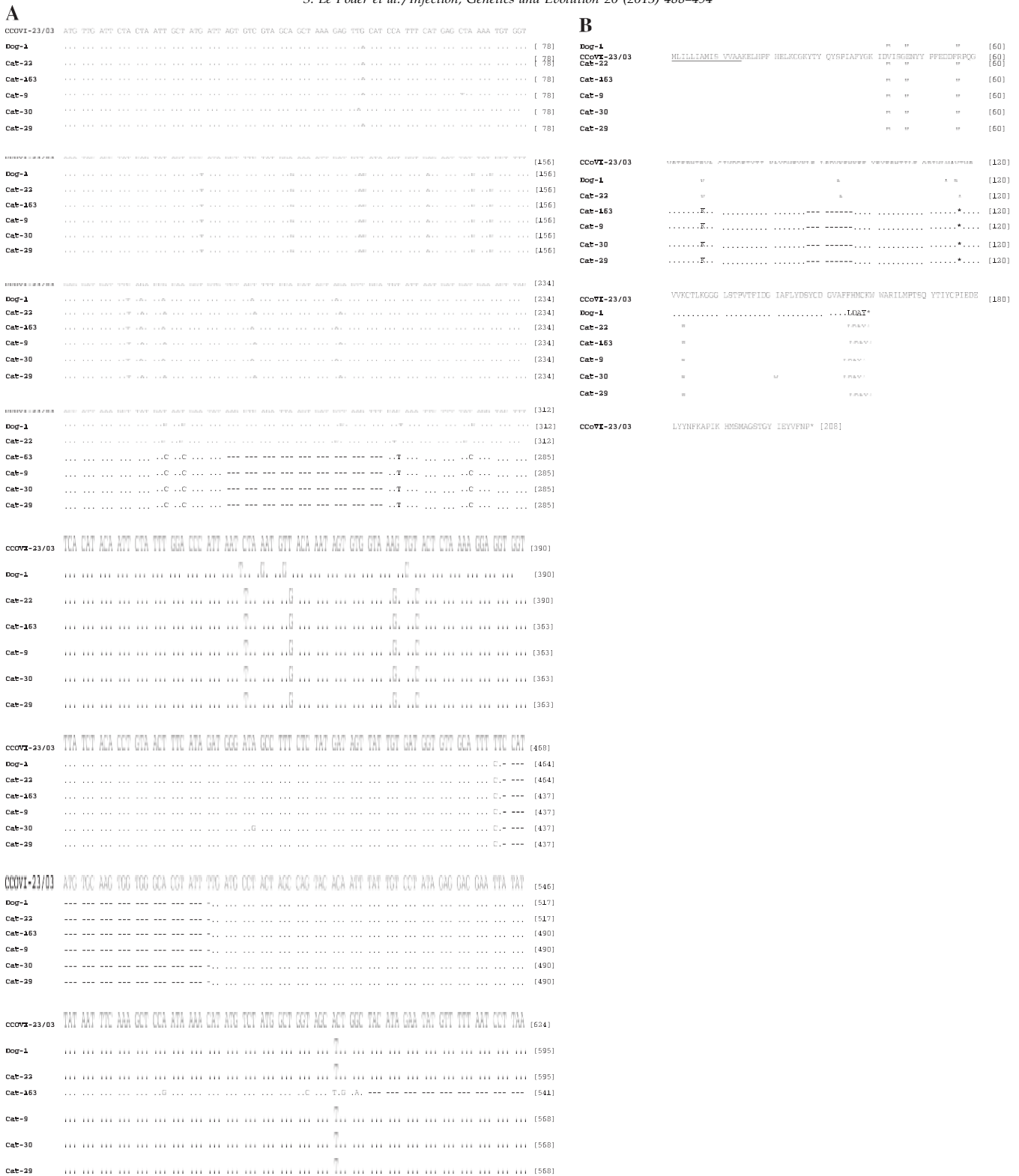


Fig. 3. Alignment of nucleotide (A) and amino acid (B) ORF3 sequences in comparison with the sequence from the reference CCoV-I 23/03 strain. A conserved potential signal peptide is underlined, a potential N-glycosylation site is indicated by an asterisk in bold (B).

the potential N-glycosylation site, described in intact gp3 proteins (Fig. 3B).

4. Discussion

The data of molecular biology of FCoV and CCoV have rapidly accumulated since the early 2000s. FCoV and CCoV strains are clas-

sified into 2 main genotypes sharing close relationships. In particular, the S protein from FCoV-II originates from CCoV-II and the S protein of FCoV-I and CCoV-I share 81% identity, which could potentially lead to interspecific transmission of coronaviruses because S mediates cell entry. By sequence analysis of fragments from the M and S genes, Benetka et al. suggested that interspecies transmission of CoVs occurred in a shelter where cats and dogs were in contact. They indeed detected atypical FCoV strains related

to CCoV-I (Benetka et al., 2006). However, since the ORF3 gene had not been described at this time, it was impossible to confirm that these atypical strains belonged to the CCoV-I genotype. *Journal of Virology* 87 (2013) 488–494

The present study was conducted on cats from private owners with the aim to investigate (i) the possible influence of the presence of dogs to the prevalence of cats coronavirus infection and (ii) the genetic characterisation of FCoV strains and CCoV strains from dogs living with CoV infected cats. We collected 88 faecal samples from healthy cats and 11 ascitic fluid from cats with clinical symptom of FIP. Among them, 19 were living with at least one dog in the same household but the percentages of infected cats and non-infected cats in close contact with a dog were not statistically different (Table 1).

Phylogenetic analysis was performed on 15 coronavirus strains collected from healthy cats, 11 FIP cats and one dog by first sequencing fragments from N and S genes (Figs. 1,2). Most of the FCoV strains were identified as FCoV-I with the N and S genes clustering with reference FCoV-I strains. Surprisingly, the N sequences from six strains recovered from Cats -9, -22, -29, -30, -163 and 179 clustered within the CCoV-I genotype whereas the spike gene of 4 of them (Cats -22, -29, -30 and 163) segregated within the FCoV-I genotype. One significant element is the presence of the accessory ORF3 gene in these atypical FCoV strains, as this gene was described to be peculiar to CCoV-I strains (Lorusso et al., 2008). Lorusso et al. hypothesised that, CCoV-I may have acquired the ORF3 gene after the divergence of FCoV-I and CCoV-I from their common ancestor, or, alternatively that FCoV-I may have lost the ORF3 gene present in the common parental virus. However, the description of atypical FCoV-I strains harbouring ORF3 gene led to the hypothesis that the ORF3 gene also persisted in some FCoV-I or that these strains may originate from a recombination between FCoV-I and CCoV-I strains. In the present study, we identified one couple of cat and dog (Cat-22 and Dog-1) harbouring coronavirus and living together. Notably, Dog-1 was infected by a CCoV-I strain and Cat-22 by an atypical FCoV-I strain comprising an ORF3 gene. Moreover, strains infecting these animals shared 99% of nucleotide identity within the N gene, the ORF3 genes exhibited the same 29-nt deletion which yielded a stop codon but the S genes significantly differed. It is therefore tempting to speculate that the strain detected in Cat-22 originated from recombination with the CCoV-I strain from the Dog-1. However, without extensive sequence analysis of the full-length genome from some atypical FCoV-I harbouring an ORF3 gene, it would be impossible to clarify whether they originate or not from recombination with CCoV-I and to identify the putative recombination sites.

Curiously, the ORF3 genes amplified herein were found to have one or two deletions, never described. All had the same 29-nt deletion which yielded a stop codon and four of the six obtained ORF3 genes shared another 27-nt in frame deletion (Fig 3). So far all sequenced ORF3 genes originated from Italy and all of 623 bp in length (Lorusso et al., 2008). More information regarding the variability of this gene will be necessary to determine if these deletions are essentially restrained to cats viruses or if they are also spread among the canine strains.

In this study, all cats, except Cat-163 were living in the same French geographic region, moreover Cat-22/Dog-1 and Cat-29/Cat-30 were in close contact, which could explain the high degree of sequence homology between the ORF3 sequences amplified herein. Only Cat-163 originated from Romania and its ORF3 sequence diverged from the others, with a shortening after the stop codon introduced by the 29-nt deletion (Fig. 3A).

ORF3 belongs to the group of accessory genes which are characteristic of each genus of the *Coronaviridae* family. The functions of accessory proteins are often unknown but they are regularly implied in tropism switch or in adaptation of a viral strain to a new host species. Thus, adaptation of SARS-CoV from the bat to human was accompanied by several mutations in different parts of the genome including a characteristic 29-nt deletion within the accessory ORF8 gene (Chinese SMEC, 2004; Oostra et al., 2007). The

function of the gp3 protein encoded by the intact ORF3 gene is still unknown. Its biochemical properties, glycosylation and molecular weight have been studied by *in vitro* translation assays (Lorusso et al., 2008). Gp3 is a 28 kDa N-glycosylated protein with a cleavable N-terminal signal, indicating that it may be a secretory protein. According to the predictive computer analysis, the truncated gp3 derived from the deleted ORF3 genes retain the same glycosylation site and the signal peptide. More studies on the biological properties of gp3 and its putative truncated counterparts described herein will be necessary to understand their role.

5. Conclusion

Finally, our data demonstrate for the first time the circulation of atypical feline coronaviruses harbouring a truncated form of the ORF3 gene, whereas it was so far described as peculiar to CCoV-I strains. Clarifications must be made upon whether these strains are widespread amongst the cat population and full-length genome sequencing will be required to deepen the phylogenetic analysis. Given the dramatic consequences of SARS-CoV infection and more recently the new MERS-CoV, greater understanding of the molecular processes leading to the emergence of coronaviruses remains crucial (Zaki et al., 2012). In this context the surveillance of animal coronaviruses remains important not only because animal coronaviruses are often responsible of major veterinary diseases but also because the knowledge accumulated have a substantial contribution to the understanding of the genetic evolution and pathobiology of coronaviruses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.meegid.2013.09.024>.

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Supplementary Table 1: Coronavirus positive cats studied, date and location of sampling, clinical data, eventual contact with dogs

Cat no.	Date of collection	Location	Clinical data	Contact with a dog
Cat-1	June 2003	Paris, France	FIP	
Cat-9	February 2009	Basse-Normandie, France	Asymptomatic	
Cat-21	October 2003	Paris, France	FIP	
Cat-22	March 2009	Basse-Normandie, France	Asymptomatic	Yes
Cat-24	November 2003	Paris, France	FIP	
Cat-29	March 2009	Basse-Normandie, France	Asymptomatic	
Cat-30	March 2009	Basse-Normandie, France	Asymptomatic	
Cat-33	April 2004	Paris, France	Asymptomatic	
Cat-41	March 2005	Paris, France	FIP	
Cat-57	June 2006	Paris, France	Asymptomatic	Yes
Cat-61	June 2005	Paris, France	Asymptomatic	Yes
Cat-74	November 2005	Paris, France	FIP	
Cat-81	January 2006	Paris, France	Asymptomatic	Yes
Cat-118	November 2006	Paris, France	FIP	
Cat-122	April 2006	Paris, France	Asymptomatic	
Cat-125	December 2006	Paris, France	Asymptomatic	
Cat-127	December 2006	Paris, France	Asymptomatic	
Cat-130	December 2006	Paris, France	Asymptomatic	Yes
Cat-139	January 2007	Paris, France	Asymptomatic	
Cat-149	February 2007	Paris, France	Asymptomatic	
Cat-163	July 2007	Iasi, Romania	FIP	
Cat-179	December 2008	Paris, France	Asymptomatic	Yes
Cat-180	January 2009	Paris, France	FIP	
Cat-181	February 2009	Paris, France	FIP	
Cat-196	September 2009	Paris, France	FIP	
Cat-198	September 2009	Iasi, Romania	FIP	

CHARACTERIZATION OF DIFFERENT FORMS OF THE ACCESSORY GP3

CANINE CORONAVIRUS TYPE I PROTEIN IDENTIFIED IN CATS

The ORF3 gene is unique to CCoV-I genotype and atypical FCoV strains described above. A previous study of the ORF3 encoded protein was performed through an *in vitro* translation assay by Lorusso et al. The study demonstrated that gp3 is an N-glycosylated protein, probably secreted as no transmembrane domain was identified. Various functions have been assigned to CoV accessory proteins and some are involved in tissue or host switch. In light of our past study, with the recovery of atypical feline strains harbouring truncated forms of the ORF3 gene, we decided to further analyse the different encoded gp3 glycoproteins, in a host-cell context: the wild-type gp3, recovered from a dog sample, gp3- Δ 1, retrieved from a cat sample and displaying one deletion in its C-terminus part and gp3- Δ 2, also recovered from a cat sample and displaying the C-terminus part deletion, as well as an additional nine amino acids deletion. All proteins retain a predicted signal peptide, a glycosylation site and are predicted to be secreted.

As the CCoV-I strain does not replicate in laboratory cell lines, nor the atypical FCoV strain, expression plasmids containing optimised sequences of the different ORF3 genes, under the control of a CMV promoter and a 3x-Flag tag in frame at their 3' ends were constructed. These plasmids were transfected either in canine A72 cells or in feline CrFK cells and expression of the different proteins were analysed.

In this second study, we have made the following observations:

- Despite the exhibited deletions, all gp3 proteins share the same post-translational process: all bear a cleavable signal sequence and possess a high mannose sugar, specific of proteins residing in the ER and assemble into covalent multimers.

- All three proteins localise in the same subcellular domain, the ER, regardless of the cell line species, canine or feline and in the absence of a specific retention signal.
- Gp3, gp3- Δ 1 and gp3- Δ 2 display similar expression levels in A72 canine cells, whereas in CrFK feline cells, only gp3- Δ 1 sustains a visible expression level, about five-fold higher than gp3 and ten-fold higher than gp3- Δ 2.
- In the absence of CCoV-I and atypical FCoV laboratory strains, A72 cells expressing gp3, gp3- Δ 1 and gp3- Δ 2 were infected with type II CCoV. No impact of these proteins on the CCoV-II viral life cycle was found.

Comparison of the different gp3 proteins lead to the conclusion that the N-terminus part contains determinants of the main features: oligomerization, glycosylation, determinants of retention in the ER. The C-terminus part influences its proper expression in feline host as well as the stretch of nine amino acids, between Lys⁸⁸ and Phe⁹⁶. Functions of gp3 and its different variants remain enigmatic. The absence of effect on the viral production in CCoV-II infected cells suggests that these proteins confer a selective advantage only to CCoV-I and the atypical FCoV strains, which contain the ORF3 genes.

Characterization of different forms of the accessory gp3 canine coronavirus type I protein identified in cats

Anne-Laure Pham-Hung d'Alexandry d'Orengiani^{1,2,3}, Lidia Duarte^{1,2,3}, Nicole Pavio^{1,2,3},
Sophie Le Poder^{1,2,3*}

¹ Université Paris-Est, Ecole Nationale Vétérinaire d'Alfort, UMR 1161 Virology, Maisons-Alfort, France.

² INRA, UMR 1161 Virology, Maisons-Alfort, France

³ ANSES, Animal Health Laboratory, UMR 1161 Virology, Maisons-Alfort, France

* corresponding author. Tel.: +33 1 43 96 73 25; fax: +33 1 43 96 73 96. E-mail address:
slepoder@vet-alfort.fr

Key words: coronavirus, accessory protein, species barrier, gp3

Abstract:

ORF3 is a supplemental open reading frame coding for an accessory glycoprotein gp3 of unknown function, only present in genotype I canine strain (CCoV-I) and some atypical feline FCoV strains. In these latter hosts, the ORF3 gene systematically displays one or two identical deletions leading to the synthesis of truncated proteins gp3- Δ 1 and gp3- Δ 2. Studies in canine and feline cells demonstrate that these different gp3 proteins oligomerize through covalent bonds, are N-glycosylated and are maintained in the ER, without any specific retention signal. However, deletions influence their level of expression. In canine cells, all proteins are properly expressed whereas in feline cells, only gp3- Δ 1 retains a similar level of expression. None of the gp3 proteins modulate the viral replication cycle of genotype II CCoV in canine cell line, leading to the conclusion that the gp3 proteins are probably advantageous only for CCoV-I and atypical FCoV strains.

Introduction

The genome of the *Coronaviridae* (CoV) family is a large single-stranded and positive-sense RNA molecule of about 30 kDa (Gorbalenya et al., 2006). For all CoVs, the first two thirds of the genome encode non-structural proteins which are essentially involved in the CoV replication and transcription machinery (Bredenbeek et al., 1990). The rest of the genome comprises genes of the structural proteins S (spike), E (envelope), M (membrane), N (nucleocapsid) and eventually HE (hemagglutinin-esterase)(de Groot, 2006). Additional genes named accessory genes are interspaced between genes encoding the structural proteins. The number and position of these genes vary from one CoV to another. With eight accessory genes, SARS-CoV possesses the highest number of them, whereas hCoV-229E contains only one (Dijkman et al., 2006).

Feline CoV (FCoV) and canine CoV (CCoV) are common pathogens of cat and dog populations, sometimes leading to fatal diseases (Pedersen, 2009)(Decaro and Buonavoglia, 2008). CCoVs are separated into two genetic clusters, CCoV-I and CCoV-II, according to the S gene sequence. One accessory gene, named ORF3 is a singularity of the CCoV genotype I (CCoV-I). Its sequence is highly conserved among CCoV-I, suggesting its importance in some aspects of the viral infection. It is the only CCoV accessory gene which has been investigated so far. *In vitro* translation assays demonstrated that ORF3 encodes an N-glycosylated protein of 28kDa, named gp3 (Lorusso et al., 2008). Still, the function of gp3 remains unknown. Our recent phylogenetic studies conducted on the characterisation of CoV strains infecting cats lead to the discovery of atypical FCoV strains harbouring a feline S gene and downstream genes genetically related to CCoV-I. Sequences of the ORF3 gene were also present in these strains but displayed deletions never described so far. All ORF3 sequences from atypical FCoV strains shared a deletion of 29-nt, which introduced a stop codon and another frequently observed deletion of 27-nt deleted the predicted protein of nine additional

a.a (Le Poder et al., 2013). The complete gp3 protein of CCoV-I is of 207 a.a. in length, whereas the deleted forms present in atypical FCoV strains are predicted to produce either a deleted protein, named gp3-Δ1 of 158 a.a. or another form, named gp3-Δ2, of 149 a.a. In comparison with gp3, gp3-Δ1 and gp3-Δ2 lost the C-terminus part from the cysteine residue in position 157 onward. Gp3-Δ2 is further truncated of 9 a.a. between Lys⁸⁸ and Phe⁹⁶. All proteins maintain a predicted signal peptide at the N-terminus extremity and a putative glycosylation site on Asn¹¹⁶. No other particular functional domain was predicted by *in silico* analysis.

Coronaviruses are well known for crossing the species barrier, sometimes leading to the emergence of new pathogens, like the SARS-CoV or the MERS-CoV. Adaptation to a new host is often accompanied by modifications of some accessory proteins, among others (McBride and Fielding, 2012). With the aim of understanding the role of the gp3 deletions in the adaptation of atypical FCoVs to the feline species, we characterised the basic properties of the different forms of gp3 in both feline and canine cells. The absence of culture-cell adapted strains of CCoV-I and of the newly discovered atypical FCoV strains impaired studies of the different gp3 proteins in cells culture infected with these specific CoVs. Through transient transfection of plasmids expressing the different proteins, we demonstrated that the gp3 deletions influence their expression. The complete gp3 protein is expressed only in canine cells, gp3-Δ1 is expressed at a high level in both canine and feline cells and gp3-Δ2 is faintly observed in feline cell lines. In contrast, all proteins assemble into covalent oligomers and are maintained in the endoplasmic reticulum (ER) despite cleavage of their peptide signal and absence of a known specific retention signal. None of the gp3 proteins influence the viral production of CCoV-II strains.

Results

Expression of gp3, gp3- Δ 1 and gp3- Δ 2 in A72 and CrFK cells

The ORF3 genes were cloned into expression plasmids under the control of the CMV promoter with a 3x-Flag tag fused in frame at the 3' end of ORF3 (Figure 1). Each plasmid was transfected either in A72 canine cells or in CrFK feline cells. Both cell lines were chosen for their susceptibility to CCoV-II and FCoV-II infections. At 24h post-transfection, cells were lysed and proteins were detected by immunoblotting with an anti-Flag antibody.

In A72 cells, all three proteins, gp3, gp3- Δ 1 and gp3- Δ 2 are detected. They migrated according to their predicted molecular weight at 28, 22 and 21 kDa, respectively (Figure 2A).

In CrFK cells, expression of gp3 and gp3- Δ 2 was low, whereas gp3- Δ 1 was still detected (Figure 2A). Quantification demonstrates that expression of the gp3- Δ 1 is about five-fold higher than gp3 and ten-fold more than gp3- Δ 2 (Figure 2B). In A72 cells, differences of expression levels between the three proteins are less pronounced. Quantity of gp3- Δ 1 is only two-fold more elevated than gp3 and gp3- Δ 2.

As gp3 possesses six cysteine residues while gp3- Δ 1 and gp3- Δ 2 contain only four, we investigated the possible formation of covalent homo-oligomers. At 24h post-transfection, cell lysates were treated and migrated onto SDS-PAGE gels with or without β -mercaptoethanol. Under non-reducing conditions, supra forms of approximately twice the molecular weight of each protein were detected. After treatment with β -mercaptoethanol, the monomeric forms were mainly observed, suggesting the assembly of gp3, gp3- Δ 1 and gp3- Δ 2 into dimeric complexes through disulfide bonds (Figure 2C). For gp3- Δ 1, higher order assemblies were observed, which could correspond to tetramers.

Subcellular localisation of the proteins

Next, the subcellular localisation of the proteins was examined by colocalisation assays of gp3 proteins with different markers of the cellular compartments in both A72 and CrFK. 24h post-transfection, dual labelling were carried out with anti-Flag antibody to visualise the gp3 proteins and specific antibodies directed against calnexin, a resident protein of the ER, or giantin, a marker of the Golgi apparatus. By apotome microscopy analyses, the fluorescence signals of the gp3 proteins and the giantin were distinct in both A72 and CrFK, indicating that the proteins had not reached the Golgi apparatus (data not shown). In contrast, the gp3 proteins colocalised with calnexin signals in canine and feline cell lines (Figures 3A and 3B).

Post-translational maturation of the proteins

The gp3 proteins bear a putative glycosylation site at the Asn¹¹⁶ position. This amino acid is conserved in all three proteins. In order to characterize the glycan, assays with glycosidases were performed. At 24h post-transfection, cell lysates were incubated either with PNGase F or Endo H or mock treated. PNGase F removes all N-linked glycans whereas Endo H cleaves only polymannose carbohydrates that have not been further processed into complex sugar. Gp3, gp3- Δ 1 and gp3- Δ 2 are all sensitive to PNGase F (data not shown) and to Endo H (Figure 4A). These results show that the different gp3 proteins are glycosylated with a high mannose glycan. The same results were obtained in both A72 and CrFK cell lines (data not shown).

In silico analyses indicated an N-terminal hydrophobic domain functioning as signal sequence cleavable after the alanine amino acid in position 15. To test whether this peptide signal is cleaved, mutants without the predicted signal peptide for each protein (Δ SP-gp3, Δ SP-gp3- Δ 1, Δ SP-gp3- Δ 2) were constructed. Mutants with the cleavage site replaced by five Leucine residues (5L-gp3, 5L-gp3- Δ 1, 5L-gp3- Δ 2) were also constructed in order to impair the potential cleavage of the signal peptide. At 24h after transfection, cells lysates were collected,

treated or not with Endo H and analysed by immunoblotting. All Δ SP proteins migrated faster than the wild type proteins. The estimate apparent molecular weight of the Δ SP proteins was about 3 kDa less than the native proteins. Without a signal peptide, proteins cannot enter the ER and therefore cannot become glycosylated, as confirmed by the same electrophoretic profile of Δ SP proteins treated or not by EndoH. 5L gp3- Δ 1 and gp3- Δ 2 proteins which harbour an uncleavable signal peptide display a slower migration and an apparent molecular weight of approximately 3 kDa higher than the wild type proteins. Some unglycosylated forms are detected as shown by comparison with lysates treated with Endo H. 5L-gp3 migrated faster than the wild-type protein.

CCoV-II infection in cells expressing the different gp3 proteins

In the absence of laboratory CoV strains harbouring ORF3, the impact of the different gp3 proteins during the viral life cycle was investigated by inoculating cells with CCoV-II strain, not bearing ORF3, onto gp3, gp3- Δ 1, or gp3- Δ 2 transfected cells. This study was conducted in A72 cell lines in which the expression of the different gp3s is the best. Twelve hours after transfection with the empty pCDNA3.1 plasmid or plasmids encoding gp3, gp3- Δ 1, or gp3- Δ 2, A72 were infected at a MOI of 10 with the CCoV-II 1-71 isolate. A72 cells were also infected at an MOI of 10, without prior transfection. At 24h, 48h and 72h post-infection, supernatants and cells were collected and analysed by quantitative RT-PCR. Quantity of viral copy genome in cells supernatant (Figure 5) and cellular extracts (data not shown) was calculated. No difference was observed between infected cells and infected cells expressing the different gp3 proteins.

Discussion

The presence of an additional accessory gene, ORF3 is specific to CCoV-I. In CCoV-II and FCoV-II, only very short sequences, less than 71-nt and corresponding to ORF3, are present and were interpreted by Lorusso *et al.* as residual sequences from a CoV ancestor. In FCoV-I, no nucleotide corresponding to ORF3 has been described until our recent study, in which the ORF3 gene was identified in atypical feline CoV strains harbouring an N gene related to CCoV-I and an S gene close to FCoV-I (Le Poder *et al.*, 2013). In these atypical feline strains, the ORF3s are 595-nt or 568-nt in length and are preceded by specific transcription-regulating sequences, suggesting the possible translation into functional proteins during infections.

All ORF3 sequences recovered in these feline CoV strains were deleted the same way, leading either to a putative gp3- Δ 1 protein, comprising the first 158 a. a. of gp3 or to gp3- Δ 2 protein, with an additional 9 a. a. deletion compared to gp3- Δ 1 (Le Poder *et al.*, 2013). A previous study explored the biochemical properties of the complete canine gp3 protein using an *in vitro* translation system and concluded that gp3 is a probably secreted, soluble glycoprotein of 28kDa (Lorusso *et al.*, 2008). Here, the expression of the different gp3, gp3- Δ 1 and gp3- Δ 2 proteins was analysed in cell culture. Proteins were expressed either in canine or feline cells in accordance to the host tropism from which they were derived. The level of expression is different not only between the two cell lines used, but also between the different proteins. In canine A72 cells, the three proteins are expressed at approximately the same level, but in feline cells, discrepancies between them are very marked. Expression of gp3- Δ 1 is five-fold higher than gp3 and gp3- Δ 2 (Figure 2B). Whether these different expressions levels are due to different degradation processes or modulations at the translation step remain to be explored. Expression levels might be influenced by cellular factors involved in regulating protein translation. Interestingly, the gp3 is expressed in canine cells but not in feline cells which may correlate with the canine virus origin of gp3.

Beside these discrepancies, the proteins share common biochemical properties. In *in vitro* translation assay, gp3 was expressed in a monomeric form (Lorusso et al., 2008). Here, in cell culture, we demonstrated that gp3, but also gp3- Δ 1 and gp3- Δ 2 oligomerize in, at least, covalent homo-dimers and probably in higher multimeric forms, as shown for gp3- Δ 1 (Figure 2C). The absence of detection of higher multimeric form other than dimers for gp3 and gp3- Δ 2 could be due to their fainter expression. The oligomerization status of CoV accessory proteins has been investigated for some of them. Multimerization has also been demonstrated for the accessory ORF3 from PEDV (Wang et al., 2012), ORF4a from HCoV-229E (Zhang et al., 2014) or ORF8ab from SARS-CoV (Oostra et al., 2007).

Post-translational modifications are also common to the three proteins in the studied cell lines. *In vitro* translation indicated that gp3 contains an N-linked glycosylation site (Lorusso et al., 2008). In cell culture and through the use of different deglycosidases, we were able to identify the glycan residue as a high polymannose (Figure 4A), which corroborates with their localisation in the ER (Figure 3). Indeed, maturation into complex sugar needs processing by enzymes from the Golgi apparatus and presence of a polymannose is characteristic of proteins residing in the ER. It is still unclear how the gp3 proteins are maintained in the ER, as no peculiar signal for ER retention has been identified (Gao et al., 2014). Retention of the gp3 proteins in the ER could have been explained by a non-cleavage of the signal peptide. This hypothesis was tested by molecular mass comparison between native gp3 proteins and proteins either deleted of the signal peptide or harbouring a non-cleavable signal peptide (Figure 4B). The migration difference between 5L mutants and native proteins strongly suggest an efficient cleavage of the signal peptide in native gp3 proteins. Indeed, 5L-gp3- Δ 1 and 5L-gp3- Δ 2 have a molecular mass higher than the native gp3- Δ 1 and gp3- Δ 2. The 5L-gp3 mutant migrated at a lower molecular mass than the native gp3 but Lorusso *et al.* had already observed that the presence of the signal peptide in gp3 induced an aberrant faster migration

similar to our observation herein. Thus, retention of the gp3 proteins is not due to a default in the signal peptide cleavage. Alternatively, the gp3 proteins could be maintained in the ER through interactions with cellular factors, as suggested by studies on the ORF8ab protein of the SARS-CoV (Oostra et al., 2007).

From the comparative study of the complete gp3 and the deleted mutant gp3- Δ 1 and Δ 2, we can deduce that the first 158 amino acids contain the necessary signals for the basic features of the proteins: the cysteine residues for oligomerization, the N-glycosylation site and the unknown retention determinant in the ER. These conserved features might be important for their function.

In the absence of laboratory strains harbouring ORF3 gene, *i.e.* CCoV-I strains or the atypical FCoV strains, the only way to study the different gp3 proteins in virus infected cells was to infect transfected cells with a CoV strain that does not encode gp3. As the gp3 proteins are better expressed in canine A72 cells rather than in feline CrFK cells, the impact of gp3 on viral replication efficiency was investigated in transfected A72 infected by a CCoV-II virus. In this context, the viral production is similar in cells expressing gp3 in comparison with the empty plasmid transfected cells or only infected cells (Figure 5). As gp3 is present only in CCoV-I strain and deleted gp3 in some atypical FCoV strains, it is plausible that the function of these proteins is crucial only for these particular CCoV and FCoV and thus could not be observed in the model developed herein. However, most accessory proteins have no effect on viral life cycle in *in vitro* assays (Hodgson et al., 2006) (Haijema et al., 2004), except those acting as viroporins like ORF3 of PEDV (Wang et al., 2012), ORF3a of SARS-CoV (Chan et al., 2009) and ORF4a of HCoV-229E (Zhang et al., 2014). However, their persistence in the viral genome suggests their biological importance *in vivo*. Yet, recent studies proved that accessory proteins might have a wide range of functions including modulation of viral pathogenicity like ORF7 of TGEV, which absence increases cell RNA degradation and viral

pathogenicity in infected piglets (Cruz et al., 2011). Others act as counteragents of the innate immunity like ORF7a of FCoV or ORF6 of SARS-CoV to name a few (Dedeurwaerder et al., 2014) (Frieman et al., 2007). Some others are crucial in viral tropism. As an example, ORF3c of FCoV is necessary for the confinement of the feline virus in the intestinal tract, whereas FCoV without this gene disperses through viremia in the feline host (Bálint et al., 2014). During host change, mutations of accessory proteins have also been observed, suggesting a possible role in adaptation of CoV to their host. Thus, during the adaptation of CCoV-II to swine, leading to the emergence of TGEV, deletion of ORF7b and ORF3b occurred (Decaro et al., 2007). In the same manner, whereas SARS-CoV strains infecting bats and civets harbour an intact ORF8ab gene, a 29-nt deletion is systematically observed in strains infecting humans, leading to the translation of two novel proteins 8a and 8b (Guan et al., 2003). Gp3 has many common features with ORF8ab. Both accessory proteins oligomerize through disulfide bonds and are maintained in the ER without any specific retention signal. The ER is a crucial cellular compartment for folding and maturation of novel synthesized proteins. ORF8ab has been demonstrated to facilitate protein folding by up-regulating the activating transcription factor 6 (ATF6) (Sung et al., 2009) . Whether gp3 could also facilitate viral protein synthesis needs to be further investigated, by studying its impact on the ER protein translation and regulation activities. Deletions in ORF8ab lead to two new putative proteins ORF8a and ORF8b which are unstable in mammalian cells and with different subcellular localisation compared to ORF8ab. In contrast, deleted forms of gp3 conserve the same basic features and could have a similar function than intact gp3.

In concordance with other studies, our data underline the importance to investigate the role of CoV accessory proteins during the viral adaptation to new hosts. In the context of emergence of high virulent CoVs such as SARS-CoV or MERS-CoV arising from animals, it remains

important to understand the mechanisms of CoV pathogenicity and cross-species transmissions.

Materials and Methods

Cell culture and transient transfection

Canine A72 and feline CrFK cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Life Technologies) supplemented with 10% foetal bovine serum, 1% penicillin-streptomycin solution, 1% sodium-pyruvate and 1% non-essential amino acids.

Subconfluent A72 and CrFK cells were transfected in 6-well plates with 2.5 µg DNA plasmid using TransIT-LTI transfection reagent (Mirus), following the manufacturer's instructions.

Plasmids

Optimised codon sequences of the complete ORF3 gene (GenBank accession numbers JN714200), and the two deleted gene forms (GenBank accession numbers JN714196 and JN714199) were synthesized in frame with the 3xFlag tag into pCDNA3.1 plasmids (Invitrogen) between restriction site BamHI and NotI, by Proteogenix (ProteoGenix SAS-France).

Mutant plasmids with deletion of the signal peptide of the ORF3 genes were subsequently generated from each parental plasmid. Briefly, plasmids consisting of residues 15 to the stop codon, were obtained by PCR assays with the forward primers (Δ SP-ORF3 F1: 5'-GGACTAGTGGATCCATGCACCCCTTTCACG-3', Δ SP-ORF3- Δ 1/ Δ 2 F1: 5'-TCCGCCGCCACCATGCTCCACCCCTTTCAC-3') and reverse primers (Δ SP-ORF3 R1: 5'-CGTGAAAGGGGTGCATGGATCCACTAGTCC-3', Δ SP-ORF- Δ 1/ Δ 2 R1: 5'-GTGAAAGGGGTGGAGCATGGTGGCGGCGGA-3'). To create gp3 proteins with uncleaved signal sequence, the amino acids critical for cleavage were changed into five Leucine residues by PCR assays using the following primers (5L-ORF3 F2: 5'-ATCAGTGTCGTCCTTTTGTATTACTCCACCCCTTT-3', 5L-ORF3 R2: 5'-

AAAGGGGTGGAGTAATAACAAAAGAAGGACACTGAT-3', 5L-ORF3-Δ1/Δ2 F2: 5'-ATGATTAGTGTGGTCCTTTTGTATTACACCCCTTTCAC-3', 5L-ORF3-Δ1/Δ2 R2: 5'-CGTGAAAGGGGTGTAATAACAAAAGGACCACACTAAT CAT-3'). PCR products were submitted to digestion by DpnI enzyme (New England Biolabs) during 1 hour at 37°C to eliminate the parental plasmid. Library Efficiency DH5α bacteria (Invitrogen) were transformed with the digestion products according to the manufacturer's recommendations.

Immunoblotting

At 24h post-transfection, cells were lysed with RIPA extraction buffer (20mM Tris-HCl, pH7.5, 0.2% Nonidet P-40, 150mM NaCl, 0.5 mM EGTA, 1.5 mM MgCl₂, 10% Glycerol) supplemented with Complete Protease Inhibitor Cocktail Tablet (Roche). Samples containing 100μg of total proteins were then mixed with an equal volume of 5X Laemli buffer and heated at 95°C for 5 min. Where mentioned, 10% of β-Mercaptoethanol was added to cell lysates. Proteins were separated on sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transferred onto a nitrocellulose membrane (GE Healthcare). After saturation with 5% milk in PBS-Tween (0.01%) buffer (PBST), the membranes were incubated with anti-Flag monoclonal antibody (Sigma-Aldrich) diluted at 1/5000 or anti-actin monoclonal antibody (Sigma-Aldrich) diluted at 1/2000 in PBST-milk buffer overnight at 4°C. The membranes were washed three times in PBST buffer and incubated for 1h at room temperature with horseradish peroxidase-conjugated anti-mouse antibody (Roche) diluted at 1/5000 in PBST-milk buffer. After three washes, blots were revealed using enhanced chemiluminescence (ECL) reagents (Pierce) and analysed with a Fusion imaging system (Bio-Rad). Quantification was performed by using the Bio-1D software.

For glycosidase assays, 20 μl of cell lysates were mixed with 1μl of either peptide-N-glycosidase F (PNGase F) or endoglycosidase H (Endo H) (Biolabs), and 3μL of 10X Glycoprotein denaturing buffer (Biolabs) prior to incubation at 37°C for 3h.

Indirect immunofluorescence microscopy

A72 and CrFK were transfected with expression plasmids of the different gp3 proteins. At 24h post-transfection, cells were washed with PBS and fixed with 4% paraformaldehyde in PBS-4% sucrose (Life Technologies). After two washes in PBS, a solution of 50mM NH₄Cl was added for 10 min at room temperature, then fixed cells were incubated with PBS-0.5% Bovine Serum Albumin (BSA) for 30 min. After one wash with PBS-1% BSA and two washes with PBS-1%BSA-0.5% saponine, a mix of primary antibody raised against the Flag tag (Sigma-Aldrich) and either antibody specific of the calnexin (Sigma-Aldrich) or the giantin (Abcam) at dilution of 1/500 in PBS-BSA-Saponine solution was added for 1h at room temperature. After three washes, cells were incubated with a mix of anti-mouse Alexa 488 and anti-rabbit Alexa 555 secondary antibodies (Invitrogen) at working dilutions of 1/600 in PBS-BSA-Saponine solution for 45min. After three washes with PBS, cells were covered with a solution of Mowiol mixed with DAPI for staining of the nucleus. Slides were examined at 63x magnification by apotome microscope (Zeiss) and images were analysed using the Axiovision software.

Infections

For infection assays, following 12 hours post-transfection as described above, subconfluent A72 cells were inoculated with type II strain CCoV-1-71, from the American Type Culture Collection (ATCC®VR-809™), at a multiplicity of infection (MOI) of 10. Cells were then incubated for 120 min. at 37°C, with gentle rocking for optimal viral adsorption. Inoculum was then replaced by standard fresh medium, supplemented with 10% FBS. Cells were left to incubate for specified amount of time. In parallel, infection of non-transfected cells using the same protocol was conducted.

Quantitative RT PCR

Viral RNA was extracted from supernatants using the QIAamp Viral RNA Mini kit (QIAGEN). RNA was reverse transcribed and amplicons were generated using the Quantitect SYBR Green RT-PCR kit (QIAGEN) and the following primers set: CCoV-7F: 5'-GGCAACCCGATGTTTAAACTGG-3' and CCoV-7R: 5'-CACTAGATCCAGACGTTAGCTC-3', following the manufacturer's instructions at an annealing step of 52°C. These primers target the accessory gene 7b of type II CCoV.

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Figures Legends

Fig.1. Schematic representation of the different gp3 proteins. Gp3 is the full length protein encoded by CCoV-I, gp3- Δ 1 and gp3- Δ 2 arise from atypical FCoV and are deleted of the C-terminus part in comparison with gp3. A deletion of 9 amino acids differs between gp3- Δ 1 and gp3- Δ 2. The potential signal peptide and N-glycosylation site are indicated by a light grey box and a star, respectively. Length of the different proteins is indicated by the number of amino acids at the C-terminus.

Fig.2. Protein expression in canine and feline cell types. **(A)** Detection of the proteins in transfected canine A72 and feline CrFK cells by immunoblotting assays. Cells were transfected with the different gp3 expression plasmids or with an empty pCDNA vector (Control). At 24h post-transfection, cells were lysed and processed onto SDS-PAGE gels for immunoblotting. Gp3 proteins were detected using an anti-Flag antibody. Cellular β -actin was revealed in parallel by a specific monoclonal anti-actin antibody. **(B)** Levels of expression of the different gp3 proteins. Immunoblots of the different gp3 proteins were quantified using the Bio-1D software. Quantity of gp3 was arbitrary set to 1 and the relative quantity of gp3- Δ 1 and gp3- Δ 2 are expressed in comparison with gp3. All data are representative of three independent experiments. Error bars represent standard deviations. **(C)** Multimerisation of the

gp3 proteins. A72 and CrFK cells were transfected with the empty vector pCDNA (Control) or plasmids encoding the different gp3 proteins and lysed at 24h post-transfection. Cell lysates were treated with (+) or without (-) β -Mercaptoethanol (β -ME), prior to migration onto SDS-PAGE gels. Proteins were detected by immunoblotting with anti-Flag antibody.

Positions and masses (in kDa) of the molecular mass protein markers are indicated on the left.

Fig.3. Subcellular localisation of gp3, gp3- Δ 1 and gp3- Δ 2. A72 cells (**A**) and CrFK cells (**B**) were transfected with an empty pCDNA plasmid (Control) or plasmids encoding the different gp3 proteins. At 24h post-transfection, cells were dual labelled with antibodies against the Flag tag (α -Flag) and against calnexin (α -calnexin), a marker of the ER. Alexa-488 anti-mouse and alexa-555 anti-rabbit were used as secondary antibodies. Cell nuclei were counterstained with DAPI. All slides were observed with a 63X oil immersion objective lens by an apotome microscope (Zeiss). At the right, a merged image of the anti-Flag, the anti-calnexin and the DAPI signal is shown. Bar chart: 50 μ m.

Fig.4. Processing of the gp3 proteins. (**A**) Maturation of the N-linked oligosaccharides. A72 cells were transfected with plasmids containing the indicated sequences and lysed at 24h post-transfection. 20 μ L of whole cell lysates were then treated (+) with Endo H or mock treated (-). After migration on SDS-Page gels, gp3 proteins were revealed by immunoblotting with the anti-Flag antibody. (**B**) Cleavage of the signal peptide of the gp3 proteins. A72 cells were transfected with expression plasmids containing different constructs of the gp3 proteins. Δ SP-gp3, Δ SP-gp3- Δ 1 and Δ SP-gp3- Δ 2 are deleted of the first 14 N-terminal amino acids. In 5L-gp3, 5L-gp3- Δ 1 and 5L-gp3- Δ 2 constructs, the putative cleavage site was replaced by five Leucine residues, to prevent signal peptide cleavage. At 24h post-transfection, cell lysates were recovered and treated with (+) or without (-) Endo H treatment, prior to migration. The different proteins were detected by immunoblotting with an anti-Flag antibody.

Positions and masses (in kDa) of the protein markers are indicated on the left.

Fig.5. Infection of transfected A72 cells with CCoV-II. A72 cells were transfected with plasmids containing the indicated sequences and infected 12h post-transfection with the CCoV-II 1-71 strain. Supernatant was collected at 24h, 48h and 72h post-infection and processed for viral genome quantification by quantitative RT-PCR. Means from triplicate experiments are given. Error bars represent standard deviations.

Fig.1.

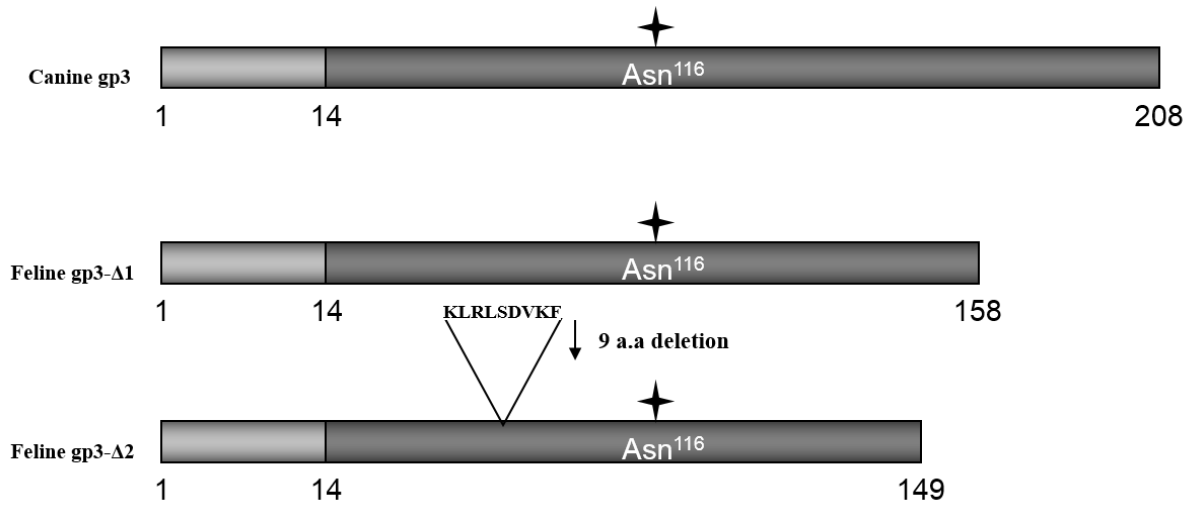
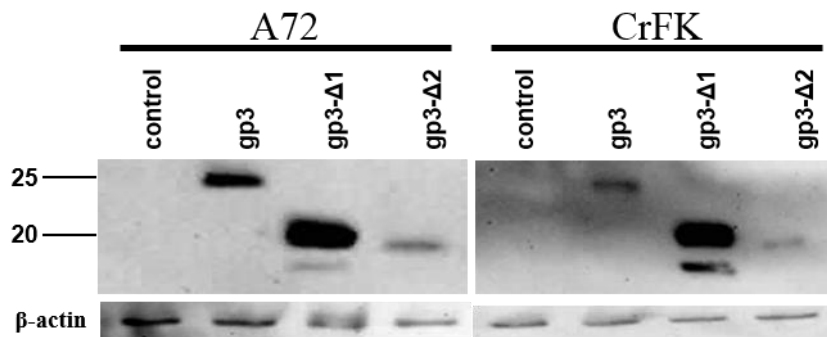
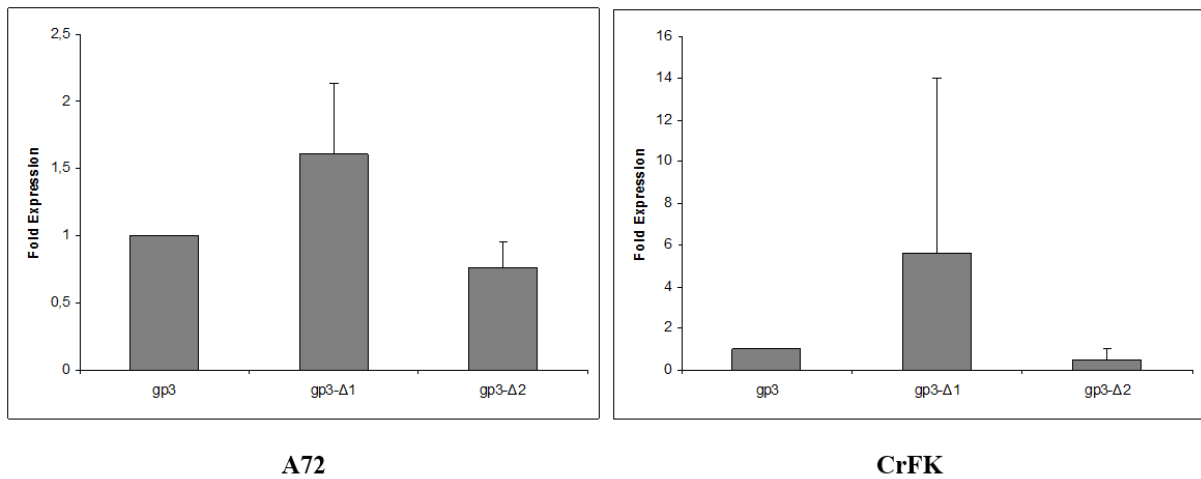


Fig.2.

A



B



C

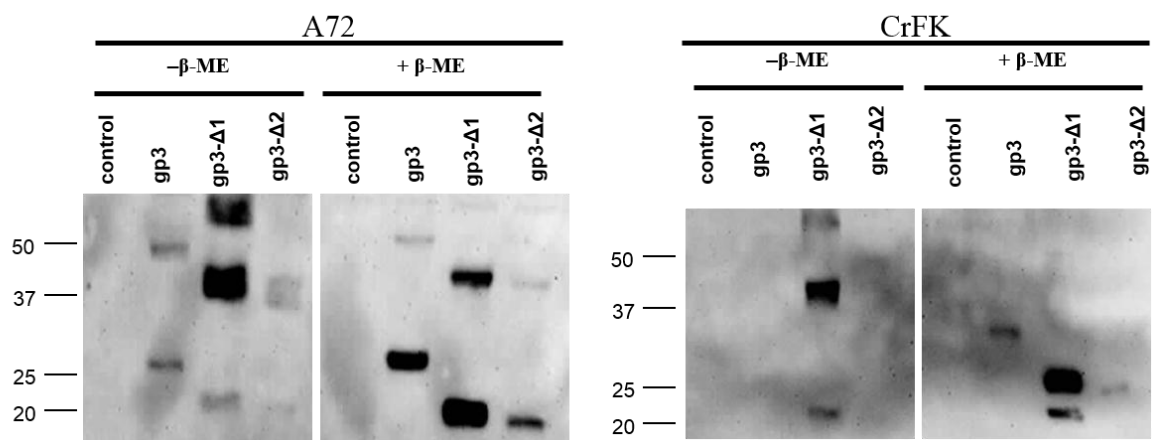


Fig.3.

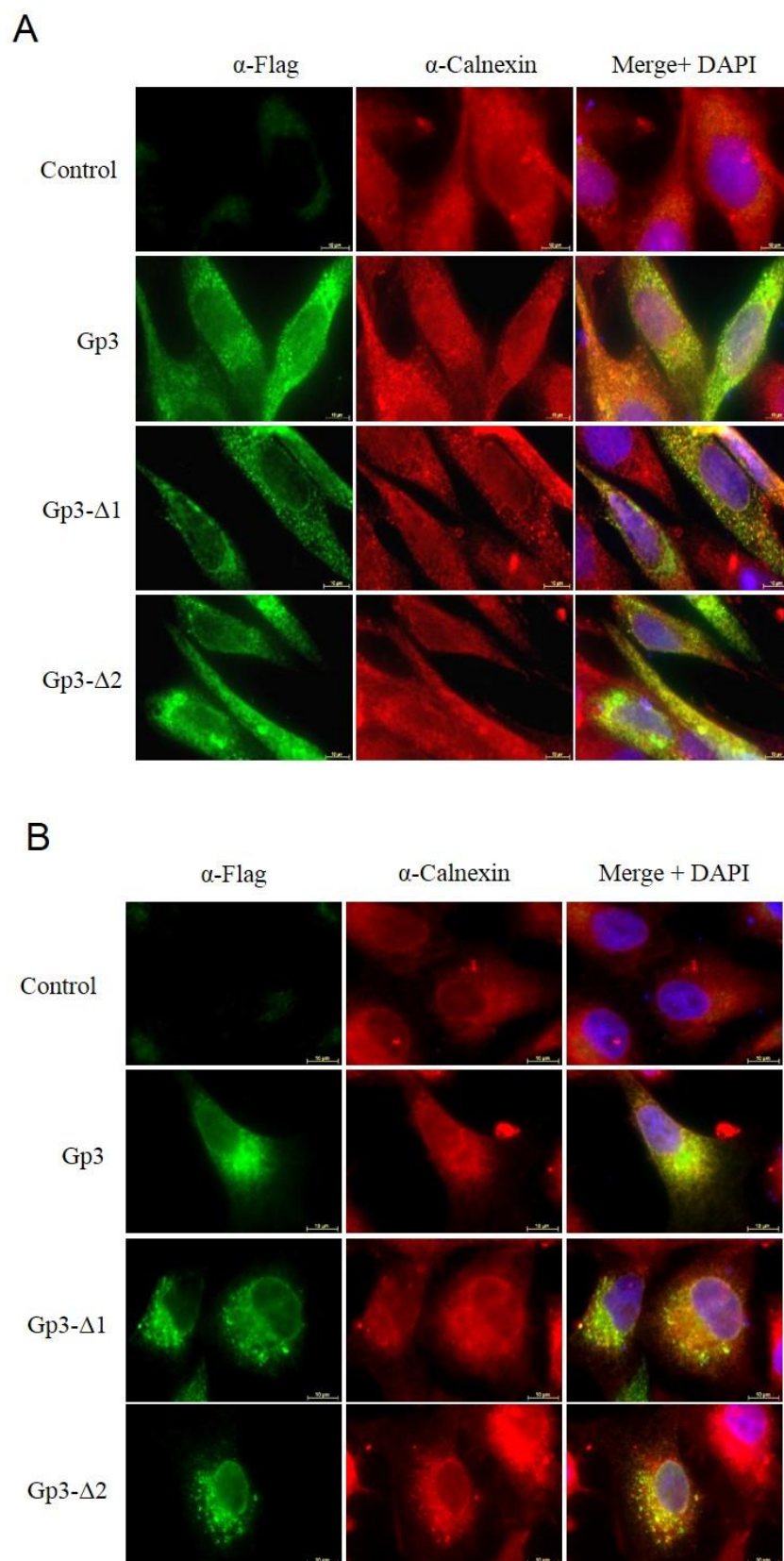


Fig.4.

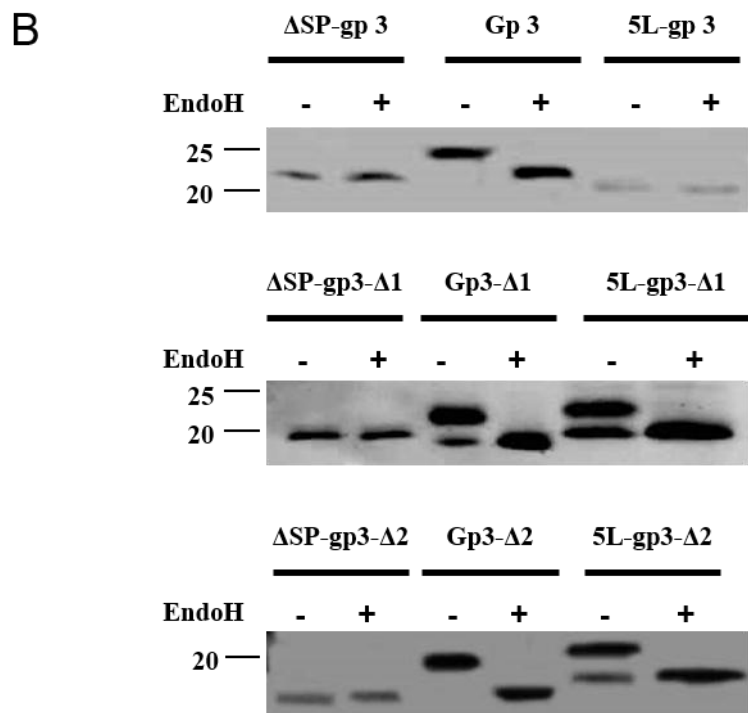
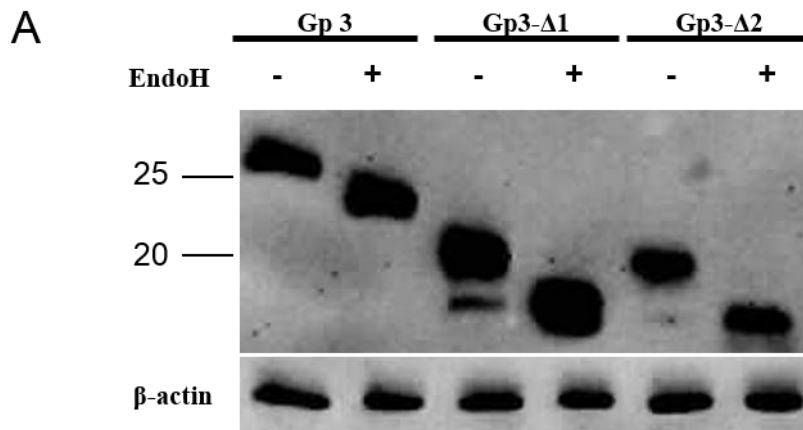
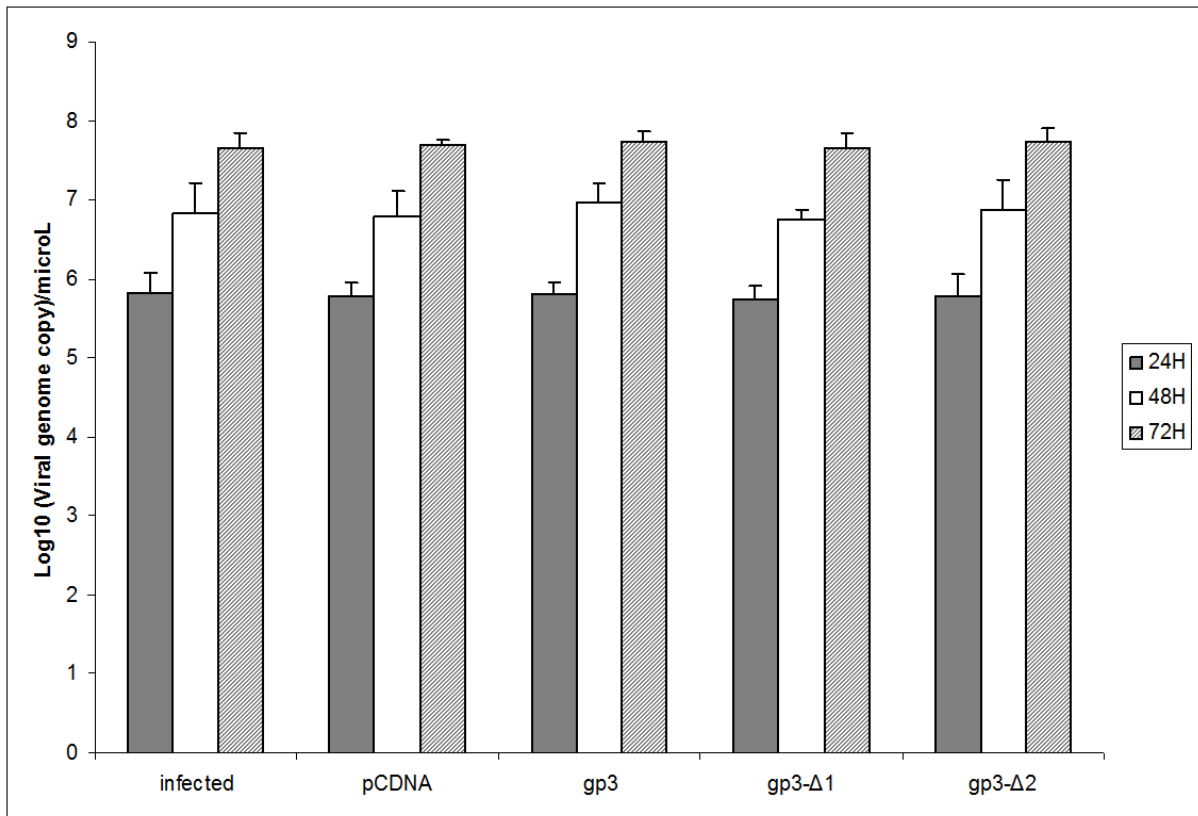


Fig.5.



DISCUSSION

I. Infections of cats by strains harbouring the CCoV-I ORF3 gene

Accumulated data of genetic characterization of FCoV and CCoV strains in the field led to the discovery of numerous different genotypes arising from recombination events between strains of different species origin as FCoV-II strains (Herrewegh et al., 1998) and CCoV-IIb (Decaro et al., 2010). Cats and dogs are the most common companion animals of developed countries and France has the highest cats and dogs populations in Europe. Moreover, both species often cohabitate under the same roof, which could favour host-jump of CoV from one species to another. Some indications suggest that cats may be more specifically susceptible to infection by non-feline coronavirus. First, the feline APN has the unique property to serve as receptor for CCoV-II, TGEV, and even the human HCoV-229E. Secondly, with an S gene of more than 80% of homology with FCoV-I, infection of cats by CCoV-I is plausible. For these reasons, we conducted a phylogenetic study in naturally infected cats with the aim of investigating the diversity of CoV strains in these animals.

Eighty-eight samples from healthy cats and 11 from cats suffering of FIP, all living in households were recovered. Samples came essentially from two different French regions, Basse-Normandie and the suburb of Paris, but also from Romania through collaboration with the veterinary university of Iasi. The purpose was to obtain a sampling from animals living in different areas in contrast to the previous studies conducted by Betneka et al (Benetka et al., 2006), who analysed samples from cats and dogs living in close contact, all in the same shelter. At the time of cats sampling, presence of dogs in the same household was inquired and as far as possible, dogs' samples were also collected to eventually compare the CoV

strains present in both species. However, among the 99 cats samples, only 19 cats were living with at least one dog and within our range of action, only three dogs were sampled.

The presence of Coronavirus in samples was assessed by RT-PCR using a set of primers targeting the 3' end of the genome, which shares a high nucleotide identity with FCoV, CCoV and TGEV. All diseased cats were tested positive, along with 15 healthy cats. This relatively low percentage of positives among healthy cats is however in accordance with previous studies (Addie and Jarrett, 1992). One among the three tested dogs was also CoV infected.

Phylogenetic characterizations of the Coronavirus strains were performed by sequence analysis of the N and S genes. The choice of N was motivated by the assumption that this ordinarily conserved gene would allow significant discrimination between strains. Amplification was performed with consensus primers of the different FCoV and CCoV strains, available in bank sequences. Sequencing of the 3' end of the S gene was attempted by using specific FCoV-I, FCoV-II, and CCoV-II primers previously described, to discriminate the different genotypes. Twenty of the analysed strains were clearly assigned to the FCoV-I genotype. No FCoV-II was identified, which is not surprising, as all previous studies in different countries demonstrates the rare occurrence of FCoV-II strains (Addie et al., 2003)(Hohdatsu et al., 1992)(Duarte et al., 2009)(Lin et al., 2009b).

The remaining six strains displayed atypical features with an N gene related to CCoV-I, whereas the S gene was assigned to the FCoV-I cluster, along with the other strains. To further characterize these strains, amplification of the ORF3 accessory gene, which is unique to the CCoV-I genotype, was attempted and succeeded for five out of six sequences. Analysis of these ORF3 sequences allowed identification of two identical deletions. One is a systematic 29 nt deletion at the 3' end of the gene that yields a stop codon and the other one, not systematically found, is a 27 nucleotides in-frame deletion.

To explain the presence of genes related to CCoV-I and an S FCoV-I type gene, two hypotheses could be considered. First, these animals could be co-infected with CCoV-I and FCoV-I. However, all six atypical samples were tested with both set of primers targeting specifically FCoV-I and CCoV-I genotypes. Only the specific FCoV-I primers succeeded to amplify the S gene, precluding the presence of CCoV-I in parallel. Thus, we favoured the second hypothesis of an atypical feline CoV strain harbouring N gene related to CCoV-I and truncated forms of ORF3, named ORF3 Δ 1 and ORF3 Δ 2 (Figure 11).

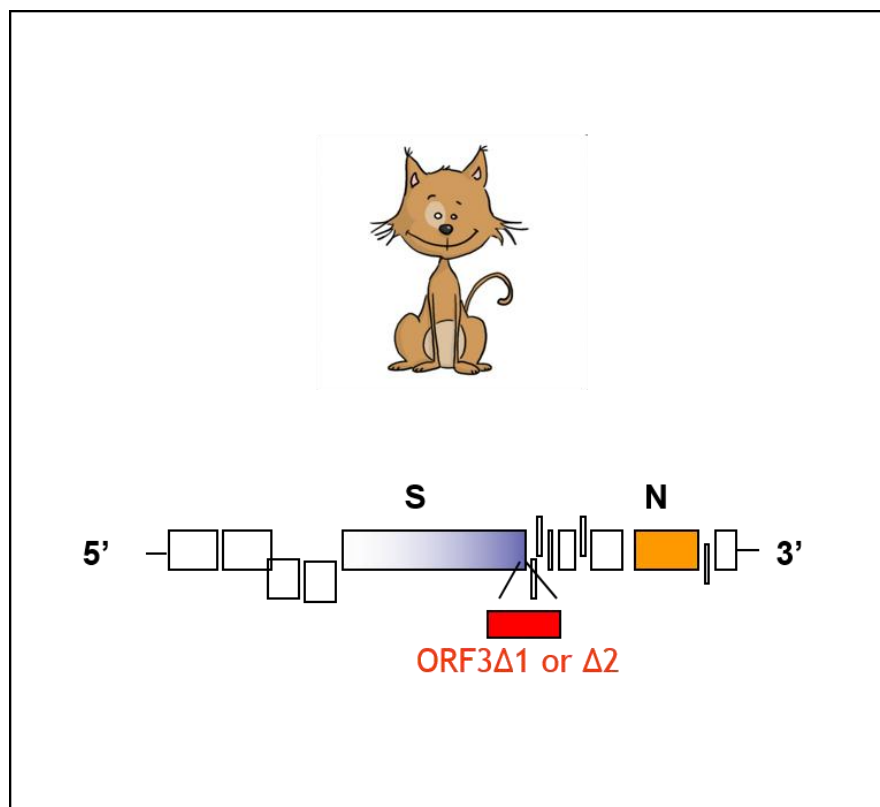


Figure 11: Representation of the atypical strain found in CoV-infected cat samples.

In white, sequences of unknown origins, in blue, sequences of feline origins, in orange, sequences of canine origin.

In their previous study in an Austrian shelter, Betneka et al. (Benetka et al., 2006) had already suggested the presence of atypical strains among cats, displaying homology with CCoV-I strains. However, it is not possible to conclude whether these Austrian strains are similar to

those described herein. Betneka et al. analysed the M and S genes but not the ORF3 gene, which was discovered after their study. Atypical strains described by Betneka harbour an M gene not clearly related to CCoV-I, but rather identified as an intermediate between FCoV-I and CCoV-I clusters. Moreover, excepted for one cat, the S gene could not be amplified, neither with FCoV-I nor with FCoV-II primers. Primers specific to a CCoV-I S gene were not attempted. These strains, with an unknown S gene, are probably different from those we characterized. Eventually, the only strain described by Betneka et al. harbouring an M gene intermediate between FCoV-I and CCoV-I, but also with an FCoV-I type S gene, could be similar to those described in our study. However, without amplification of ORF3, definitive conclusion is impossible.

Whether these atypical strains described herein are widespread among the cat population remain to be investigated with larger sample collection. Here, we detect such strains in a minority of analysed FCoV, not only from cats recovered in France but also from one cat living in Romania. Like FCoV-II, it is possible that these strains circulate in different cat populations but with a low prevalence, in regards to FCoV-I.

Without the full length genome sequence of the newly discovered strains, we can only speculate about their origin. Given sequence analyses of the intergenic regions between S and the ORF3 genes group in different FCoV and CCoV genotypes, it was suggested that ORF3 was somehow, either gained by the CCoV-I strains shortly after its demarcation with FCoV-I, or represents a remnant of a common ancestor between CCoV-I and FCoV-I, further lost by FCoV-I. Here, with the description of atypical FCoV strains harbouring an ORF3 gene, it could be possible that ORF3 persisted in some FCoV-I strains. However, this hypothesis does not explain the presence of an N gene closely related to CCoV-I. Alternatively, these atypical strains may originate from a double recombination event between FCoV-I and CCoV-I. One argument could be the phylogenetic link between strains recovered from the couple of cat and

dog living together in the same household. The infecting strain of the dog was identified as CCoV-I but the ORF3 gene displayed the same 29-nt deletion as described in the cat sample and both N genes shared 99% of nucleotide identity. It is therefore tempting to hypothesise that the strain detected in the cat originated from a recombination with the CCoV-I strain from the dog. Extensive full-length genome will be necessary to clarify whether the FCoV atypical strains originate or not from recombination and the identification of the putative recombination sites. Recombination events are a hallmark of the *Coronaviridae* family evolution process. It was estimated that recombination frequencies may be as high as 25% during mixed infection due to their discontinuous RNA transcription mechanism (Fu and Baric, 1994). Recombination events frequently occur within the S gene, as illustrated by FCoV-II, CCoV-IIb and different antigenic variants of IBV (Jackwood et al., 2010).

Taken together, we propose to add to the representation of the phylogenetic evolution of FCoV and CCoV suggested by Lorusso et al., a novel recombination event between CCoV-I and FCoV-I, as illustrated in Figure 12.

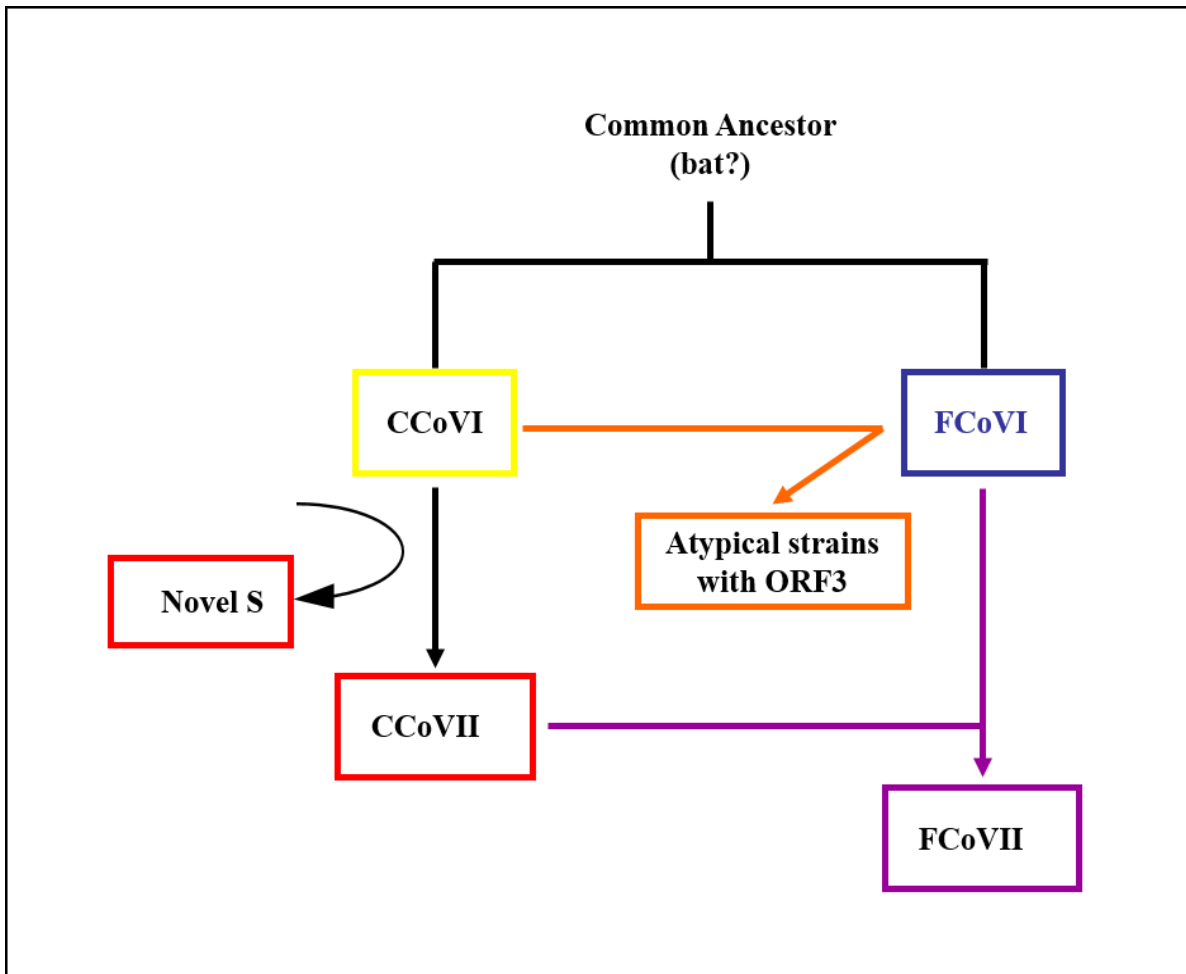


Figure 12 : Representation of the possible evolution of canine and feline Coronaviruses

In yellow, strains of CCoV-I. In blue, strains of FCoV-I. In red, strains of CCoV-II with newly acquired sequences. In orange, result of the recombination event between CCoV-I and FCoV-I. In purple, result of the recombination event between CCoV-II and FCoV-I. Adapted from Lorusso et al. (Lorusso et al., 2008)

In contrast with the recombination event of FCoV-II that gave rise to strains with an S gene of canine origins inserted into a feline genome, the recombination in this case, would lead to a strain harbouring a feline S gene, whereas others genes come from canine virus.

One of the striking features of the atypical FCoV strain is the presence of common deletions within the ORF3 gene. As seen in chapter II, during the interspecies host switch, past studies have observed deletions in accessory proteins, in parallel of mutations within S. Functions and properties of the encoded gp3 protein are still unknown. As truncated forms of this protein

have been found in the context of inter-species transmissions, impact of the deletions during the infection by atypical FCoV of the feline host remain to be inquired.

II. Characterisation of the deleted forms of the accessory glycoprotein 3 (gp3)

In our first study, we observed in atypical strains infecting cats the presence of the ORF3 gene harbouring systematically one or two deletions. ORF3- Δ 1 with a 29-nt deletion, is predicted to be translated in a protein of 158 a.a., named gp3- Δ 1 and truncated of the C-terminus part of gp3 from a.a. 157. ORF3- Δ 2 with an additional 27-nt deletion, leads to a 149 a.a. length protein, named gp3- Δ 2, which lost 9 a.a. between the positions Lys⁸⁸ and Phe⁹⁶. All gp3 proteins maintain the predicted signal peptide and the N glycosylation site. *In silico* analysis with PSORT software, predicts a secretion of the proteins with a probability of 44 %, or an alternative localisation in the ER, but with a probability of only 22 %. As the gp3 deletions are essentially present in feline strains, we wondered about their putative roles during the adaptation of these strains to cats, especially since deletions in accessory proteins have already been observed during tissue or host switch tropism, as seen in Chapter II of the Introduction. For instance, ORF8ab of SARS-CoV was found unmodified in infected civets and humans during the early stages of the epidemic. It is later, in the middle phase of the epidemic that ORF8ab was found truncated. It subsequently led to two smaller ORFs, ORF8a and ORF8b, and disrupted the proper expression of the ORF8ab protein (Oostra et al., 2007). To assess the putative role of the deleted forms of gp3, we compared their properties with the complete gp3 only present in infected dogs. Through *in vitro* translation system, gp3 has already been characterized as a 28 kDa glycoprotein with a potentially cleavable signal peptide. Without any identified transmembrane domain or other retention signals, gp3 was thought to be either secreted or associated with plasma membranes (Lorusso et al., 2008). The

function of gp3 remains unknown. Here, gp3 and the deleted forms were studied in cell culture to explore their post-translational processes and their subcellular localisation. In accordance with the host origin from which arise the different gp3 proteins, canine and feline cells susceptible to CoV infection were selected. In the absence of specific antibody against gp3, proteins were expressed through transfection of plasmids with a 3xFlag tag added at the C-terminal extremity. The three different cloned sequences were chosen for their high nucleotides sequence identity between them (72,52% between ORF3 and ORF3- Δ 1 and 94,30% between ORF3- Δ 1 and ORF3- Δ 2). This choice was motivated to assign eventual differences between the three proteins to the different deletions and not to the eventual amino acids substitutions.

Despite the deletions, the three proteins share common properties, which are the same in both feline and canine cell lines. All proteins oligomerize into covalent dimers and probably into higher multimeric forms, at least for gp3- Δ 1. This characteristic was not observed in the previous *in vitro* translation system. Considering the different deletions, oligomerization is due to the four cysteine residues (at amino acids positions 25, 71, 124 and 149) common to all proteins. Oligomerization in high multimeric forms has already been demonstrated for others CoV accessory proteins such as ORF4a of HCoV-229E, ORF8ab and ORF3a of SARS-CoV (Zhang et al., 2014) (Oostra et al., 2007) (Chan et al., 2009).

All proteins are N-glycosylated with a high polymannose sugar, consistent with their ER localisation observed in the studied cell lines. Without any specific predicted ER retention signal or any transmembrane domain, we hypothesized that the gp3 proteins could be maintained by a non-cleaved signal peptide, as it was observed for the accessory protein 3a of IBV. Mutants of each protein, deleted of the signal peptide or displaying mutations of the cleavage site to prevent parting of the signal peptide were constructed and molecular weights, were compared to the wild-types. For gp3- Δ 1 and gp3- Δ 2, mutants with non-cleaved signal

peptide migrated a little slower than the wild-type forms, indicating that their signal peptide is effectively cleaved. For gp3, the wild form had a molecular weight superior to the mutant form with uncleaved signal peptide. Lorusso et al, had already demonstrated that the signal peptide of gp3 induced an aberrant migration with an apparent molecular weight lower than gp3 without signal peptide. Exchange of the signal peptide sequence restored a coherent migration. Consistent with these results, we concluded that the signal peptide of gp3 is also cleaved. Curiously, aberrant migrations were not observed for mutant gp3- Δ 1 and gp- Δ 2 which yet, harbour the same signal peptide as gp3. Combined with results from Lorusso et al., it is probable that the C-terminus part of gp3 also influences the migration of gp3 with an uncleaved signal peptide.

Proteins with a cleavable signal peptide are usually translocated in the secretory pathway, like the accessory protein 7b of FCoV. In this case, retention of the proteins in this specific compartment could be due to interactions with cellular factors resident of the ER. Many accessory proteins of CoV are located in the ER, like the ORF3a of IBV, ORF6, ORF7a and ORF8ab of the SARS-CoV (Pendleton and Machamer, 2005)(Geng et al., 2005)(Fielding et al., 2004)(Oostra et al., 2007). Exception made for this latter, all contain transmembrane domains, which could explain their localisation. For the ORF8ab encoded protein, similar to the gp3 proteins, no peculiar ER retention signal was identified.

Beside their common properties, the expression levels among the proteins differ according to the cell line. In canine cells, the three proteins are properly expressed, gp3- Δ 1 a bit more than the two others. In feline cell lines, only gp3- Δ 1 expression is easily detected. The different observed expression may be due either by excessive degradation process or specific translation inhibition.

From this study, it appears that the C-terminus part of gp3 and the nine amino acids between Lys⁸⁸ and Phe⁹⁶ are key factors for their expression in feline cells, whereas the N-terminus

part, common to all three proteins, contains all determinants for oligomerization, glycosylation and ER retention of the proteins. These conserved features, despite the deletions, suggest their importance for the functions of these proteins.

Impact of these proteins on the viral replication cycle was also sought. A72 canine cells were used for this study, as all gp3 proteins seem to be expressed at a similar and visible level in a canine host cell environment. As neither CCoV-I nor atypical FCoV isolates are available, infecting cells expressing the different gp3 proteins, with a CCoV strain not harbouring any of the ORF3 sequences, was the only possible way to study their effect on the viral course. Despite their expression, it did not have any impact on the replication cycle of the type II CCoV 1-71 strain used. This absence of effect might not be completely surprising for different reasons.

First, it has been demonstrated that a majority of accessory proteins of Coronaviruses, such as those of FCoV, IBV and SARS-CoV are dispensable for the viral replication *in vitro* (Hodgson et al., 2006)(Haijema et al., 2004) (Yount et al., 2005)(Shen et al., 2003). Yet, only accessory proteins that act as viroporins, confer a viral production advantage when present in the viral genome, such as the ORF3 of PEDV (Wang et al., 2012), ORF3a of SARS-CoV (Chan et al., 2009) and ORF4a of HCOV-229E (Zhang et al., 2014).

Second, function of the gp3 proteins could benefit only to their original viral strain, in which their ORF are present, that is CCoV-I and the atypical FCoV strains. Here, the main difference between CCoV-I and CCoV-II lies in the sequence of the spike protein. The different gp3 proteins may have a hypothetical function linked to the presence of the CCoV-I S protein. Also, infection of feline CrFK cells expressing the gp3 proteins could also help, as the study was carried out in a canine host cell environment only. In feline cells, the levels of expression differ between gp3, gp3- Δ 1 and gp3- Δ 2. Thus, it is plausible that their impact on the viral life cycle may also differ and correlate with their expression. However, in the

absence of laboratory atypical FCoV strains which harbour gp3- Δ 1 and gp3- Δ 2, only imperfect models using FCoV-I or FCoV-II could be used and the issue encountered in the model of canine cells infected by CCoV-II described above could be raised.

We can only speculate about the function of these gp3 proteins. As the gp3 proteins are retained without any retention signal in the ER, they could have a specific role regarding this cellular component, especially when one considers its tribute to the viral replication and transcription steps. For instance, ORF6 of SARS-CoV participates to the reorganisation of the ER compartment membranes, leading to the formation of the DMVs, known to be associated with CoV replication (Knoops et al., 2008). Alternatively, ORF8ab of the SARS-CoV activates the ER resident factor ATF6, which in turn, facilitates protein folding and perhaps, viral production (Sung et al., 2009).

Whether the deleted forms gp3- Δ 1 and gp3- Δ 2 provide a selective advantage to the atypical FCoV strains in infected feline cell remain unclear. In general, the deleted forms of accessory proteins seem not to be functional. Studies of the 29-nt deletion of the ORF8ab of the SARS-CoV, leading to the expression of two smaller ORF8a and ORF8b, concluded that ORF8ab is probably the only functional encoded protein and advantageous in bat and civet hosts, the integral form being dispensable in humans. The proper expression in canine cell lines of the complete gp3, present only in strains infecting dogs, may suggest that the biological importance of this protein is relevant in a canine cellular environment. If their functions correlate with their expression, only gp3- Δ 1 could be advantageous in infected feline cell lines.

III. Perspectives

FCoVs and CCoV are common pathogens and readily evolve through mutation and recombination like other members of the *Coronaviridae* family. Phylogenetic studies of infected dogs have led to the discovery of new genotypes: CCoV-I, CCoV-IIa, CCoV-IIb and one of its virulent biotype, the canine pantropic Coronavirus. Here, by phylogenetic analyses of infected cats, we demonstrated for the first time the circulation of atypical feline Coronaviruses, which could have arisen from recombination between FCoV-I and CCoV-I. Further phylogenetic investigations are needed in order to clarify whether these strains are widespread amongst the cat population, as well as their full-length genome for identification of these strains origins. With the emergence of deep-sequencing technologies, acquisition of the full-length genome should be easier and would probably lead to the discovery of more FCoV strains.

The discovered atypical strains harbour truncated forms of the canine CCoV-I ORF3 gene, leading to the synthesis of either gp3- Δ 1 or gp3- Δ 2. In the aim to explore the molecular processes of adaptation of these strains to the feline host, we explored the basic properties of gp3, gp3- Δ 1 and gp3- Δ 2. However, in the absence of culture cell adapted CCoV-I and atypical FCoV strains, our characterisation was carried out through transient expression of proteins alone. Beside the discrepant levels of expression, all proteins retain the same biological properties: covalent oligomerization, N-glycosylation, cleavage of the signal peptide and retention in the ER. In the absence of laboratory CoV strains encoding gp3, the impact of gp3s on the viral life cycle was investigated in A72 cells expressing gp3 and further infected by a CCoV-II strain. In this context, none of the gp3 proteins seem to influence the viral replication efficacy. Gp3 may have an influence only on CoV strains from which they arise. To assess this hypothesis, one strategy would be to construct an infectious clone with

the advent of the reverse genetic system. The reverse genetic system exists for FCoV, either derived from FCoV-I or FCoV-II strains, but not for any CCoV(Dedeurwaerder et al., 2014)(Thiel et al., 2014). FCoV-I would share the closest relationship with atypical FCoV harbouring truncated ORF3 genes. Adding the ORF3 gene inside the FCoV-I infectious clone would be one possibility to further study the role of ORF3 during the viral life cycle. In light of the emergence of SARS-CoV and more recently the MERS-CoV, the surveillance of animal Coronaviruses remains important, not only because animal CoVs are often responsible of major veterinary diseases, but also because knowledge accumulated could bring a substantial contribution to the general understanding of the genetic evolution and host adaptation process of Coronaviruses.

GENERAL CONCLUSION

Coronaviruses are pathogens of importance, first recognised in the veterinary medicine. They are widely spread in wildlife, birds, farm and companion animals. They lead to important economic losses in pigs and birds industries. In companion animals, they are the causative agents of fatal diseases: the feline infectious peritonitis and the canine pantropic Coronavirus disease. Since the emergence of SARS-CoV in 2002, CoVs are also a source of concern in human medicine. With the emergence of MERS-CoV, six different human Coronaviruses are now described. SARS-CoV and MERS-CoV are the most pathogenic, leading respectfully to 10 and 30% of mortality. Moreover, these two viruses arise from inter-species transmissions. SARS-CoV results from a cross-species transmission from a bat-CoV and MERS-CoV is transmitted to humans from contact with infected dromedaries, which in turn, could probably be contaminated by a bat-CoV.

These examples illustrate one of the striking features of the CoV: their ability to switch species. At least three characteristics of the CoV genome may explain these features. First, it is their potential high mutation rates associated with RNA replication, despite the presence of a non-structural protein, nsp14 with a proof-reading activity. Second, due to their discontinuous mechanism of RNA transcription, recombination events are frequent and can reach a frequency of 25% during mixed infections. Third, with the largest genome of about 30 kB, Coronaviruses have numerous possibilities of genomic variations and encode many different proteins that could help to adapt to a new host.

FCoV and CCoV are commonly widespread among cat and dog populations. They are subdivided in different genotypes, which also witness different inter-species transmissions between those two hosts. Three CCoV genotypes, and probably a fourth one, have been described in dogs and to date, only two for FCoV genotypes. Through phylogenetic analyses,

we discovered new atypical FCoV strains, harbouring an S gene related to FCoV but with an N gene related to the type I CCoV as well as the presence of the accessory ORF3, also characteristic of CCoV-I. Numbers and functions of the accessory proteins of Coronaviruses highly differ from one species virus to another. They have not all been characterised but some of them have been shown to display deletions during the switch of tissue tropism, such as the ORF3a of TGEV or the ORF3c of FCoV and during the inter-species transmission, like the ORF8ab of the SARS-CoV.

Here, the ORF3 in strains infecting cats presents one or two deletions that are systematically the same. All ORF3 recovered in cats are putatively translated either into gp3- Δ 1, deleted of the C-terminus part of gp3, or gp3- Δ 2, which lost an additional nine amino acids, between Lys⁸⁸ and Phe⁹⁶. With the aim to investigate the putative role of gp3, gp3- Δ 1 and gp3- Δ 2 in different host environments, the three proteins were expressed and analysed in both canine and feline cells. Despite deletions, all proteins share the same properties of oligomerization and localised in the ER in the absence of specific retention signals. In contrast, deletions influence the proteins expression in different cells. All proteins are properly expressed in canine cells but only gp3- Δ 1 is easily detected in feline cells. As no CCoV-I or atypical FCoV isolates exist, the only possible way to study the gp3 proteins in a viral context, was to infect cells already expressing the different gp3s through transfection, with a CCoV strain that does not harbour any of the ORF3 genes. Results demonstrated that the expression of the gp3 proteins had no impact on the viral replication.

In this particular context of new Coronaviruses emergence representing a threat for humans, models of CoV inter-species transmissions are still needed. FCoV and CCoV improve the general understanding of Coronavirus. They readily evolve in cats and dogs populations and their study could greatly benefit to a better comprehension of their interactions between the virus and host determinants.

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The different genotypes of canine (CCoV-I/II) and feline (FCoV-I/II) Coronaviruses share a close phylogenetic relationship, suggesting inter-species transmissions between cats and dogs. Through sequence analyses of cat samples, atypical FCoV strains, harbouring an S gene related to FCoV-I, an N gene close to the CCoV-I cluster and the ORF3 gene, peculiar to CCoV-I, were discovered. This ORF3 gene was systematically truncated in feline samples, displaying either one or two identical deletions, leading to the translation of gp3- Δ 1 and gp3- Δ 2. As deletions in accessory proteins have already been involved in host-switch, studies of the different variants of gp3 were conducted. Results demonstrate that all proteins oligomerize through covalent bonds and are retained in the ER, without any specific retention signal. Deletions influence the expression level with a proper expression of the three proteins in canine cells, whereas only gp3- Δ 1 expression is sustained in feline cells. As no isolates of Coronavirus harbouring the ORF3 gene exists, cells expressing the different gp3 proteins have been infected with a CCoV-II strain. In this model, the gp3 proteins do not influence the viral life cycle. In the light of emergence of new Coronaviruses, investigations on their molecular mechanisms during the host-switch are crucial and canine and feline Coronaviruses could represent a useful model.

Keywords: Coronavirus, atypical feline Coronavirus strains, accessory protein gp3, host-jump

Les différents génotypes de Coronavirus canins (CCoV-I/II) et félins (FCoV-I/II) sont phylogénétiquement proches, suggérant des transmissions inter-espèces entre chiens et chats. Lors d'analyses de séquences menées sur des chats infectés, des souches félines atypiques ont pu être mises en évidence, contenant un gène S de type FCoV-I, un gène N de type CCoV-I, ainsi que la présence du gène ORF3, spécifique à CCoV-I. Dans ces souches, le gène ORF3 est présent avec une ou deux délétions toujours identiques, conduisant à la synthèse de protéines tronquées gp3- Δ 1 et gp3- Δ 2. Les délétions de protéines accessoires étant déjà impliquées dans les transmissions inter-espèces, une étude de caractérisation de la protéine gp3 et de ses différentes formes a été menée. Les trois protéines s'oligomérisent de manière covalente et sont retenues dans le réticulum endoplasmique, en absence de signal spécifique de rétention. Les délétions influencent le niveau d'expression des protéines en cellules félines, où seule l'expression de gp3- Δ 1 est visible, alors qu'elles conservent toutes une expression optimale en cellules canines. En l'absence de souches de Coronavirus cultivables en laboratoire contenant le gène ORF3, des cellules canines exprimant l'une des protéines gp3 ont été infectées par une souche CCoV-II. Dans ce modèle, les protéines gp3 ne modifient pas le cycle viral. Dans un contexte d'émergence de nouveaux Coronavirus, la compréhension des mécanismes moléculaires de changement d'hôte est cruciale et les Coronavirus félins et canins peuvent représenter un modèle d'étude utile.

Mots-clés: Coronavirus, souches félines atypiques, protéine gp3, transmissions inter-espèces

UMR 1161 de Virologie, ENVA-ANSES-INRA
7 Avenue du Général De Gaulle
94700, Maisons-Alfort

PÔLE : MICROBIOLOGIE / THERAPEUTIQUES ANTIINFECTIEUSES

UNIVERSITÉ PARIS-SUD 11
UFR «FACULTÉ DE PHARMACIE DE CHATENAY-MALABRY »
5, rue Jean Baptiste Clément
92296 CHÂTENAY-MALABRY Cedex