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CHAPTER SIX

Feline Coronaviruses: Pathogenesis of Feline Infectious Peritonitis

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Contents

Abstract

Feline infectious peritonitis (FIP) belongs to the few animal virus diseases in which, in the course of a generally harmless persistent infection, a virus acquires a small number of mutations that fundamentally change its pathogenicity, invariably resulting in a fatal outcome. The causative agent of this deadly disease, feline infectious peritonitis virus (FIPV), arises from feline enteric coronavirus (FECV). The review summarizes our current knowledge of the genome and proteome of feline coronaviruses (FCoVs), focusing on the viral surface (spike) protein S and the five accessory proteins. We also review the current classification of FCoVs into distinct serotypes and biotypes, cellular receptors of FCoVs and their presumed role in viral virulence, and discuss other aspects of FIPV-induced pathogenesis. Our current knowledge of genetic differences between FECVs and FIPVs has been mainly based on comparative sequence analyses that revealed "discriminatory" mutations that are present in FIPVs but not in FECVs. Most of these mutations result in amino acid substitutions in the S protein and these may have a critical role in the switch from FECV to FIPV. In most cases, the precise roles of these

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mutations in the molecular pathogenesis of FIP have not been tested experimentally in the natural host, mainly due to the lack of suitable experimental tools including genetically engineered virus mutants. We discuss the recent progress in the development of FCoV reverse genetics systems suitable to generate recombinant field viruses containing appropriate mutations for in vivo studies.

1. FELINE CORONAVIRUSES

1.1 Taxonomy and Genome Organization

Together with the *Arteriviridae*, *Mesoniviridae*, and *Roniviridae*, the family *Coronaviridae* (subfamilies *Coronavirinae* and *Torovirinae*) make up the order *Nidovirales*. Coronaviruses belong to the subfamily *Coronavirinae* which has been divided into four genera: *Alpha-*, *Beta-*, *Gamma-*, and *Deltacoronavirus*. Within the genus *Alphacoronavirus*, feline coronaviruses (FCoVs) are part of the species *Alphacoronavirus 1*, the latter also containing a few other closely related viruses, such as canine coronaviruses (CCoVs) and the porcine transmissible gastroenteritis virus (TGEV). Other more distantly related species in the genus *Alphacoronavirus* include *Porcine epidemic diarrhea virus* (PEDV), *Human coronavirus 229E* (HCoV-229E), and *Human coronavirus NL63* (HCoV-NL63) (de Groot et al., 2012).

The positive-strand RNA genome of FCoVs has a size of approximately 29 kb and shows the typical genome organization of coronaviruses (Fig. 1). The 5′ untranslated region (UTR) comprises about 310 nucleotides (nts) and contains the leader sequence as well as the transcription regulatory sequence (TRS) with the core-TRS motif. This 5′-CUAAAC-3′ core-TRS motif is conserved in all FCoVs (de Groot et al., 1988; Dye and Siddell, 2005; Tekes et al., 2008). The 3′ UTR consists of around 275 nts and is followed by a poly(A) tail. The replicase gene covers around two-thirds of the genome and comprises open reading frames (ORFs) 1a and 1b. The translation of the FCoV replicase gene leads to the production of polyproteins (pp) 1a and pp1ab, which are processed by virus-encoded proteinases (Dye and Siddell, 2005; Ziebuhr et al., 2000). By analogy with other alphacoronaviruses, FCoV pp1a/pp1ab is thought to be cleaved by virus-encoded papain- and 3C-like proteases at 3 and 11 sites, respectively (Ziebuhr, 2005). Accordingly, proteolytic processing of the FCoV pp1a/1ab gives rise to 16 nonstructural proteins (nsps) that form the replication/transcription complex and, in some cases, are involved in interactions with host cell factors and functions. The 3′-terminal one-third of the FCoV

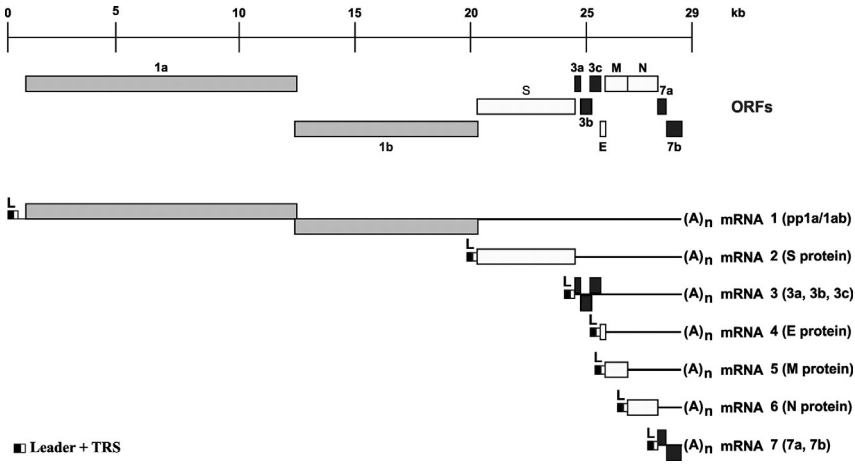

Fig. 1 Genome organization of FCoVs. Schematic representation of the FCoV genome, open reading frames (ORFs), and the characteristic set of subgenomic (sg) mRNAs. The predicted translated regions of each sg mRNA are indicated with *boxes*. The leader (L) sequence together with the transcription regulatory sequence (TRS) located at the 5′ end of all mRNAs is depicted as a *black* and *white boxes,* respectively.

genome contains (i) four genes encoding the four structural proteins called spike (S), envelope (E), membrane (M), nucleocapsid (N) protein, respectively, and (ii) several accessory genes.

1.2 Accessory Genes and Proteins

FCoVs possess five accessory genes called 3a, 3b, 3c, 7a, and 7b. Like in other alphacoronaviruses, these genes are located at two different genome positions (Dye and Siddell, 2005; Haijema et al., 2007; Tekes et al., 2008). Between the S and E genes, FCoVs and CCoVs possess three ORFs (3a, 3b, and 3c), while TGEV contains only two ORFs (3a and 3b). Recently, an additional ORF named ORF3 was described in this region for CCoV (Lorusso et al., 2008). Other members of the *Alphacoronavirus* genus possess only one ORF 3. Sequence analyses suggest that FCoV ORF 3a is homologous to CCoV ORF 3a and TGEV ORF 3a, while the FCoV ORF 3c is a homolog of CCoV ORF 3c, TGEV ORF 3b, and ORF 3 of all other alphacoronaviruses (Narayanan et al., 2008). CCoV ORF 3b represents the only known homolog of FCoV ORF 3b. Furthermore, all members of the species *Alphacoronavirus 1* harbor various numbers of additional accessory genes downstream of the N gene. TGEV has only one ORF (called ORF 7), which is homologous to ORF 7a of FCoVs and CCoVs. The latter

two contain yet another ORF, called 7b, which precedes the 3′ UTR. Deletions of the entire FCoV ORF 3 and 7 genome regions showed that the accessory genes are dispensable for viral growth in vitro; they were suggested to be important for virus replication and virulence in vivo (Haijema et al., 2004). However, the functions of the accessory proteins remain still to be investigated.

FCoV ORF 3a is predicted to encode a 72-amino acid(aa)-long protein without any known or predicted function. This protein is thought to be expressed from the subgenomic (sg) RNA 3, which has been detected in infected cells (Dye and Siddell, 2005; Tekes et al., 2008) and the synthesis of which involves the canonical core-TRS motif upstream of the translational start signal of 3a. However, until now, the expression of this protein in infected cells has not been demonstrated. ORF 3b overlaps with ORF 3a and is supposed to encode an approximately 9-kDa protein with currently unknown functions. Similar to 3a, expression of 3b protein has not been demonstrated in infected cells. It is generally thought that the 3b protein is translated by a noncanonical mechanism from the second ORF present in the 5′-unique region of sgRNA 3. ORF 3c is predicted to code for a protein of 238 amino acids which likely represents a membrane protein with three transmembrane regions. The predicted topology of the 3c protein transmembrane domains is similar to that of the viral M protein (Oostra et al., 2006). Thus far, the expression of 3c protein in infected cells could not be shown. Also, it is unclear whether the 3c protein is expressed from the same sgRNA3 (as predicted for the 3a and 3b proteins) or from a separate sgRNA that, however, has not been identified to date. The existence of an additional sgRNA from which 3c could be expressed receives some support by the observation that the genomes of most FCoV isolates contain a core-TRS motif or a very similar sequence immediately upstream of the ORF3c translational start codon. The transient expression of 3c in a cat cell line revealed a perinuclear localization (Hsieh et al., 2013). Based on the sequence analyses of FCoV field isolates, it has been proposed that 3c is essential for viral replication in the gut but dispensable for systemic infection (Chang et al., 2010). Furthermore, the FCoV 3c protein homologs conserved in PEDV and HCoV-229E were suggested to be incorporated into virus particles, to function as ion channels and to enhance virus production (Wang et al., 2012; Zhang et al., 2014). It remains to be determined whether FCoV 3c protein has similar functions.

Although the synthesis of the FCoV 7a protein in infected cells has not been confirmed experimentally, the protein is expected to be expressed from

sgRNA 7 as was shown previously for its TGEV homolog. FCoV ORF7a is predicted to encode a 71-amino acid (~10 kDa) protein with an N-terminal signal sequence and a C-terminal transmembrane domain (Haijema et al., 2007). Using a plasmid construct expressing 7a with a C-terminal GFP tag, the 7a protein was shown to colocalize primarily with the endoplasmic reticulum (ER) and Golgi apparatus. Using the same plasmid construct and a recombinant virus lacking the entire ORF7, a specific function of the 7a protein in counteracting IFN-α-induced antiviral responses was suggested (Dedeurwaerder et al., 2014). In contrast to all other FCoV accessory proteins, the expression of 7b in infected cells has been confirmed experimentally and the detection of FCoV 7b-specific antibodies in sera obtained from infected cats indicates that the protein is produced in vivo (Herrewegh et al., 1995b; Kennedy et al., 2008; Vennema et al., 1992, 1993). Together with 7a, the 7b protein is expected to be expressed from sgRNA7; however, the translation mechanism used to initiate translation from this second ORF remains to be determined. The 7b protein has a molecular mass of ~26 kDa, it is secreted from the cell, and contains (i) an N-terminal signal sequence, (ii) a potential N-glycosylation site at aa position 68, and (iii) a C-terminal KDEL-like ER retention signal (Vennema et al., 1992). The presence of an internal stop codon or a deletion in the 7b gene has been suggested to indicate cell culture adaptation and a possible (partial) loss of virulence in vivo (Herrewegh et al., 1995b). The precise function of the 7b protein in the FCoV life cycle remains to be elucidated in further studies.

1.3 FCoV Serotypes and Cellular Receptor Usage

Based on serological properties, FCoVs are classified into two serotypes. The vast majority of the natural infections (80–95%) in Europe and America are caused by serotype I FCoVs, while serotype II FCoVs are less common in the field (Benetka et al., 2004; Kummrow et al., 2005). Furthermore, serotype II FCoVs have predominantly been observed in Asia and they were reported to be responsible for up to 25% of the natural infections in those countries (Amer et al., 2012; An et al., 2011; Sharif et al., 2010). There is consistent evidence from independent studies that serotype II viruses emerge via double homologous recombination between serotype I FCoV and CCoV (Decaro and Buonavoglia, 2008; Haijema et al., 2007; Herrewegh et al., 1998; Lin et al., 2013; Lorusso et al., 2008; Terada et al., 2014). As a consequence of the recombination, approximately one-third (~10 kb) of the serotype I FCoV genome including the S gene and the neighboring

regions are replaced with the equivalent parts of the CCoV genome (Decaro and Buonavoglia, 2008; Haijema et al., 2007; Herrewegh et al., 1998; Lin et al., 2013; Lorusso et al., 2008; Terada et al., 2014). Detailed sequence analyses of numerous serotype II FCoVs revealed that the 5′-recombination event occurs in the polymerase gene while the 3′-recombination site is located in the E or M genes. However, the exact locations of these recombination sites vary in the different isolates, indicating that serotype II FCoVs continuously arise through independent recombination events (Haijema et al., 2007; Herrewegh et al., 1998; Lin et al., 2013; Terada et al., 2014). It is considered likely that the described recombination occurs in cats that are coinfected with serotype I FCoV and CCoV. However, the exact source of serotype II FCoVs is unclear. It is believed that serotype II FCoVs are more virulent (Lin et al., 2013; Wang et al., 2013).

The most important biological consequence of the recombination is the integration of the CCoV S gene into serotype I FCoV. The coronaviral S protein is the major determinant for viral attachment and host cell type specificity. While the S1 domain of the S protein is responsible for receptor binding, the S2 domain is required for fusion of the viral and cellular membranes (Bosch et al., 2003; Kubo et al., 1994; Yoo et al., 1991). The poor sequence identity (∼30%) of the S1 domains of FCoVs serotype I and II strongly suggests that the two serotypes use different receptors for cell entry. Early studies showed that serotype II FCoVs employ as a cellular receptor the feline aminopeptidase N (fAPN) (Tresnan and Holmes, 1998; Tresnan et al., 1996), a 150-kDa glycoprotein with metalloprotease activity that is expressed in many host tissues, including epithelial cells from the intestinal brush border (Kenny and Maroux, 1982; Look et al., 1989; Semenza, 1986). These early studies suggested that fAPN may also facilitate the entry of serotype I FCoVs, albeit less efficiently. Subsequent experiments showed that an fAPN-specific monoclonal antibody is able to block infection by serotype II FCoVs (as well as CCoV and TGEV), but not by serotype I FCoVs (Hohdatsu et al., 1998), suggesting that the two serotypes use different receptors for cell entry. This hypothesis was supported by experiments using pseudotyped retroviruses containing the spike protein of FCoV serotypes I and II, respectively, to transduce different continuous cat cell lines. The data obtained in this study provided evidence that serotype I spike fails to recognize fAPN as a receptor for attachment and entry, suggesting that fAPN is not a functional receptor for serotype I FCoVs (Dye et al., 2007). In line with this, recombinant serotype I FCoVs generated by reverse genetics and expressing serotype I and serotype II S proteins, respectively,

were used to demonstrate that the S protein alone is responsible for the different receptor usage of serotype I and serotype II FCoVs (Tekes et al., 2010). It is now generally accepted that serotype I FCoVs employ another cellular receptor. Other studies suggest that feline C-type lectin dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (fDC-SIGN) has a role in cellular attachment and may serve as a coreceptor for both FCoV serotypes in vitro (Regan and Whittaker, 2008; Regan et al., 2010; Van Hamme et al., 2011). The identification of the cellular receptor for serotype I FCoVs remains an important topic in FCoV research.

The usage of different cellular receptors by the FCoV serotypes is reflected in the characteristics of these viruses in vitro. Whereas serotype II FCoVs replicate well in feline tissue culture cells in vitro, serotype I FCoVs grow poorly, if at all, in cell culture, except for a few cell culture-adapted isolates. Accordingly, in the last decade, most studies on FCoVs were based on serotype II viruses, while the more prevalent serotype I FCoVs were largely neglected (de Haan et al., 2005; Dye and Siddell, 2005; Haijema et al., 2003, 2004; Rottier et al., 2005; Tekes et al., 2012).

2. INFECTION WITH FELINE CORONAVIRUSES

2.1 Feline Enteric Coronavirus

FCoVs can cause infections in domestic and wild *Felidae* worldwide (Hofmann-Lehmann et al., 1996; Leutenegger et al., 1999; Munson et al., 2004; Paul-Murphy et al., 1994). Approximately 20–60% of domestic cats are seropositive, with seropositivity rates approaching 90% in animal shelters or multi-cat households (Hohdatsu et al., 1992; Pedersen, 2009, 2014). As pointed out earlier, most of the natural infections are caused by serotype I FCoVs (Addie et al., 2003; Hohdatsu et al., 1992; Kennedy et al., 2002; Kummrow et al., 2005). According to pathogenicity, FCoVs are separated into two biotypes that are generally referred to as feline enteric coronavirus (FECV) and feline infectious peritonitis virus (FIPV). These two biotypes exist in both serotypes I and II.

The vast majority of FECV infections are benign and they either remain undetected or cause a mild diarrhea. However, FECVs can occasionally induce severe enteritis (Kipar et al., 1998b). Convincing evidence for persistent infections caused by FECVs was first provided in the late 1990s (Herrewegh et al., 1997). In these experiments, naturally infected cats were isolated and monitored for virus shedding in the feces. In several cases, FECVs remained detectable in the feces of the cats for more than 15 weeks,

although with decreasing viral loads. To investigate the course of infection in more detail, FECV infection experiments have also been performed under controlled conditions (Desmarets et al., 2016; Kipar et al., 2010; Pedersen et al., 2008; Vogel et al., 2010). These studies showed that FECVs induce symptomless persistent infections similar to natural infections. The virus could be detected a few days after infection in the feces, and virus shedding was confirmed to last for several months (Pedersen et al., 1981b; Vogel et al., 2010). Furthermore, similar to natural infections viral RNA was also found in the blood (Gunn-Moore et al., 1998; Herrewegh et al., 1995a, 1997; Kipar et al., 2006a,b; Meli et al., 2004; Simons et al., 2005; Vogel et al., 2010). Seroconversion of the animals started approximately 10 days postinfection and the antibody titers remained at a relatively low level. Postmortem analyses showed that, in acute infections, FECVs have a tropism to the apical epithelium of the intestinal villi from the lower part of the small intestines to the caecum (Pedersen et al., 1981b). Although coronaviral RNA can be detected in persistently infected cats in the entire gastrointestinal tract, blood, and different tissues, experimental infections revealed that the lower part of the gastrointestinal tract is the major site for viral replication and FECV persistence (Herrewegh et al., 1997; Kipar et al., 2010; Vogel et al., 2010). These observations confirm that FECVs are primarily associated with the gastrointestinal tract but they are also capable of infecting monocytes, albeit less efficiently, and thereby spread throughout the body (Dewerchin et al., 2005; Kipar et al., 2006a, 2010; Meli et al., 2004; Porter et al., 2014).

FECVs are highly contagious and are transmitted horizontally via the fecal–oral route (Pedersen, 2009, 2014; Pedersen et al., 1981b). Usually, kittens become infected with FECVs at a young age in the litter, most probably through viruses in the feces of the mother (Addie and Jarrett, 1990, 1992; Pedersen et al., 1981b). Since persistently infected cats shed the virus in their feces for extended periods of time, they play a central role in spreading and maintaining FECVs in cat populations and therefore represent a threat to other animals.

2.2 Feline Infectious Peritonitis Virus

In sharp contrast to FECVs, FIPV causes a lethal disease called feline infectious peritonitis (FIP). The disease is characterized by fibrinous and granulomatous serositis, protein-rich serous effusion in body cavities, and/or granulomatous lesions (pyogranulomas) (Hayashi et al., 1977; Kipar and

Meli, 2014; Kipar et al., 1998a, 2005; Pedersen, 1987, 2009; Weiss and Scott, 1981a,b). The cellular composition, the level of viral antigen expression and the distribution of the FIP-characteristic lesions in different organs can vary in individual cases (Kipar and Meli, 2014). For the development of these lesions, FIPV-infected monocytes and macrophages have been identified as major target cells of FIPVs and are assumed to play a pivotal role (Haijema et al., 2007). FIPVs are able to efficiently infect and replicate in monocytes/macrophages (Dewerchin et al., 2005; Rottier et al., 2005; Stoddart and Scott, 1989) and to trigger an activation of these cells (Regan et al., 2009). Circulating activated monocytes heavily express cytokines such as tumor necrosis factor -α, IL-1β, and adhesion molecules (e.g., CD11b and CD18) (Kipar et al., 2006b; Kiss et al., 2004; Regan et al., 2009; Takano et al., 2009, 2007a,b); the latter facilitate the interaction of monocytes with activated endothelial cells in the small- and medium-sized veins. Moreover, it has been suggested that the increased expression of enzymes such as matrix metalloproteinase-9 by the activated monocytes contributes to endothelial barrier dysfunction and subsequent extravasation of monocytes (Kipar and Meli, 2014; Kipar et al., 2005). Furthermore, the production of vascular endothelial growth factor produced in FIPV-infected monocytes and macrophages was proposed to induce increased vascular permeability and hence effusion in body cavities (Takano et al., 2011). Although leukocytes are not susceptible to FIPV infection, they appear to become activated during FIPV infection by as-yet-unknown mechanisms, thereby probably contributing to endothelial cell damage and the development of FIP lesions (Olyslaegers et al., 2013).

Based on the presence or absence of protein-rich effusions in the abdominal and pleural cavities, wet (effusive), dry (noneffusive), and a combination of these two clinical forms (mixed form) of FIP can be distinguished (Drechsler et al., 2011; Hartmann, 2005; Kipar and Meli, 2014; Pedersen, 2009). In natural infections, the wet form seems to be more prevalent than the dry and mixed form, respectively (Pedersen, 2009). The development of the various clinical forms is believed to be dependent on the host immune response. Although the underlying mechanisms are not completely understood, it is generally accepted that the balance between cellular and humoral immune responses in infected animals critically determines the clinical progression of the disease. While strong cellular immune responses may control the disease (Pedersen, 2009, 2014), weak cellular but vigorous B cell responses have been associated with the wet form and somewhat stronger T cell immune responses are thought to cause the dry form of FIP

(Pedersen, 2009, 2014). It has been observed in field cases of FIP that the wet form often develops during the terminal stage of dry FIP, probably reflecting a collapse of the immune system (Pedersen, 2009, 2014).

Factors that may trigger the progression of the disease have been described for both naturally and experimentally infected cats. It was shown that stress or superinfections with feline leukemia virus and feline immunodeficiency virus, respectively, increase the risk for FIP development (Poland et al., 1996). The underlying mechanism for this phenomenon is not completely understood, but it is assumed that immunosuppression favors the generation of escape mutants and thereby, the probability of clinical manifestation of FIP. Furthermore, genetic predisposition to FIP was suggested (Golovko et al., 2013; Hsieh and Chueh, 2014; Pedersen, 2009; Pesteanu-Somogyi et al., 2006; Wang et al., 2014; Worthing et al., 2012).

Despite the existence of serotype I and II FIPVs, the characteristics of the disease caused by these serotypes appear to be very similar. The incubation time for naturally occurring FIP cases is difficult to assess, but a number of studies using experimentally infected specific pathogen-free (SPF) cats revealed incubation times of 2–14 days for the wet form and several weeks for the dry form (Kiss et al., 2004; Pedersen and Black, 1983; Pedersen et al., 1981a, 1984; Tekes et al., 2012). Following experimental infections with the prototype serotype II FIPV 79-1146 strain or a recombinant form of this virus, respectively, cats were shown to develop fever after a few days and lost weight rapidly. Shortly after infection, viral RNA became detectable in the feces and blood; serum antibody titers increased rapidly and remained at a high level during the entire course of infection. In some cases, infected animals seemed to recover after the first week of clinical signs, but subsequently developed pronounced clinical signs of the disease including fever, lack of appetite, weight loss and a progressively worsening condition. Although most of the cats died within 4–5 weeks after experimental exposure to serotype II FIPV strain 79-1146, a low number of animals survived for a few months and succumbed to the disease at a later time point (de Groot-Mijnes et al., 2005). The overall survival time of cats can vary significantly, depending on the amount and virulence of the virus used for the experimental infection (Kiss et al., 2004; Pedersen and Black, 1983; Pedersen et al., 1981a; Tekes et al., 2012). It should also be noted that experimental infections of cats with FIPVs always lead to clinical signs, but approximately 20% of the animals can survive and recover (de Groot-Mijnes et al., 2005; Dean et al., 2003; Kipar and Meli, 2014; Tekes et al., 2012).

Naturally occurring FIP usually affects cats at a young age of less than 2 years; the incidence of FIP dramatically decreases with increasing age (Foley et al., 1997; Pedersen, 2009). It is generally accepted that FIP occurs sporadically. Unlike FCoVs, FIPVs are usually not transmitted horizontally from cat to cat, even though FIPV shedding into feces has been detected under experimental infection conditions (Bank-Wolf et al., 2014; Pedersen, 2009; Pedersen et al., 2012; Tekes et al., 2012; Thiel et al., 2014). However, FIPV shedding in the feces does not lead to FIP in contact animals (Pedersen et al., 2012) and horizontal transmission of FIPV resulting in FIP is thought to have no epidemiological role.

2.3 Origin of FIPV

FIP develops in approximately 5% of cats that are persistently infected with FECV (Chang et al., 2011; Haijema et al., 2007; Pedersen, 2009). Over many years, the origin of FIPVs was unclear and discussed quite controversially. In early investigations, FECVs and FIPVs were considered different virus species. In subsequent studies, FECVs and FIPVs were proposed to be closely related viruses with distinct virulence properties. Sequence analyses of both biotypes revealed much higher sequence similarity of FIPV and FECV isolates collected in the same cattery compared to FCoV sequences from distinct catteries/geographical regions (Herrewegh et al., 1995b; Pedersen et al., 1981b; Poland et al., 1996; Vennema et al., 1998). These observations led to the hypothesis that FIPV evolves from FECV by specific mutations occurring in the viral genomes in individually infected cats. This "internal mutation" hypothesis received further support from a series of animal experiments (Poland et al., 1996; Vennema et al., 1998). Based on other data, an alternative "circulating virulent–avirulent FCoV" hypothesis that contradicted the widely accepted theory was also proposed (Brown et al., 2009). The study suggested the independent coexistence of virulent and avirulent FCoVs in a cat population. The authors claimed that cats develop FIP only upon infection with the virulent FCoV type. However, this hypothesis has failed to receive any backing, and since then additional experiments and further analyses have strengthened the "internal mutation" theory (Bank-Wolf et al., 2014; Barker et al., 2013; Chang et al., 2010, 2011, 2012; Lewis et al., 2015; Licitra et al., 2013; Pedersen et al., 2012; Porter et al., 2014). It is now widely accepted that FIPV emerges during persistent infection through mutations from the harmless FECV. However, it is not understood which mutation(s) occur(s) at which stage during the

development of FIP. As mentioned earlier, FECVs show a pronounced tropism toward epithelial cells in the gut, but they are also able to infect monocytes, albeit inefficiently. It was suggested that in monocytes—rather than in intestinal epithelial cells—FECVs acquire mutations that can convert them into FIPVs (Pedersen et al., 2012). The resulting FIPVs display an altered cell tropism; they infect and replicate efficiently in monocytes and macrophages. This property is considered a key step in the development of FIP.

3. MOLECULAR PATHOGENESIS OF FIP

3.1 Differences Between FECV and FIPV

In the past decades, many studies were aimed at identifying mutations responsible for the biotype switch. Mutations in accessory genes and the S gene of FCoVs have been associated with FIP development. In this regard, accessory gene 3c was one major focus. Early studies showed that FECVs always contain an intact 3c gene, while more than two-thirds of FIPV-derived 3c sequences were found to contain mutations (e.g., deletion or point mutation) that prevented translation of an intact full-length protein. Therefore, mutations in 3c were initially thought to be a general virulence marker indicative of FIP (Pedersen et al., 2009; Vennema et al., 1998). More recent studies confirmed these earlier observations, with 3c being heavily mutated in the majority of FIPV isolates and possibly involved in FIP development. Comprehensive sequence analyses of FECVs isolated from the gut and FIPVs isolated from the gut, organ lesions, and effusions, respectively, suggested that an intact 3c gene is required for viral replication in the gut but nonessential for systemic replication of FIPVs (Bank-Wolf et al., 2014; Chang et al., 2010, 2011, 2012; Pedersen et al., 2012). It is currently considered likely that the mutations in 3c are no virulence markers for FIP, but rather a consequence of systemic spread and enhanced replication of FIPVs. Nevertheless, it cannot be excluded that (frameshift and other) mutations that affect 3c protein expression contribute to an increased viral fitness in monocytes/macrophages and, thereby, to the development of FIP.

Based on sequence analyses of the 7a gene of FECV/FIPVs obtained from Persian cats, one study proposed that deletions in the 7a gene are associated with the development of FIP (Kennedy et al., 2001). However, these data did not receive support from others. Mutations in the 7a gene are not currently considered to be crucial for the biotype switch.

Deletions in the 7b gene have also been proposed to play an important role in FIP development. However, consecutive analyses revealed that

deletions in 7b primarily evolve during cell culture adaptation and are associated with loss of virulence (Herrewegh et al., 1995b, 1998; Takano et al., 2011). The existence of deletions in the 7b gene in naturally occurring FECVs argues against a major involvement of mutations in 7b in FIP development (Lin et al., 2009).

Lately, the focus of research on FIP pathogenesis shifted toward the investigation of the S gene. The coronaviral S protein is crucial for receptor binding and virus entry. Since the FECV–FIPV transition involves a switch of target cell tropism, mutations in the S gene alone or in combination with changes in other genes may contribute to the biotype switch. To address this possibility, recent studies investigated the involvement of S gene mutations in FIP pathogenesis. An analysis of 11 FECV and 11 FIPV full-length genome sequences identified two point mutations in the S gene that can distinguish the vast majority of FIPVs from FECVs (Chang et al., 2012). To confirm this observation, the same research group investigated additional FECV and FIPV S gene sequences; the outcome was basically identical. The analyses showed that either one or both mutations were present in approximately 96% of the FIPV sequences while they were absent in all examined FECVs, providing strong evidence to suggest that these mutations correlate with the occurrence of FIP. One of the mutations leads to a Met-to-Leu substitution at amino acid position 1058 in the S protein (M1058L) and the other causes a Ser-to-Ala substitution (S1060A) (Chang et al., 2012). Since the affected residues are located in the putative fusion peptide of the S protein, it is tempting to speculate that amino acid changes in this region affect the cellular tropism of the virus, resulting in enhanced monocyte/macrophage tropism, a hypothesis that remains to be confirmed in additional experiments. Based on the observed sequence differences between the S genes of FECVs and FIPVs, a diagnostic assay for FIP diagnosis has been developed.

Porter et al. sequenced a short fragment of the S gene derived from fecal and tissue samples of both FECVs and FIPVs. In the majority of the fecal samples, the authors found methionine at position 1058 and in the majority of tissue samples leucine at position 1058, regardless of whether the cats were infected with FECV or FIPV. They concluded that the M1058L substitution represents a marker for systemic FCoV infection rather than a marker for FIP (Porter et al., 2014).

Another study investigating FECV–FIPV discriminatory mutations determined 3 FECV and 3 FIPV full-length genome sequences (Lewis et al., 2015). Similar to the observations described earlier (Chang et al., 2012), M1058L was

identified as a fully discriminatory mutation between FECVs and FIPVs because it was exclusively present in the analyzed FIPV but not in any of the FECV samples. Interestingly, this work identified one more substitution suitable to discriminate between FECVs and FIPVs, an Ile-to-Thr substitution at position 1108 (I1108T) in the heptad repeat 1 (HR1) region, which was exclusively found in FIPVs. Amino acid substitutions in the HR1 region of FIPVs (but not of FECVs) have also been described by others (Bank-Wolf et al., 2014). As discussed by Lewis et al., it seems plausible that changes in the HR 1 region result in an altered fusogenic activity of the S protein which may affect the cellular tropism of the virus. However, it remains unclear whether the described mutations are relevant to FIP development.

In a recent study, the furin cleavage site located between the S1 and S2 domains of the S protein was investigated in FECV and FIPV samples (Licitra et al., 2013). While all FECVs were found to contain an intact and functional furin cleavage motif, as many as 10 out of 11 FIPVs contained amino acid substitutions at the cleavage site itself or in close proximity to the furin cleavage site. Fluorogenic peptide assays showed that the mutations identified in FIPVs affect the efficiency of furin-mediated S protein cleavage. Because the fusion activity of the coronaviral S protein generally requires activation by cellular proteases, substitutions at the protease cleavage site may indirectly affect viral spread and, thus, disease progression and the development of FIP (Bosch and Rottier, 2008).

The point mutations described earlier were detected in different regions of the S gene. Obviously, there is a strong correlation between the genetic changes and the occurrence of FIPV. However, it is important to emphasize that the FECV–FIPV substitutions were identified only via comparative sequence analyses and, so far, none of the assumed functional changes concerning cell tropism and biotype switch for FIP pathogenesis have been proved experimentally.

4. REVERSE GENETICS OF FELINE CORONAVIRUSES

Reverse genetics approaches are extremely valuable tools to produce recombinant FCoVs containing genetic changes suitable to investigate the role of specific viral proteins in the molecular pathogenesis of FIP. So far, three different reverse genetics systems have been described for FCoVs.

The very first system is based on targeted RNA recombination and was established for the highly virulent serotype II FIPV strain 79-1146 (Haijema et al., 2003). This system proved to be a very useful tool for modifying the

FCoV genome but, for technical reasons, only the 3′-terminal third of the genome is amenable to mutagenesis via this approach. By deleting the entire ORF3 (FIPV-Δ3), the entire ORF7 (FIPV-Δ7), or both (FIPV-Δ3Δ7), recombinant viruses were generated that displayed similar properties in cell culture to the parental virus. However, all of these recombinant viruses were attenuated in vivo and did not induce FIP. Furthermore, cats inoculated with FIPV-Δ3 or FIPV-Δ7 mutants were protected against a challenge with the parental virus, demonstrating that the accessory genes are dispensable for viral growth in vitro but contribute to virulence in vivo (Haijema et al., 2004). The same group investigated the genetic determinants for macrophage tropism of FIPV strain 79-1146. Parts of the S gene or the entire S gene of the FIPV strain 79-1146 were replaced with the corresponding S gene sequences derived from a cell culture-adapted serotype II FECV. Infection of macrophages with the recombinant viruses and the parental virus, respectively, revealed that the S protein alone was responsible for efficient macrophage infection and replication. Moreover, the C-terminal domain of the S protein was suggested as a key determinant for target cell tropism (Rottier et al., 2005).

Others have also used the recombinant viruses FIPV-Δ3, FIPV-Δ7, and FIPV-Δ3Δ7 to study certain aspects of FCoV biology. Dedeurwaerder et al. investigated the role of ORF3 and ORF7 for replication of FIPV in peripheral blood monocytes (Dedeurwaerder et al., 2013). They were able to show that only the FIPV-Δ3 and the parental virus but not FIPV-Δ3Δ7 and FIPV-Δ7 were able to maintain replication in monocytes. Accordingly, it was suggested that ORF7 is crucial for FIPV replication in monocytes and macrophages. In another study, this group addressed the question of whether 7a and 7b proteins interfere with the cellular innate immune system. Using the recombinant FIPV-Δ7 virus and a 7a-expressing plasmid construct, data were obtained to suggest a function of the 7a protein in counteracting IFN-α-induced antiviral responses (Dedeurwaerder et al., 2014).

Balint et al. established a bacterial artificial chromosome (BAC)-based reverse genetic system for serotype II FIPV strain DF2 (Balint et al., 2012). In contrast to the targeted RNA recombination system, the BAC system is suitable to mutagenize the entire FCoV genome. However, instability of the cloned FCoV cDNA in BAC may hinder the efficient generation of recombinant FCoVs. Strain DF2 is the cell culture-adapted variant of strain 79-1146, which is used in the only available vaccine against FIP (Kipar and Meli, 2014; Pedersen, 2009). The strain DF2 contains a 338-nt-long

deletion in ORF3, resulting in truncated ORF3a and ORF3c proteins and deletion of the entire ORF3b. Using the BAC-based reverse genetic system, the authors generated a recombinant DF2 identical to the parental virus and a virus with fully restored ORF3 derived from CCoV. All recombinant viruses showed similar characteristics in established cell lines. However, after infection of peripheral blood monocytes, the virus with the fully restored ORF3 showed significantly lower replication compared to the virus in which ORF3 was deleted, suggesting that the ORF3 deletion may promote efficient virus replication in monocytes. In follow-up studies, the virulence of the recombinant viruses and the role of a fully restored ORF3 were assessed in experimental infections (Balint et al., 2014a,b). Only the recombinant virus containing an intact ORF3 was associated mainly with the gut and did not cause systemic infection. Accordingly, a pivotal role of ORF3 in establishing efficient infection of the intestine in vivo was suggested.

The third type of reverse genetic system relies on the integration of the entire coronaviral genome as a cDNA into the vaccinia virus genome, with the resulting recombinant vaccinia viruses serving as vectors to clone and manipulate the coronavirus cDNA insert (Casais et al., 2001; Coley et al., 2005; Thiel et al., 2001). The advantages of this system are that desired changes can be introduced at any position of the viral genome and, in contrast to *Escherichia coli*-based cloning systems, genetic instabilities of the full-length FCoV cDNA insert in the vaccinia virus genome have never been observed. However, complex and time-consuming procedures are required to manipulate the FCoV cDNA by vaccinia virus-mediated recombination and to produce (wild-type or mutant) genome-length FCoV RNA to be transfected into susceptible cells. Such a vaccinia virus-based system was reported for the serotype I FCoV strain Black (Tekes et al., 2008). This virus was isolated from a cat with FIP (Black, 1980). Interestingly, the recombinant serotype I FCoV strain Black and the virus isolate that was used to assemble the FCoV Black sequence did not induce FIP in SPF cats. Most likely, the propagation of the virus in tissue culture led to adaptive mutations that resulted in a nonpathogenic virus. The serotype I FCoV strain Black contains a stop codon in the accessory gene 7b that was thought to be an adaptive mutation responsible for the nonvirulent phenotype of this virus. Accordingly, a recombinant virus with a fully restored ORF 7b was generated and used for animal experiments; however, FIP was still not induced. In an attempt to generate a recombinant FCoV that reproducibly induces FIP, increasing portions of the recombinant FCoV Black genome were replaced with the homologous genome regions derived from the highly virulent

serotype II FIPV strain 79-1146 (Tekes et al., 2012; Thiel et al., 2014). One of the chimeric viruses contained the S-3abc region, and another additionally possessed most of the ORF 1b of the serotype II FIPV in the serotype I virus backbone. Both viruses led to a systemic infection and induced high serum antibody titers, but FIP was not induced. Accordingly, the introduced parts of the serotype II genome were not sufficient to convert the nonpathogenic virus into a FIP-inducing virus, possibly because the virus contained additional attenuating mutations elsewhere in the serotype I backbone. Another explanation is an "incompatibility" of specific genome regions derived from serotype I FCoV strain Black and serotype II FIPV strain 79-1146, respectively, in inducing FIP. Finally, in order to generate a full-length recombinant serotype II FIPV strain 79-1146, the remaining parts of the serotype I backbone were replaced with the homologous regions of the serotype II FIPV. As expected, the recombinant serotype II FIPV and its parental virus induced FIP in experimentally infected SPF cats. In both the recombinant and the parental serotype II FIPV strain 79-1146, the ORF 3c contained a mutation causing a premature translational termination of this protein. Remarkably, sequence analyses of viral RNA originating from tissues of diseased cats revealed the restoration of ORF 3c. At first glance, this finding contradicts previous reports since an intact 3c is thought to be required for replication in the gut but dispensable for systemic infection (Chang et al., 2010; Pedersen et al., 2012). Interestingly, FIPV RNA with intact ORF 3c was detected in the fecal samples shortly before the cats succumbed to the disease, suggesting that FIPV replication also took place in the gut prior to death. It remains to be determined whether the restoration of ORF 3c is required to induce FIP after experimental infection with FIPV strain 79-1146.

5. PERSPECTIVES

Over the past few years, many aspects of FCoV biology have been studied, providing interesting new insight into FIP pathogenesis; however, a number of important questions remain to be addressed. This also applies to the emergence of FIPVs. It is now generally accepted that FIPV evolves from FECV through mutations being acquired in persistently infected animals. However, mutations that can convert a nonpathogenic FECV into a deadly virus have not been determined unambiguously, for example, by using reverse genetics approaches to produce and characterize mutants containing specific genetic changes predicted (by previous sequence analyses of

FCEV/FIPV pairs) to convey a FIP-inducing phenotype. In the past, various reverse genetic systems have been developed, but all of them are based either on cell culture-adapted serotype I or serotype II FCoVs that are not suitable for detecting mutations responsible for the biotype switch. Accordingly, there is an urgent need for a robust reverse genetic system that allows the production, characterization, and manipulation of serotype I field isolates.

There are two promising strategies for identifying mutations that can turn a nonpathogenic FECV into a FIP-inducing virus. One possibility is to introduce the described FECV–FIPV discriminatory mutations into a FECV field isolate using reverse genetics. So far, the most relevant data concerning FECV–FIPV discriminatory mutations originate from extensive comparative sequence analyses of serotype I FECV and FIPV field isolates. The identified mutations correlate with the switch of biotype and primarily concern the S gene. Accordingly, these discriminatory mutations should be introduced into the S gene of a serotype I FECV field isolate using reverse genetics and the virulence of the resulting viruses should be assessed in animal experiments.

We hypothesize that mutations in one gene or a combination of changes in different parts of the FCoV genome can lead to the emergence of FIPV. This assumption is supported by published data, which locate the mutations in four different regions of the S protein (predicted fusion peptide, HR1 region, furin cleavage site, and C-terminal region). All of these S gene mutations may lead to an altered cell tropism and finally to the development of FIP. However, sequence analyses also revealed substitutions located at many positions throughout the genomes of the corresponding FECV and FIPV pairs. Accordingly, it should be possible to identify mutations responsible for the biotype switch by generating and studying chimeric FCoVs. The starting materials for these viruses are "infectious clones" of genetically defined FECV–FIPV pairs. By replacing increasing parts of the FECV genome with the corresponding FIPV genome segments, the region of the FIPV genome that is able to convert the nonvirulent FECV into a FIP-inducing virus will be localized. However, this approach requires the growth of field viruses in standard cell culture systems, which has not been achieved so far. There is only one recently described feline enterocyte cell line that apparently allows propagation of serotype I FCoVs (Desmarets et al., 2013). It remains to be seen whether such a cell line can be used to grow FCoV field isolates to high titers or even for the identification of the cellular receptor(s) of serotype I FCoVs.

Another intriguing question relates to the role of accessory proteins in the FCoV life cycle. Due to the lack of appropriate tools, our knowledge about these proteins remains limited. So far, only the expression of 7b gene could be demonstrated in infected cells (Herrewegh et al., 1995b; Vennema et al., 1992, 1993). Furthermore, one publication suggested a role for the 7a protein as an interferon antagonist (Dedeurwaerder et al., 2014). There is increasing evidence that the accessory proteins are important for virulence in vivo, but the underlying molecular mechanisms are not understood. Also, FCoV accessory proteins may be required for viral persistence in specific cell types. Future studies are required to elucidate the functions of the accessory proteins and are expected to provide interesting insight into the molecular mechanisms that determine the pathogenesis, progression, and outcome of FCoV-induced diseases.

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