Title: Feline Infectious Peritonitis: challenges and promises of coronavirus RNA detection.

Authors: Wadim J. Kapulkin ^{1,3,4)}, Pawel Kita ²⁾

¹⁾ Department of Infectious Diseases, Microbiology and Parasitology, Faculty of Veterinary Medicine, Grochowska 272, 03-849 Warsaw, Republic of Poland

²⁾ Department of Epizootiology, Faculty of Veterinary Medicine, Faculty of Veterinary Medicine, Grochowska 272, 03-849 Warsaw, Republic of Poland

10 ³⁾ Department of Virology and Immunopathology, National Institute of Public Health-PZH, Chocimska 24, 00-791 Warsaw, Republic of Poland

⁴⁾ contact information: Dr. Wadim J. Kapulkin mRCVS, DVM, PhD [e-mail: wadim_kapulkin@yahoo.co.uk]

15

Significance and perspective article.

ABSTRACT:

Our perspective article covers major findings concerning Feline Infectious Peritonitis (FIP) - a fatal
20 coronaviral disease of cats. In the context of FIP pathogenesis, we outline disease signalment and
focus on the challenges and promises of FIP invoking coronavirus RNA detection. In particular, we
outline critical aspects of coronavirus RNA replication and biogenesis. We infer the replicative
intermediates of feline coronavirus may constitute an underappreciated factor triggering the
progression of the maladaptive immune response underlying FIP pathogenesis.

25

30

35 **Feline Infectious Peritonitis (FIP) is a terminal disease resulting from infection with**
entero-tropic Feline Coronavirus (FCoV). Considering FIP as a transmissible disease of cats,
there are two major antigenically distinct serotypes of Feline Coronavirus involved: most common
FCoV type I and FCoV type II (Uzal et al. 2016). Below we outline the events and processes
underlying the above diversity in the context of critical biogenesis steps in coronaviral replicative
40 cycle.

Coronaviruses, including FIP pestilent inhabiting feline niche, are undoubtedly the largest
and most complex RNA viruses. Encapsidated and infectious RNA genome consists of a single (+)
strand RNA molecule, approaching 27-33kb in length (Hegemeijer et al. 2012; Hartenian et al.
45 2020). Most of the region following the untranslated portion at 5' end of coronaviral genomic RNA
encodes two polyprotein precursors translated of one open reading frame disjointed by programmed
ribosomal frameshift (Plant et al. 2008). Encoded polyprotein precursors referred to as ORF1a and
ORF1b, together occupy about two-thirds of the RNA genome. In a coronaviral replication cycle
ORF1 (i.e. replicase encoding ORF) translate directly of infectious (+) RNA strand - immediately
50 after uncoated coronaviral RNA enters a cell. ORF1 translates into a viral replicase RNA-dependent
RNA-polymerase (and several other non-structural proteins with RNA modifying functions –
including RNA helicase, exoribonuclease, uridylate specific endoribonuclease, and ribose 2'-O-
methyltransferase - required for the completion of coronavirus replication) proteolytically cleaved
of a fairly large polyprotein precursor. RNA-dependent RNA-polymerase (along with auxiliary
55 proteins providing RNA modifying functions) is critical for the synthesis of complementary anti-
genomic (-) strand RNA. Anti-genomic (-) RNA strand constitutes the essential replicase co-factor
required for infectious (+) RNA strand synthesis (and for the synthesis of viral mRNAs, as well as
minor subgenomic coronaviral RNAs of both positive and negative polarities; see Hegemeijer et al.

and Hartenian et al. for details and relevant references).

60

This mode of copying of the genetic information is common to all non-reverse transcribing RNA viruses and immediately suggest the presence of intermediate dsRNA in the replicative cycle.

In addition to RNA-dependent RNA-polymerase i.e. replicase genes, RNA genome embeds
65 8-10 open reading frames encoding for other coronaviral proteins. The other than ORF1 coronaviral genes, located toward the 3' portion of the genome, encode for structural capsid proteins and few other proteins referred to as accessory, are translated after the completion of synthesis of (-) RNA strands (both major anti-genomic cRNA strand and minor, subgenomic RNAs of negative polarity (Hegemeijer et al. 2012; Hartenian et al. 2020)). While coronaviruses may have evolved machinery
70 ensuring the replicative fidelity, because of the large genome size; the RNA replication involving anti-genomic cRNA strand synthesis (with the inevitable formation of dsRNA intermediates) is regarded as an error-prone process, resulting in replicative infidelities, hence contributing factor promoting coronaviral variant diversity. The replicative infidelities associated with RNA replication involving dsRNA intermediates are also known to contribute to the process of RNA-RNA
75 recombination. It has been proposed that the mechanisms of genomic RNA-RNA recombination promote exchange and mediate shuffling of the coronaviral RNAs between replicating molecules (Terada et al. 2014). Indeed, coronavirus diversity seems also to origin from frequent replication-associated genomic RNA-RNA recombinations expectedly increasing the variability of replicating RNA molecules (Hartenian et al. 2020).

80

The genomic structure of FCoV and its pernicious FIP-invoking derivative is typical for the family of coronaviruses. In terms of the overall genomic architecture, where kinship is based on the RNA sequence homology, FIP causing feline coronavirus appears related and most similar to

coronaviruses infecting canids. As represented by branches radiating at the top of the cladogram
85 shown in (Fig.1), most of the FIP virus genomes clearly form a distinct clade within feline
coronaviruses. In contrast, coronaviruses infecting other species, (where genomes of canine isolates
cluster with porcine coronaviruses) are represented by branches radiating at the bottom of the tree.
Only four annotated isolates appear as side branches radiating at intermediate positions – located in
the middle of the presented cladogram – positioned between distinct FIP/FCoV feline coronavirus
90 clade and canine coronavirus branches. Those four annotated feline coronavirus genomes represent
type II FIP/FCoV isolates. This pattern of branching is consistent with the accepted perspective that
the later type II FCoV represents a product of the inter-specific RNA-RNA recombination between
the aforementioned type I feline coronavirus with canine coronavirus (Herrewegh et al. 1998;
Terada et al. 2014; Jaimes et al. 2020).

95

Circulating feline coronaviruses are ubiquitous in the environment; still minimally resistant
to environmental damage - easily inactivated by most of the disinfectants and common detergents.
Primary FCoV infection is largely subclinical or only associated with transient (usually mild)
enteric symptoms (and reviewed elsewhere Addie et al. 2009, 2019; Pedersen 2014a: 2014b; Uzal et
100 al. 2016). Infections with mostly benign entero-tropic pathotypes of feline coronaviruses are
prevalent in the cat population. There seems to be strong evidence that FIP causing coronavirus is
not transmitted horizontally; instead, the pathogen evolves independently within each cat that
eventually develops symptoms of infectious peritonitis. An increase in pathogenicity, in that case,
observed as FIP is assumed to be balanced by the loss of infectivity. The pathomechanism of FIP,
105 sporadic transmissible disease of wild and domesticated felids, seems insufficiently defined.
According to the accepted perspective, FIP is a systemic form of immune-mediated idiosyncratic
reaction invoked by coronavirus replication, with poorly defined proximal disease triggers.

The detailed pathomechanism of FIP remains cryptic. Clinical picture is not reconciled easily with a modality of the immune response observed upon feline coronavirus replication. The disease may manifest in two extreme forms: effusive and non-effusive (the later referred also as a granulomatous or 'dry' form), depending on the type and the intensity of the adverse immune reactions. In the course of infection, acquired entero-tropic coronavirus replicates initially within the epithelial cells lining the enteric mucosa. The replication of the entero-tropic coronavirus limited to the subpopulation of cells implicates the replicase genes expressed first from infectious (+) RNA strands released from coronavirus capsids are restricted to the intestinal epithelium. Therefore, at that stage translated coronaviral RdRp will engage with genomic (+) RNA and synthesize antigenomic cRNA copy – an essential replicase co-factor required for (+) strand amplification and completion of the coronaviral replicative cycle – in a mode confined within the alimentary tract. That early stage of infection is consistent with enteric coronavirus transmission and explains how the shedding of the infectious (+)RNA strand embedded in coronavirus capsids – into the intestinal lumen occurs.

However, in a small proportion of infected cats mostly innocuous enteric coronavirus acquires hyper-replicative properties resulting in systemic spread of pervasive, FIP-invoking RNA. After crossing the intestinal wall the spread of pervasive RNA invoking FIP is accompanied by adverse immuno-pathological response involving many organs. Again for poorly defined reasons, factors, or conditions enteric coronavirus infection elicits a strictly maladaptive immune-mediated response of an inflammatory type. Quoting after Uzal et al. '*A requirement for the development of FIP is likely the capacity of the virus to replicate within monocytes of the host.*' Clearly, the replicative cycle proceeds through complementary-RNA intermediate regardless of the cell type supporting coronavirus replication. Expectedly both genomic and subgenomic cRNA replicons are required for the completion of the replication cycle, hence the replicative byproducts consisting of

dsRNA will form in the infected mononucleated phagocytes. During the systemic spread, feline
135 coronavirus is localized in the liver, spleen, lymph nodes, and wherever else macrophages are
found. The release of the virus infecting mononucleated phagocytes results in the release of
coronaviral molecules acting as epitope presenting antigens. Nonetheless, clinically observed is a
form of adverse immune reaction that leads to the formation of the excess immunoglobulin
complexes. The infection course is thought to depend on the type and strength of the maladaptive
140 immune reaction triggering the inflammatory response rather than particular feline coronavirus
subtype. In the infection course, as outlined above, coronavirus affected cats develop either effusive
(‘wet’) or non-effusive (‘dry’) forms of FIP, dominated by symptoms resulting from vasculitis and
pyogranulomatous inflammation respectively. The factors or conditions involved in wet/dry
transition presently remain elusive. Similarly, factors determining when and where benign enteric
145 coronavirus evolves into pervasive FIP-invoking RNA, remain covert.

The most common, effusive form (case presented in Fig. 2 and Fig. 3) is therefore regarded
as a transmissible inflammatory condition of visceral serosa and omentum. Coronavirus induced
serositis with vascular and perivascular lesions may manifest in many organs. Exudation into the
150 peritoneal cavity - signalment hallmark of FIP – severe abdominal distention rarely poses diagnostic
doubts. Additionally, pleural effusion may be present. The palpable peritoneal fluid accumulation
may be confirmed with diagnostic imaging. Definitive diagnosis requires abdominal cavity
puncture. Up to a liter of coronavirus RNA containing exudate could be drawn from a peritoneal
cavity of a single cat affected with an effusive form of FIP (Fig.4). The aspirated fluid is typical of
155 high specific-gravity (1.017–1.047), aseptic, and protein-rich (often above 50g/l), and of moderate
cellularity (Hsieh & Burney 2014). Further, the aspirate is typically translucent, opaque fluid, from
light yellowish to gold in color (Fig.5), intrinsically viscous, it may contain strands of fibrin, and
may appear flocculent.

160 The dominant symptom of ascites may be accompanied by other symptoms not
pathognomonic for FIP. In particular, dyspnea can be present in cats with pleural involvement.
Disease signalment may also involve apathy, malaise, fatigue, inappetence, weight loss, fluctuating
antibiotic irresponsive fever, jaundice, in addition to lymph node enlargement, ocular manifestations
(Fig. 6), and neurological symptoms. Leukocytosis and neutrophilia are concluded with routine
165 hematology counts. Hyperbilirubinaemia, hypergammaglobulinemia, and altered albumin-to-
globulin (A:G) ratio are concluded with serum biochemistry. Effusive FIP must be differentiated
from non-coronaviral peritonitis (e.g. bacterial).

 In contrast non-effusive i.e. granulomatous FIP, a disease of insidious onset, poses a
170 considerable diagnostic challenge. Most of the time only a presumptive diagnosis can be reached.
Symptoms caused by granuloma formation are grossly non-specific; rather reflect the malfunction
of the particular coronavirus affected organs (involving: hepatic icterus, nervous system symptoms,
renal symptoms, with concomitant fever - present singly or in combination). None of the above
symptoms justifies the legitimate diagnosis of the non-effusive form of coronaviral FIP.
175 Considerably altered albumin-to-globulin ratio (A:G) may be indicative, but may also be observed
in other, unrelated disorders (below). Intravital diagnosis may involve granuloma biopsy (of lymph
nodes or kidneys) followed by histopathology. However, due to technical impediments, the
intravital granuloma collection (given the variable spatiotemporal formation of granulomas) could
be challenging and expectedly biased toward false-negative results. Until recently (and still of
180 considerable concern), the conclusive diagnosis could only be ascertained post-mortem (Dunbar et
al. 2019).

 Altered albumin-to-globulin ratio, consisting of concomitant hypoalbuminemia with

hypergammaglobulinemia, is a common feature in both FIP forms. Hypergammaglobulinemia,
185 however, seems not directly related to elevated feline coronavirus antibody titers (Paltrinieri et
al.1998), foreshadowing the non-specific maladaptive immune-complex mediated process
underlying FIP pathogenesis. Serologically circulating antibodies recognizing feline coronavirus
epitopes appear detectable also in clinically unaffected cats (hence reasonable doubts concerning
the clinical utility of serological FIP examinations). The other relevant indication is based on certain
190 confirmed FIP cases that appear seronegative. Together, the consensus concerning causality
between the systemic spread of FIP coronavirus and the type of immune-complex mediated lesions
observed in the disease course is lacking. Clinically gammopathies could be mistaken for the
neoplastic process, as could granulomas may mimic a neoplasm.

195 Interestingly FIP, coronaviral disease of cats, bears considerable similarity to murine disease
caused by naturally contracted MHV observed in IFN- γ (-/-) deficient mice (France et al. 1999).
MHV induced pleuro-peritonitis in mice with targeted disruption into interferon- γ gene can be
regarded as an important model for serosal inflammation in coronaviral feline infectious peritonitis.
Moreover, both feline and murine coronaviruses infection result in either enteric or systemic
200 disease, where RdRp-dependent replication in macrophages is considered a key event in the
pathogenesis of coronaviral infection.

Above considerations prompted our interests in developing an improved methodologies for
detection of pervasive FIP invoking coronavirus-derived RNA species present in diseased felids.
205 While encapsidated (+)RNA strand is present in coronavirus virions it is possible that increased
load of coronavirus derived dsRNAs – themselves formed of coronavirus replicative intermediates
detectable in infected cats – may contribute to the observed immunopathology.

The sampling of the effusions is regarded as the most useful diagnostic step, especially in cases of effusive form, providing the ample of diagnostic material for the detection of coronaviral RNA (Addie et al 2019). Provided our early efforts in cDNA synthesis involved the implementation of recombinant *Thermus thermophilus* (rTth) DNA Polymerase I (Myers et al. 1991; used instead of retroviral reverse transcriptases) for reverse transcription step in amplification reactions of SL-RNA isolated from non-coronaviral enteric pathogen (Magalska A. 2000) were productive (Kapulkin WJ et al. 2016), we next turned our attention toward the detection of RNAs isolated from viral pathogens. Above methodologies were immediately successful in attempts of detection of feline coronaviral RNA in the ascitic fluid of naturally infected cats presented to us clinically (Kita P. 2002; also independently validated on canine distemper virus samples described in Jozwik A. 2004). Since both forward and reverse primers are included in the reverse transcriptase step, so both sense (+) RNA and anti-genomic complementary (-) strand RNA may initiate cDNA amplifications. This step has the advantage of simplicity in clinical settings; the entire reaction is confined to a single tube which minimizes the risk of cross-contamination and other errors of mishandling. However, in spite of initial enthusiasm, we (Kita et al. 2002) and others (Vennema et al. 1998; Pedersen 2012; 2014) collectively failed to discern between mostly avirulent FCoV and pathogenic FIP invoking feline coronavirus strains.

Since our early efforts focused on the detection of FIP-coronavirus RNA were reported and embedded into clinical guidelines (Addie et al. 2004) below we present certain relevant advances and formulate further suggestions.

230

i.) FIP virus RNA detection. Since the detection of anti-genomic (-) strand could be taken as *bona fide* evidence for coronaviral RdRp activity, we recommend that two separate strand-specific RT-reactions are initiated. Ideally, this could be done in a genome-wide manner (with 12-24 primer

pairs sparsed across genomic RNA), discriminating between (+) strand genomic RNA and
235 antigenomic cRNA (-) strand. This approach has been validated with several other than coronavirus
RNA viruses including Respiratory Syncytial virus (RSV), Influenza virus, and Hepatitis C virus
(HCV) preparations (Kapulkin WJ, Belino P, Godzik P, Szkoda T, et al. unpublished observations).
We further note additional primer pairs may be required to discern polyadenylated coronaviral
mRNAs and negative-sense subgenomic replicons derived from FIP virus RNA. Moreover, we
240 remark, the terminal 5' and 3' end sequences (not accessible with conventional amplification format)
may need to be determined to fully evaluate the diversity of circulating FIP replicons. This could be
achieved with a T4 RNA ligase-based viral RNA ligation used for circularization or concatenation
of the terminal 5' and 3' ends of coronaviral replicons (as described by Wang et al. 2014,
perceivably including the prior removal of 7-methyl guanosine-caps). While aware above steps may
245 generate additional costs (precluding the routine diagnostics), given the strand stoichiometry favors
(+) strand synthesis in the coronaviral replicative cycle, we collectively suggest the ratio of (+) and
(-) RNA strands may have a diagnostic value.

ii.) Approaches toward quantification of FIP RNA load. Quantitative RT-PCR reactions are
250 presently regarded as gold-standard (Claassen J. 2005) in viral RNA quantifications. However, qRT-
PCR approaches seem to suffer from a number of limitations (Chetverina et al. 2002), hence
Molecular Colony Technique (MCT) has been proposed as a convenient diagnostic alternative
(Chetverina & Chetverin 2015). Molecular Colony Technique is a nucleic acid amplification carried
in the RT-PCR compatible semi-solid format; typically a thin gel layer sufficiently flattened to
255 provide 2-D readout. Given the spatial separation of amplification products, viral nucleic acids
amplified in such settings form distinct foci comprised each of many copies descended of the initial
RNA molecule. An accurate representation of sampled coronavirus RNA load could be quantified
by simply counting the number of observed amplification foci reflecting the FIP RNA titer. MCT

should be useful in clinical settings when samples often contain a massive excess of other target and
260 non-target nucleic acids and when the presence of viruses other than coronavirus is expected.
Indeed combinatorial FIP infections have been reported, in cats involved with Feline
Immunodeficiency virus (FIV) and Feline Leukemia virus (FeLV), or other concomitant infectious
agents complicating the disease picture.

265 **iii.) FIP coronavirus RNA sequencing.** In recent years PCR primer-targeted sequencing of FIP
variants resulted in the characterization of polymorphisms tentatively associated with the systemic
spread of coronavirus infection (reviewed in Pedersen 2014). However, sequence-level FIP variant
characterization appears to lack the expected clear consensus (Pedersen et al. 2012) concerning
variants specifically involved with pathogenesis (Addie et al. 2019). Several RT-PCR limitations
270 may contribute; first and foremost entire coronavirus genome may not be amplified in a single
reaction. Additionally, error-prone RNA replication of the coronavirus genome may result in an
increase of the variant diversity (perceivably expected during the systemic spread of the pervasive
FIP RNA at a hyper-replicative disease stage). While PCR-MCT may overcome some of those
limitations, and enrich for some variants disfavoured by in vitro amplification reactions, recent
275 technological advances in RNA sequencing and computational data analysis (Dolja & Koonin 2018,
with relevant references therein) hold the promise of unbiased detection of coronaviral RNAs in
complex samples. In a recent example, the metagenomic analysis identified the pangolin niche for
zoonotic coronavirus (Wahba et al. 2020, with relevant discussion and references on metagenomic
virome analysis). Collectively, advances in sequence-based technology make the implementation of
280 unbiased FIP RNA detection methods into clinical sequencing increasingly feasible, providing a
mean of evaluation for loads of (+) and (-) RNA strands. Hereby we foresee unbiased detection of
FIP replicons should be clinically informative particularly in the settings when multiple samplings
are collected in a disease course to monitor FIP progression.

285 **iv.) Feline coronavirus-derived D-RNA or DI-RNA?** Perhaps a most confounding aspect of FIP
pathogenesis and one so far under-explored FIP research direction concern the detection of atypical
coronavirus-derived subgenomic replicons in diseased felids. Atypical RNA species smaller than
genomic RNA have been described to emerge in certain coronaviruses other than FIP: including
Murine Hepatitis virus (MHV) Makino et al. 1989; avian Infectious Bronchitis virus (IBV) Penzes
290 et al. 1996; and Transmissible Gastro-Enteritis virus (TGE) Masters & Perlman 2013. These smaller
RNA species are collectively referred to as defective RNAs (D-RNA) or defective-interfering RNAs
(DI-RNA). D-I-RNA (defective-or-interfering RNAs) are efficiently a form of parasitic RNA-
replicons, replicating at the convenience of coronaviral replicase, involving the synthesis of
replicative dsRNA intermediates. The emergence of D-I-RNA may interfere with coronavirus
295 replication hence may confer variable disease susceptibility. Sequence-based diagnostic
technologies will expectedly facilitate the discovery and aid in the characterization of atypical
subgenomic FIP replicons as well as permit for feline D-I-RNA characterization. Feline
coronavirus-derived D-RNA and/or DI-RNA emergence during infection and its association with
FIP progression remains still pending and an open question.

300

Significance and perspective

In this article we argue that feline coronavirus derived dsRNA act as mediator (or
305 **modulator) of idiosyncratic reaction underlying FIP pathogenesis.** Our article outlines critical
biogenesis steps of coronaviral replication perceivably involved in dsRNA formation in FIP
pathogenesis. While coronaviral agents may limit dsRNA formation, e.g. imposing strand-specific
RNA secondary structures, coupling RNA degradation with RNA strand sequestration, or some

other intricate means (Kindler &Thiel 2014; Nelemans &Kikkert 2019), the products of (+) and (-)
310 RNA strand collapse can constitute a key, while still underappreciated, event in FIP pathogenesis.

Therefore, conceivably FIP-borne dsRNA, of either genome or sub-genome size, may
constitute one of the key proximal pro-inflammatory mediators relevant in the coronaviral disease
of cats. This is because of well recognized side effects, known to occur after parenteral
315 administration of double-stranded RNAs acting as a potent inducer of the interferon response.
Induction of the interferon response pathway by dsRNA involve the recognition and sensing of viral
replicative intermediates by several components of the innate immune system. The innate immune
recognition of dsRNA has been extensively studied and characterized (Hilleman et al. 1971; Fire A.
2005; Gantier &Williams 2007; Nellimarla &Mossmann 2014 and elsewhere in cited literature)
320 albeit in animal systems other the felids, hence not specifically covered here. Of note, FIP treatment
with the available type of recombinant feline interferon accounts for viable therapeutic modality, of
some reported curative potential (Ishida et al. 2004). Collectively, we propose replicative
intermediates of feline coronavirus consisting of dsRNA may constitute previously unforeseen pro-
inflammatory mediator or modulator relevant in maladaptive immune response triggering the
325 progression of observed immunopathology.

Induction of the endogenous feline innate immune-response pathways by coronaviral
replicative intermediates corroboratively assumes to engage in dsRNA sensing pathways
characterized in other mammals. Innate immune dsRNA response observed in viral infections
330 (Kawai &Akira 2006) typically consider sensing of replicative intermediates (regarded as pathogen-
associated-patterns, PAMP) by certain types of pattern recognition receptors (PRR) - a dedicated
dsRNA sensing structures confined mostly to subcellular compartments supporting the coronaviral
replication. While coronavirus replication is confined to intracellular structures assembled FIP virus

may also be released by the lysis of the infected feline cells (Uzal et al. 2016). Extracellular viral
335 and monocytic antigens were indeed seen in the foci with scattered intercellular necrosis (Paltrinieri
et al.1998), suggesting the necrotic process may have affected FIP coronavirus infected cells. This
type of disordered release of encapsidated coronavirus (+) RNA strand would expectedly guide an
inadvertent release of dsRNA replicative intermediates into the extracellular compartment.
Moreover, other mechanisms leading to eventually lytic processes involving FIP virus-infected cells
340 may efficiently contribute to the extracellular release of coronavirus dsRNA. Here, we infer the
release of undegraded coronaviral replicative intermediates (consisting of complementary (+) and
(-) RNA strands) into the extracellular compartment may plausibly constitute the critical pathogenic
event underlying the systemic spread of feline coronavirus clinically presenting as FIP.

345 The extracellular exposure of coronavirus-derived dsRNA rises yet another unanticipated
possibility. The particular previously under-explored pathway concern dsRNA acting as a
remarkably efficient antigen engaging the detectable humoral immune response (Bonin et al. 2000).
Indeed experimental immunization (via parenteral administration of certain dsRNA formulations)
was shown to elicit specific types of anti-dsRNA antibodies in rabbits (Schwartz &Stollar 1969;
350 Kitagawa &Okuhara 1980), mice, rats, guinea-pigs, dogs, and baboons (Cunnington &Naysmith
1975). Moreover, several distinct murine monoclonal anti-dsRNA antibodies were developed
(Schönborn et al. 1991) presumably recognizing unique dsRNA epitopes. Those anti-dsRNA
antibodies were used to confirm the demonstrable reactivity against the number of RNA viruses
(Stollar et al. 1970; Silverstein &Schur 1970; Stollar &Stollar 1970; Miller et al. 1975; Weber et al.
355 2006; Son et al. 2015) replicating through dsRNA intermediates (i.e. complementary RNA strand
synthesized by RNA-dependent-RNA-polymerase). Markedly themselves instructive examples of
the practical use of anti-dsRNA antibody reactivities include certain, other than feline, coronaviral
agents (Comar et al. 2019; Hackbart et al. 2020 – with the relevant discussion on dsRNA epitopes).

In particular, Hackbart et al. study provides innovative approaches involving dsRNA-antibody
360 immunoprecipitation followed by RNA sequencing clearly applicable to FIP coronavirus.

In that context dsRNA-antibody formation in response to extracellular exposure of dsRNA
has not been considered in coronavirus infection. However, circulating dsRNA-reactive antibodies
were seen in certain autoimmune cases reported in humans and mice (Schur & Monroe 1969;
365 Koffler et al. 1974; Payne & Kalmakoff 1975; Winfield et al. 1975; Klassen et al. 1979). Elevated
levels of anti-dsRNA antibodies could be suggestive of the previous dsRNA exposures, hence
represent an unperceived factor contributing to coronavirus pathogenesis. If confirmed, broadly
reactive circulating anti-dsRNA antibodies may explain the mechanisms that trigger maladaptive
immune-complex mediated reactions observed in FIP progression. Perceivably undegraded dsRNAs
370 can act as multivalent ligands for itself dimeric anti-dsRNA antibodies resulting in extracellular
formation of ribonucleoprotein nets consisting of anti-dsRNA antibodies complexed with exposed
coronavirus-derived dsRNAs. This may explain immune-mediated systemic reactions involving
vicious antibody response resulting in maladaptive immune-complex formation. Provided
antibodies confer opsonizing properties, formation of dsRNA containing, circulating, immune-
375 complexes may explain certain aspects of macrophage involvement in FIP – suggesting a novel
mechanism for engulfment of dsRNA. Preferential replication in macrophages, by the same token,
perceivably could be a factor in the seemingly paradoxical phenomenon known as Antibody-
Dependent-Enhancement (ADE) postulated in certain viruses (Kulkarni 2019) including FIP
invoking feline coronavirus and coronaviruses infecting man (Negro 2020; Paltrinieri et al. 2020).
380 Postulated opsonizing properties of circulating anti-dsRNA antibodies may mediate viral RNA
engulfment by monocytes, hence underly certain unexplained features of maladaptive ADE activity
(Tirado et al. 2004), implicating the role of viral replicative dsRNA intermediates in that process.

Together, in an absence of the direct evidence, we suggest that certain viral replicative intermediates i.e. coronaviral dsRNA may elicit anti-dsRNA antibody response resulting in the formation of circulating dsRNA containing immune-complexes. Viral dsRNAs complexed with anti-dsRNA immunoglobulins may confer opsonizing properties (either direct or through interactions with certain complement components) mediating dsRNA phagocytosis. Acting as opsonins, circulating anti-dsRNA immunoglobulins, mediating macrophage invasion can lead to maladaptive activation of several components of the immune system. Since either genomic or subgenomic replicative intermediates (including DI-RNA replicons) of either partial or perfect complementarity could serve as engulfment cargo, immunological mischief can be envisioned. Elevated levels of anti-dsRNA immunoglobulins could be a contributing factor explaining the variability in the response to viral infection. Klassen et al. hypothesized *'it is possible that spontaneous production of anti-dsRNA in mammals may be caused by "immunization" with viral nucleic acid'*. In our view, this unsuspected possibility needs to be considered in terms of both clinical and scientific relevance. Notably, postulated 'immunization' with viral nucleic acids resulting in the formation of anti-dsRNA immunoglobulin could be perceived as an inter-specific phenomenon; previous exposure to certain viral-borne dsRNA can have a measurable effect on subsequent infection with unrelated dsRNA producing viral agents. More broadly, we suggest the release of nucleic acids into extracellular space could represent an unrecognized event in pathogenesis of viral infections, here specifically postulated as a proximal trigger of idiosyncratic reaction observed in FIP progression.

Acknowledgments

We thank professor Halina Wedrychowicz (SGGW) for providing us with the essential laboratory facilities, and professor Tadeusz Frymus (SGGW) for encouraging our interests in feline coronaviruses. We wish to thank Andrew Fire (Stanford) for insightful discussions concerning viral dsRNA. Some figures were reproduced with permission from an early version of this article that appeared in *Magazyn Weterynaryjny* 2000; 5: 19-21, Kita P, Kapulkin WJ. We also thank Iwona Malecka-Tepicht for assistance with graphical documentation.

References

- Addie DD, Paltrinieri S, Pedersen NC; Second international feline coronavirus/feline
415 infectious peritonitis symposium. Recommendations from workshops of the second
international feline coronavirus/feline infectious peritonitis symposium. *J Feline Med Surg.*
2004;6(2):125-130. doi:10.1016/j.jfms.2003.12.009
- Addie D, Belak S, Boucraut-Baralon C, Egberink H, Frymus T, Gruffydd-Jones T, et al
420 (2009): Feline infectious peritonitis. ABCD guidelines on prevention and management. *J*
Feline Med Surg 11, 594–604. [updated 2019: Diane D. Addie, Katrin Hartmann, Séverine
Tasker, Regina Hofmann-Lehmann, Herman Egberink, Karin Möstl et al.
<http://www.abcdcatsvets.org/feline-infectious-peritonitis/>
- 425 Bonin, M., Oberstrass, J., Lukacs, N., Ewert, K., Oesterschulze, E., Kassing, R., & Nellen, W.
(2000). Determination of preferential binding sites for anti-dsRNA antibodies on double-
stranded RNA by scanning force microscopy. *RNA (New York, N.Y.)*, 6(4), 563–570.
<https://doi.org/10.1017/s1355838200992318>
- 430 Chetverina HV, Samatov TR, Ugarov VI, Chetverin AB. Molecular colony diagnostics:
detection and quantitation of viral nucleic acids by in-gel PCR. *Biotechniques.* 2002
Jul;33(1):150-2, 154, 156. doi: 10.2144/02331md03. PMID: 12139240.
- Chetverina HV, Chetverin AB. Identifying RNA recombination events and non-covalent RNA-
435 RNA interactions with the molecular colony technique. *Methods Mol Biol.* 2015;1240:1-25.

doi: 10.1007/978-1-4939-1896-6_1. PMID: 25352133.

Claassen J. (2005). The gold standard: not a golden standard. *BMJ : British Medical Journal*, 330(7500), 1121.

440

Comar, C. E., Goldstein, S. A., Li, Y., Yount, B., Baric, R. S., & Weiss, S. R. (2019). Antagonism of dsRNA-Induced Innate Immune Pathways by NS4a and NS4b Accessory Proteins during MERS Coronavirus Infection. *mBio*, 10(2), e00319-19. <https://doi.org/10.1128/mBio.00319-19>

445

Cunningham PG, Naysmith JD. Naturally occurring double-stranded RNA and immune responses. III. Immunogenicity and antigenicity in animals. *Immunology*. 1975 Dec;29(6):1001-17. PMID: 811555; PMCID: PMC1446031.

450 Dolja VV, Koonin EV. Metagenomics reshapes the concepts of RNA virus evolution by revealing extensive horizontal virus transfer. *Virus Res*. 2018 Jan 15;244:36-52. doi: 10.1016/j.virusres.2017.10.020. Epub 2017 Nov 8. PMID: 29103997; PMCID: PMC5801114.

Dunbar D, Kwok W, Graham E, Armitage A, Irvine R, Johnston P, McDonald M,
455 Montgomery D, Nicolson L, Robertson E, Weir W, Addie DD. Diagnosis of non-effusive feline infectious peritonitis by reverse transcriptase quantitative PCR from mesenteric lymph node fine-needle aspirates. *J Feline Med Surg*. 2019 Oct;21(10):910-921. doi: 10.1177/1098612X18809165. Epub 2018 Nov 8. PMID: 30407137.

460 Fire A. Nucleic acid structure and intracellular immunity: some recent ideas from the world

of RNAi. *Q Rev Biophys.* 2005 Nov;38(4):303-9. doi: 10.1017/S0033583505004117. Epub 2006 Mar 6. PMID: 16515739.

France MP, Smith AL, Stevenson R, Barthold SW. Granulomatous peritonitis and pleuritis in
465 interferon-gamma gene knockout mice naturally infected with mouse hepatitis virus. *Aust Vet J.* 1999 Sep;77(9):600-4. doi: 10.1111/j.1751-0813.1999.tb13199.x. PMID: 10561796; PMCID: PMC7159722.

Gantier MP, Williams BR. The response of mammalian cells to double-stranded RNA.
470 *Cytokine Growth Factor Rev.* 2007 Oct-Dec;18(5-6):363-71. doi: 10.1016/j.cytogfr.2007.06.016. Epub 2007 Aug 14. PMID: 17698400; PMCID: PMC2084215.

Hackbart, M., Deng, X., & Baker, S. C. (2020). Coronavirus endoribonuclease targets viral
polyuridine sequences to evade activating host sensors. *Proceedings of the National Academy
475 of Sciences of the United States of America*, 117(14), 8094–8103.
<https://doi.org/10.1073/pnas.1921485117>

Hagemeijer MC, Rottier PJ, de Haan CA. Biogenesis and dynamics of the coronavirus
replicative structures. *Viruses.* 2012 Nov 21;4(11):3245-69. doi: 10.3390/v4113245. PMID:
480 23202524; PMCID: PMC3509692.

Hartenian E, Nandakumar D, Lari A, Ly M, Tucker JM, Glaunsinger BA. The molecular
virology of coronaviruses. *J Biol Chem.* 2020 Sep 11;295(37):12910-12934. doi:
10.1074/jbc.REV120.013930. Epub 2020 Jul 13. PMID: 32661197; PMCID: PMC7489918.

485

- Herrewegh AA, Smeenk I, Horzinek MC, Rottier PJ, de Groot RJ. Feline coronavirus type II strains 79-1683 and 79-1146 originate from a double recombination between feline coronavirus type I and canine coronavirus. *J Virol*. 1998 May;72(5):4508-14. PMID: 9557750; PMCID: PMC109693.
- 490
- Hilleman M.R. et al. (1971) Double-Stranded RNA's in Relation to Interferon Induction and Adjuvant Activity. In: Beers R.F., Braun W. (eds) *Biological Effects of Polynucleotides*. Springer, Berlin, Heidelberg. https://doi.org/10.1007/978-3-642-85772-0_3
- 495 Hsieh B, Burney DP. Feline Infectious Peritonitis [2014]. *Clinician's Brief* 2014. <https://www.cliniciansbrief.com/article/feline-infectious-peritonitis-2014>
- Ishida T, Shibana A, Tanaka S, Uchida K, Mochizuki M. Use of recombinant feline interferon and glucocorticoid in the treatment of feline infectious peritonitis. *J Feline Med Surg*. 2004 Apr;6(2):107-9. doi: 10.1016/j.jfms.2003.08.011. PMID: 15123155; PMCID: PMC7129501.
- 500
- Jaimes JA, Millet JK, Stout AE, André NM, Whittaker GR. A Tale of Two Viruses: The Distinct Spike Glycoproteins of Feline Coronaviruses. *Viruses*. 2020;12(1):83. Published 2020 Jan 10. doi:10.3390/v12010083
- 505
- Jozwik A. 2004. PhD Thesis. Faculty of Veterinary Medicine. University SGGW -Warsaw.
- Kapulkin WJ, Magalska A, Janecka E, Ciesielski A, Lobočka M, Behnke J, Wedrychowicz H. Recombinant DNA resources for the comparative genomics of *Ancylostoma ceylanicum*. [CSHL-BP] bioRxiv 092494; Dec 17, 2016; doi: <https://doi.org/10.1101/092494>
- 510

- Kawai T, Akira S. Innate immune recognition of viral infection. Nat Immunol. 2006 Feb;7(2):131-7. doi: 10.1038/ni1303. PMID: 16424890.**
- 515 **Kindler, E., & Thiel, V. (2014). To sense or not to sense viral RNA-essentials of coronavirus innate immune evasion. Current Opinion in Microbiology, 20. <https://doi.org/10.1016/j.mib.2014.05.005>**
- Kita P. 2002. PhD Thesis. Faculty of Veterinary Medicine. University SGGW -Warsaw.**
- 520 **Kita, P., Frymus, T., Kapulkin, W., 2002 Detection of feline coronavirus RNA in ascitic fluid and blood of naturally infected cats by reverse transcriptase PCR. Second International Feline Coronavirus/Feline Infectious Peritonitis Symposium, Glasgow, Scotland.**
- 525 **Kitagawa Y, Okuhara E. Demonstrations of the production of specific antibodies to poly(I).poly(C) in rabbits. J Biochem. 1980 Dec;88(6):1607-13. doi: 10.1093/oxfordjournals.jbchem.a133136. PMID: 6780544.**
- Klassen LW, Williams GW, Reinertsen JL, Gerber NL, Steinberg AD. Ribavirin treatment in**
- 530 **murine autoimmune disease. I. Therapeutic efficacy and effect on the immune response. Arthritis Rheum. 1979 Feb;22(2):145-54. doi: 10.1002/art.1780220207. PMID: 33680.**
- Koffler, D., Agnello, V., & Kimkel, H. G. (1974). Polynucleotide immune complexes in serum and glomeruli of patients with systemic lupus erythematosus. The American journal of**
- 535 **pathology, 74(1), 109–124.**

- Kulkarni R. (2019). Antibody-Dependent Enhancement of Viral Infections. Dynamics of Immune Activation in Viral Diseases, 9–41. https://doi.org/10.1007/978-981-15-1045-8_2**
- 540 **Magalska A. 2000. Diploma Thesis. Interfaculty School of Biotechnology/Faculty of Veterinary Medicine. University SGGW -Warsaw.**
- Makino S, Lai MM. High-frequency leader sequence switching during coronavirus defective interfering RNA replication. J Virol. 1989 Dec;63(12):5285-92. doi: 10.1128/JVI.63.12.5285-5292.1989. PMID: 2555555; PMCID: PMC251194.**
- 545 **Masters PS, Perlman S. Coronaviridae. In: Knipe DM, Howley PM, eds. Fields Virology. Philadelphia, PA.: Lippincott Williams & Wilkins; 2013**
- 550 **Miller, J. R., Calliguri, L. A., & Tamm, J. (1975). Reaction of poliovirus RNAs with antibodies to double-stranded RNA demonstrated by an immunochemical binding assay. Journal of virology, 16(2), 290–297. <https://doi.org/10.1128/JVI.16.2.290-297.1975>**
- 555 **Myers TW, Gelfand DH. Reverse transcription and DNA amplification by a Thermus thermophilus DNA polymerase. Biochemistry. 1991 Aug 6;30(31):7661-6. doi: 10.1021/bi00245a001. PMID: 1714296.**
- Negro F. Is antibody-dependent enhancement playing a role in COVID-19 pathogenesis? Swiss Med Wkly. 2020 Apr 16;150:w20249. doi: 10.4414/smw.2020.20249. PMID: 32298458.**
- 560

- Nelemans T, Kikkert M. Viral Innate Immune Evasion and the Pathogenesis of Emerging RNA Virus Infections. *Viruses*. 2019 Oct 18;11(10):961. doi: 10.3390/v11100961. PMID: 31635238; PMCID: PMC6832425.
- 565 Nellimarla S, Mossman KL. Extracellular dsRNA: its function and mechanism of cellular uptake. *J Interferon Cytokine Res*. 2014 Jun;34(6):419-26. doi: 10.1089/jir.2014.0002. PMID: 24905198.
- 570 Paltrinieri S, Cammarata MP, Cammarata G, Comazzi S. Some aspects of humoral and cellular immunity in naturally occurring feline infectious peritonitis. *Vet Immunol Immunopathol*. 1998 Oct 23;65(2-4):205-20. doi: 10.1016/s0165-2427(98)00155-x. PMID: 9839875; PMCID: PMC7119882.
- 575 Paltrinieri, S., Giordano, A., Stranieri, A. and Lauzi, S. (2020), Feline infectious peritonitis (FIP) and coronavirus disease 19 (COVID-19): are they similar?. *Transboundary and Emerging Diseases*. Accepted Author Manuscript. doi:10.1111/tbed.13856
- 580 Pedersen NC, Liu H, Scarlett J, Leutenegger CM, Golovko L, Kennedy H, Kamal FM. Feline infectious peritonitis: role of the feline coronavirus 3c gene in intestinal tropism and pathogenicity based upon isolates from resident and adopted shelter cats. *Virus Res*. 2012 Apr;165(1):17-28. doi: 10.1016/j.virusres.2011.12.020. Epub 2012 Jan 17. PMID: 22280883; PMCID: PMC7114484. [updated at <https://ccah.vetmed.ucdavis.edu/cats/resources/general-feline-infectious-peritonitis-resources>]
- 585 Pedersen NC. An update on feline infectious peritonitis: diagnostics and therapeutics. *Vet J*.

2014 Aug;201(2):133-41. doi: 10.1016/j.tvjl.2014.04.016. Epub 2014 May 2. PMID: 24857253;
PMCID: PMC7110619.

Pedersen NC. An update on feline infectious peritonitis: virology and immunopathogenesis.
590 Vet J. 2014 Aug;201(2):123-32. doi: 10.1016/j.tvjl.2014.04.017. Epub 2014 May 2. PMID:
24837550; PMCID: PMC7110662.

Pénzes Z, Wroe C, Brown TD, Britton P, Cavanagh D. Replication and packaging of
coronavirus infectious bronchitis virus defective RNAs lacking a long open reading frame. J
595 Virol. 1996 Dec;70(12):8660-8. doi: 10.1128/JVI.70.12.8660-8668.1996. PMID: 8970992;
PMCID: PMC190960.

Plant EP, Dinman JD. The role of programmed-1 ribosomal frameshifting in coronavirus
propagation. Front Biosci. 2008;13:4873-4881. Published 2008 May 1. doi:10.2741/3046
600

Schönborn, J., Oberstrass, J., Breyel, E., Tittgen, J., Schumacher, J., & Lukacs, N. (1991).
Monoclonal antibodies to double-stranded RNA as probes of RNA structure in crude nucleic
acid extracts. Nucleic acids research, 19(11), 2993–3000. <https://doi.org/10.1093/nar/19.11.2993>

605 Schur PH, Monroe M. Antibodies to ribonucleic acid in systemic lupus erythematosus. Proc
Natl Acad Sci U S A. 1969;63(4):1108-1112. doi:10.1073/pnas.63.4.1108

Schwartz EF, Stollar BD. Antibodies to polyadenylate-polyuridyate copolymers as reagents
for double strand RNA and DNA-RNA hybrid complexes. Biochem Biophys Res Commun.
610 1969 Apr 10;35(1):115-20. doi: 10.1016/0006-291x(69)90490-2. PMID: 4976191.

- Silverstein SC, Schur PH. Immunofluorescent localization of double-stranded RNA in reovirus-infected cells. *Virology*. 1970 Jul;41(3):564-6. doi: 10.1016/0042-6822(70)90178-9. PMID: 4912824.
- 615
- Son KN, Liang Z, Lipton HL. Double-Stranded RNA Is Detected by Immunofluorescence Analysis in RNA and DNA Virus Infections, Including Those by Negative-Stranded RNA Viruses. *J Virol*. 2015 Sep;89(18):9383-92. doi: 10.1128/JVI.01299-15. Epub 2015 Jul 1. PMID: 26136565; PMCID: PMC4542381.
- 620
- Stollar BD, Stollar V. Immunofluorescent demonstration of double-stranded RNA in the cytoplasm of Sindbis virus-infected cells. *Virology*. 1970 Sep;42(1):276-80. doi: 10.1016/0042-6822(70)90270-9. PMID: 4918274.
- 625
- Stollar V, Stollar BD. Immunochemical measurement of double-stranded RNA of uninfected and arbovirus-infected mammalian cells. *Proc Natl Acad Sci U S A*. 1970 Apr;65(4):993-1000. doi: 10.1073/pnas.65.4.993. PMID: 5266168; PMCID: PMC283014.
- 630
- Terada, Y., Matsui, N., Noguchi, K., Kuwata, R., Shimoda, H., Soma, T., Mochizuki, M., & Maeda, K. (2014). Emergence of pathogenic coronaviruses in cats by homologous recombination between feline and canine coronaviruses. *PloS one*, 9(9), e106534. <https://doi.org/10.1371/journal.pone.0106534>
- Tirado SM, Yoon KJ. Antibody-dependent enhancement of virus infection and disease. *Viral Immunol*. 2003;16(1):69-86. doi: 10.1089/088282403763635465. PMID: 12725690.
- 635

- Uzal, F. A., Plattner, B. L., & Hostetter, J. M. (2015). Alimentary System. In Jubb, Kennedy and Palmer's Pathology of Domestic Animals: Sixth Edition (Vol. 2, pp. 1-257.e2). Elsevier Inc.. <https://doi.org/10.1016/B978-0-7020-5318-4.00007-3>
- 640
- Vennema H, Poland A, Foley J, Pedersen NC. Feline infectious peritonitis viruses arise by mutation from endemic feline enteric coronaviruses. *Virology*. 1998 Mar 30;243(1):150-7. doi: 10.1006/viro.1998.9045. PMID: 9527924; PMCID: PMC7131759.
- 645 Wahba L, Jain N, Fire AZ, Shoura MJ, Artiles KL, McCoy MJ, Jeong DE. An Extensive Meta-Metagenomic Search Identifies SARS-CoV-2-Homologous Sequences in Pangolin Lung Viromes. *mSphere*. 2020 May 6;5(3):e00160-20. doi: 10.1128/mSphere.00160-20. PMID: 32376697; PMCID: PMC7203451.
- 650 Wang R, Xiao Y, Taubenberger JK. Rapid sequencing of influenza A virus vRNA, cRNA and mRNA non-coding regions. *J Virol Methods*. 2014 Jan;195:26-33. doi: 10.1016/j.jviromet.2013.09.005. Epub 2013 Oct 4. PMID: 24096269; PMCID: PMC4149180.
- Weber F, Wagner V, Rasmussen SB, Hartmann R, Paludan SR. Double-stranded RNA is
655 produced by positive-strand RNA viruses and DNA viruses but not in detectable amounts by negative-strand RNA viruses. *J Virol*. 2006 May;80(10):5059-64. doi: 10.1128/JVI.80.10.5059-5064.2006. PMID: 16641297; PMCID: PMC1472073.
- Winfield, J. B., Koffler, D., & Kunkel, H. G. (1975). Specific concentration of polynucleotide
660 immune complexes in the cryoprecipitates of patients with systemic lupus erythematosus. The

665

670

675

680

685

FIGURES

695 **Figure 1.** Feline Infectious Peritonitis/Feline coronavirus whole-genome alignment presented as the radial tree of distance (Fast Minimum Evolution method; default parameters applied over BLAST homology search with gb NC_002306.3). Feline coronavirus/FIP isolates cluster at the top of the presented tree. Canine coronavirus isolates appear distant as separate branches at the bottom of the tree, along with PRCV (porcine coronavirus) clade.

700

705 **Figure 2.** Clinically presented coronaviral FIP ascites. Symmetric distention in abdominal region.

715

720

Figure 3. Clinically manifested coronaviral FIP ascites. Lateral presentation (same cat as in Fig.2). Severe abdominal distention.

725

730

735

740

745

Figure 4. Up to a liter of coronavirus RNA containing exudate could be drawn from the peritoneal cavity of a single cat affected with an effusive form of FIP.

750

755

760

765

770

Figure 5. Clinically manifested coronaviral FIP ascites. The typical appearance of coronavirus RNA containing effusions (see text for details) aspirated with a 20ml syringe.

800 **Figure 6.** Ocular manifestations of FIP.