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Title: Advances in molecular diagnostics and treatment of feline infectious peritonitis

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Synopsis (1-paragraph)

Feline infectious peritonitis (FIP) is a common differential for disease in, often younger, cats. Obtaining a definitive diagnosis by minimally-invasive means can be challenging, and a balance of probability might need to be used to guide further investigation or treatment. Although treatment is currently limited, novel anti-viral agents show real promise for the future.

Key words (5-8)

- Feline coronavirus
- Reverse-transcriptase polymerase chain reaction
- Pyogranulomatous inflammation
- Effusions
- Protease inhibitors
- Nucleoside analog
- GS-441524

Key Points

- Appreciation of the relationship between feline coronavirus (FCoV) and feline infectious peritonitis (FIP) is vital in interpreting guidance on diagnosis, treatment and prevention
- Presumptive diagnosis in most cases is relatively straightforward; however, achieving confidence in a diagnosis in some cats is more complex as is definitive confirmation of FIP
- Molecular diagnostics (especially FCoV-targeted reverse-transcriptase quantitative polymerase chain reaction on tissue or effusion samples) can increase our confidence in a diagnosis of FIP, but an appreciation of their methodology is necessary to understand their limitations
- Recently some novel therapeutics have been shown to be effective in the treatment of FIP (viral protease inhibitors; nucleoside analogs); however, more studies are required

Introduction

Background

Feline coronavirus (FCoV) is ubiquitous worldwide. Infection is common among the domestic cat population, usually only causing mild enteric signs (e.g. diarrhea). In a small percentage of FCoV-infected cats, viral mutations, systemic spread and aberrant immune response results in a syndrome of serositis, vasculitis and pyogranulomatous lesions known feline infectious peritonitis (FIP). A presumptive diagnosis of FIP is often made in sick, particularly young, cats with the effusive disease; however, variability in presentation and test limitations can make obtaining a definitive diagnosis or even a presumptive diagnosis using non- or minimally-invasive approaches difficult. In the absence of treatment using novel anti-viral agents, FIP is fatal in the overwhelming majority of cases.

Viral properties

- FCoV is an enveloped, single-stranded, positive-sense RNA coronavirus of the *Alphacoronavirus* genus (**Figure 1**)

    - Other viral species within this genus include transmissible gastroenteritis virus (TGEV) in pigs, canine coronavirus (CCoV) in dogs and human coronaviruses (HCoV-NL63; HCoV-229E)
    - Human pathogens severe acute respiratory syndrome-coronavirus (SARS-CoV), Middle East respiratory syndrome-coronavirus (MERS-CoV), and SARS-CoV-2 (the cause of COVID-19) are of the *Betacoronavirus* genus
- Coronavirus genomes are relatively large (for an RNA virus) (**Figure 2**)[1] and encode:
    - A large, non-structural polyprotein (pp1a; pp1ab), which is cleaved into smaller proteins (including proteases and the viral RNA polymerase)
    - Spike (S) glycoprotein – a trimeric transmembrane protein involved in host-cell receptor binding and cell entry; forms part of the viral envelope
    - Envelope (E) protein – forms part of the viral envelope
    - Membrane (M) protein (a.k.a. Matrix protein) – forms part of the viral envelope
    - Nucleocapsid (N) protein – interacts with the viral genomic RNA
    - Non-structural proteins (3abc and 7ab) – the function of these proteins is poorly understood; however, it is suspected that they play a role in viral replication and release, as well as interfering with the host cellular response to infection (e.g. inhibition of apoptotic pathways)
- Like other RNA viruses, FCoV exhibits a high rate of mutation during replication and exist as clusters of genetically diverse populations
- FCoV infects domestic and wild felids
    - FCoV is not transmissible to humans
- Two biotypes of FCoV are described [2, 3]:
    - Feline enteric coronavirus (FECV) – the ‘avirulent’ enteric form of FCoV; replicates mainly within enterocytes; can cause enteric clinical signs; is shed in feces
    - Feline infectious peritonitis virus (FIPV) – the ‘virulent’ systemic form of FCoV; replicates within monocytes and tissue macrophages, leading to systemic spread; results in the development of FIP in a minority of infected cats; shedding in feces is possible

    - FIPV is considered to arise from FECV as a result of mutation within individual cats ('internal mutation' hypothesis in comparison to the 'distinct circulating avirulent and virulent strains' hypothesis)
    - Genetic analysis of FIPV isolates reveal them to be most closely related to the FECV from which they arose (rather than to other FIPV isolates)
- Two serotypes of FCoV exist:
    - Type 1 – predominates worldwide; difficulties in cultivation in vitro have limited research
    - Type 2 – arose following genetic recombination between FCoV and CCoV; genetic analyses have demonstrated that this has occurred on multiple separate occasions [4-6]; extensively studied as can be cultured in vitro
    - Serotypes are differentiated primarily based upon the S glycoprotein, either by the immunological response they trigger (e.g. detection of virus neutralizing antibodies) [3], or more recently, by gene amplification and sequencing (as the change in antigenicity is due to genetic recombination detectable through this method) [7]
    - Infection with either serotype has been associated with both enteric disease and FIP, therefore either serotype can be present as either biotype
- Feline infection with other coronaviruses
    - Following detection of antibody cross-reactivity between closely related coronavirus species (incl. FCoV serotypes 1 & 2, CCoV, and TGEV) [8, 9], the potential role of cats as a vector of these infections or whether exposure to these infections conferred either protection against or enhancement of subsequent infection with FCoV was explored in early experimental studies
    - Following exposure to TGEV cats developed transient, subclinical infection with shedding in feces [10, 11]; cross-reactive antibodies were produced; protection against infection with FCoV, and subsequent development of FIP, was not documented
    - Following exposure to CCoV cats developed transient, subclinical infection with no fecal shedding detected [12]; cross-reactive antibodies were produced; neither protection against nor antibody-dependent enhancement (ADE) of infection with FCoV (and subsequent development of FIP) was documented

- Exposure to HCoV 229E did not result in clinical signs, nor the production of cross-reactive antibodies, although virus-specific antibodies were produced [13]; neither protection against nor ADE of infection with FCoV (and subsequent development of FIP) was documented

Prevalence

- FCoV is found in cats worldwide, other than on a small number of isolated islands
- FCoV frequently circulates in multicat households
  - Seroprevalence (reviewed elsewhere [14]): is significantly greater in multicat households (26-87%) than in single-cat households (4-24%)
  - In environments in which FCoV is endemic, most cats experience repeated cycles of infection and subsequent viral elimination [15, 16]
  - In some cats, the initial infection persists and is chronic (+/- intermittent) shedding may occur [16, 17]
- Incidence of FIP is low in comparison:
  - 1 in 5000 cats affected in one or two cat households
  - 5-10% of cats affected in some catteries [18, 19]
  - FIP is usually sporadic; rarely epidemics can occur, and can possibly be explained by a combination of:
    - High population density settings (e.g. breeding catteries; rescue shelters; feral cat populations)
    - Shared genetic background
    - Shared challenges to immune function (e.g. stress; limited resources)
    - Shared viral factors

FIP risk factors

- Some of the risk factors for the development of FIP likely relate to risk factors for FCoV infection

- Some studies have indicated increased risk of FIP in multicat households; however, a recent study noted that the majority of cats were living in a single- or two-cat households at the time of diagnosis [20], although this would not necessarily have reflected their situation at time of exposure to FCoV
- Male cats are at slightly higher risk of FIP [20]
- Genetic susceptibility
    - Siblings of cats with FIP are considered to be at increased risk of developing FIP (~2x risk)
    - Some studies have indicated increased risk in specific pedigree breeds [21, 22]; however, this is not borne-out by every study [20]. and there was geographical variation in the breeds identified as increased risk [21, 22]. In addition there may be a degree of reporting bias (positive and negative) from the cat fancy community
- There is an increased incidence of FIP in kittens and young adult cats (55% cases ≤2 years), with a secondary peak in older cats (>10 years). However, FIP can affect cats of all ages
    - Experimental work has shown that resistance to infection increases from 6 months' age to >1 year [23]
- Stress is often a prominent historical feature e.g. recent rehoming, neutering, vaccination etc.

Transmission

- FECV is transmitted horizontally between cats, primarily via the feco-oral route
    - Litter trays are the primary source of infection
    - Contaminated fomites (e.g. grooming equipment; soft furnishings) may also play a role
- Oronasal route, via saliva and respiratory secretions, may also play a role [24, 25]; however, further investigation is required to characterize this further
- Whether fecally-shed FIPV shed is competent of horizontal transmission of is unclear [26]
- Vertical transmission *in utero* (i.e. of FIPV from the queen with FIP to her kitten(s)) is considered possible but rare [27, 28]
- Iatrogenic transmission, via parenteral injection or aerosolization of FIPV derived from cats with FIP, has been demonstrated experimentally [13, 29]

- In large, endemically-infected, multi-cat households, kittens commonly become infected at a young age, mostly at 5-6 weeks, as maternally-derived-antibodies (MDA) wane below protective levels [18]
    - The queen is suspected to be the most common source of infection, followed by other breeding or non-breeding cats (especially older litters of kittens)
- FCoV survives 1-2 days at room temperature; but may survive up to 7 weeks in a dry environment (e.g. in feces) [30]; Fuller's earth-based cat litters appeared to be most effective at inactivating FCoV in vitro, but they failed to prevent transmission in vivo [31]
- FCoV is inactivated by most disinfectants

Pathogenesis

- The exact pathogenesis of the development of FIP is still under investigation
- It suspected that FCoV strains of variable virulence, or variable potential for virulence, are circulating in the general feline population; this could, in part, account for some outbreaks
- Ingestion of FCoV (as FECV) → small intestinal villi enterocytes are the primary site of host cell entry, with spread to colonic enterocytes
    - Viral spike protein binds to serotype-specific cell entry receptors → internalization of virus
        - The cell receptor for serotype 1 FCoV is unknown
        - Aminopeptidase N (APN; CD13) is the cell receptor for serotype 2 FCoV, for macrophages at least [32]
    - Replication within enterocytes
        - Local inflammatory reaction → immune response → infection may be cleared or persist in chronic infections (esp. in colonic enterocytes)
        - Shedding in feces within 7 days → duration of shedding is highly variable (weeks; months; lifelong) [17]
    - Intestinal macrophages acquire FCoV from infected enterocytes [33] (exact mechanism unknown) → regional lymph nodes (e.g. mesenteric) → monocyte-associated viremia in most cats [34, 35]

- FCoV mutates (i.e. FECV → FIPV), resulting in progressive acquisition of enhanced tropism for, and increased ability to replicate within, monocytes / macrophages → further systemic spread (monocyte-associated viremia)
- In an estimated 10% of cats with systemic FCoV (as FIPV) infection [36], an aberrant immune response develops whereby activated monocytes / macrophages infected with FCoV interact with endothelial cells [37] → granulomatous phlebitis and periphlebitis = FIP

- FCoV has also been detected in conjunctival, nasal, and oropharyngeal tissue [25]; its role in upper respiratory tract disease is unknown
- Role of the host immune-response in FIP pathogenesis [38]
  - A poor cell-mediated immune response results in vasculitis, particularly affecting serosal surfaces; this vasculitis / serositis leads to fluid accumulations in one or more body cavity (i.e. peritoneal > pleural > pericardial) and is termed effusive FIP
  - A partial cell-mediated immune response leads to pyogranulomatous or granulomatous lesions in organs (often kidneys, liver, lungs, eyes, CNS, mesenteric LNs and gastro-intestinal tract), and in the absence of effusions is termed non-effusive FIP
  - These likely reflect a continuum:
    - Some cats with initially non-effusive disease will develop effusions
    - Cats with effusive disease often have granulomas present in parenchymal organs
- Viral factors in FIP pathogenesis:
  - Viral mutations
    - *Spike* gene
      - Functional mutations (M1058L and S1060A) within the putative fusion peptide of serotype 1 FCoV were able to differentiate 95.8% isolates of FECV and FIPV in one study [39]; the FCoV isolates used were either tissue-derived from cats with FIP (i.e. FIPV) or feces-derived from healthy cats (i.e. FECV). This led to the suggestion that presence of either functional mutation is diagnostic for FIP (see *FCoV mutation analysis* and **Table 3**). A more recent study found that 12 of 45 (26.7%) cats without FIP had at least one

tissue or effusion sample that was positive for FCoV, and of the 18 samples from these 12 cats where *Spike* gene sequencing was successful 16 (88.9%) had functional mutations consistent with FIPV [7]

- Functional mutations within the putative furin cleavage site of serotype 1 FCoV were able to differentiate 92.7% isolates of FECV and FIPV in another study [40]; again, the FCoV isolates used were either tissue-derived from cats with FIP or feces-derived from healthy cats

- *Non-structural protein 3c* gene – mutations encoding a truncated protein are present approximately 2 in 3 cats with FIP, whereas the 3c genes are intact in cats without FIP [41-43]; again, the FCoV isolates used were either tissue-derived from cats with FIP or feces-derived from healthy cats. This has led to the conclusion that intact 3c is a requirement for enterocyte infection, but not systemic spread.
- *Non-structural protein 7b* gene – mutations are present in FCoV derived from both cats with FIP and cats without FIP; their role in the development of FIP is unknown

- Viral mutations are thought to occur during bursts of viral replication (e.g. following a period of immunosuppression)
- Some cats experience waves of clinical disease (e.g. fever and weight loss) that coincide with T-cell depletion and increased viral loads in the blood [38]
- Acquired mutations are also suspected to have a role in tissue tropism – a functional genetic mutation in the *Spike* gene was only found in viral RNA extracted from the neurological tissue of a cat with neurological FIP but not in viral RNA extracted from other organs from the same cat [44]. The same mutation was found in FCoV purified from the neurological tissue from another cat with neurological FIP

Clinical signs of enteric FCoV infection

- Often subclinical
- Replication within enterocytes may cause mild enteric-associated signs (e.g. inappetence, diarrhea, vomiting); rarely causes severe enteritis

- FCoV has been detected in conjunctival, nasal, and oropharyngeal swab samples in cats with upper respiratory tract signs [25]; however, the role of FCoV in upper respiratory tract disease requires further investigation

Clinical signs of FIP

- Two clinical variants of FIP disease are recognized
    - Effusive ('wet') form – where effusions develop in one or more body cavity as a result of vasculitis/serositis; accounts for ~80% of cases of FIP [20]
    - Non-effusive ('dry') form – where pyogranulomatous lesions are present in one or more parenchymal tissue
    - At post-mortem examination this distinction is often less clear, with many cats diagnosed with effusive disease having pyogranulomatous lesions within parenchymal tissue, and some cats diagnosed with non-effusive disease having clinically inapparent effusions present
- The incubation period, from initial FCoV infection to development of FIP is highly variable; clinical signs of effusive disease typically present earlier than those of non-effusive disease [45]
    - Following parenteral administration of FIPV, clinical signs of effusive disease developed after 2-14 days, whilst it took several weeks for clinical signs of non-effusive disease to develop
    - In specific-pathogen-free (SPF) cats, infected 'naturally' by exposure to cats known to be shedding FCoV, the first clinical signs of FIP occurred from 6 weeks post-exposure [46]
    - MDA against FCoV typically decline at around 4-8 weeks age; but kittens as young as 2-weeks of age have been diagnosed with FIP (based upon either histological diagnosis or effusion analysis with immunostaining) although it is not known how these kittens acquired FCoV nor whether they had MDA [20]
- Effusive disease typically progresses more rapidly than non-effusive disease
    - 6 to 42 days (average, 14 days) from onset of clinical signs to death in naturally-infected SPF cats with effusive disease compared with weeks to months for non-effusive disease [34]
    - When FIP is a differential diagnosis, a careful search for cavitary effusions should be made (and likely repeated if initially unsuccessful; especially following rehydration)

    - The non-effusive form of FIP is typically more difficult to diagnose
    - Cats with effusive disease (cf. non-effusive disease) are: more likely to have pyrexia, lymphopenia, and icterus; and less likely to have ocular or neurological signs, azotemia and hyperproteinemia [20, 45]

- The range of presenting signs and abnormalities on physical examination associated with FIP are variable due to the number(s) and type of organs involved in individual patients [20, 45]
    - Non-specific signs including pyrexia (non-responsive to antibiotics), lethargy and inappetence are common, although some cats remain bright until the fulminant stages of disease
    - Icterus of sclera and mucous membranes (often mild)
    - Mucous membrane pallor, due to anemia (often mild)
    - Abdominal distention, associated with ascites and /or abdominal organomegaly (often representing mesenteric lymphadenopathy, gastro-intestinal masses with focal infiltration, or renomegaly)
    - Respiratory signs (including dyspnea, tachypnea, and cough) may be associated with pleural effusion and / or pulmonary infiltration (NB: pericardial effusions are sometimes seen, but are rarely associated with cardiac tamponade)
    - Evidence of ocular involvement: uveitis (keratic precipitate formation, anisocoria, dyscoria and blepharospasm); chorioretinitis with perivascular cuffing; retinal detachment (→ acute loss of vision); hyphema; hypopyon
    - Neurological signs, attributed to meningoencephalitis or meningomyelitis, with or without obstructive hydrocephalus, are often multifocal and can include ataxia, seizures, vestibular signs (e.g. head tilt, nystagmus), cranial nerve deficits, and behavioral change (e.g. obtundation)
    - Cutaneous lesions (rare), due to perivascular pyogranulomatous dermatitis, include papular, non-pruritic lesions

Diagnostic tests for FCoV exposure or shedding

- Serology

- The uses and limitations of serological testing of cats for coronavirus antibodies has been extensively reviewed elsewhere [47, 48]
- Antibodies may be detected in serum by ELISA (e.g. FCoV/FIP Immunocomb, Biogal), immunofluorescence antibody test (IFAT, various), or immunochromatographic test (e.g. Speed F-Corona, Virbac)
    - Some of these assays (e.g. FCoV/FIP Immunocomb; Speed F-Corona) are point-of-care and give qualitative or semi-quantitative results; most are very sensitive to detect even low antibody titers [49]
    - Other assays (typically offered by commercial laboratories) give quantitative results, that can facilitate monitoring over time; due to potential of variation between laboratories it is important that the same laboratory is used when comparing results
    - Coronavirus IFAT comprise virus-infected cells fixed upon slides onto which test sera are applied; a secondary fluorophore-labeled antibody is then used to determine the presence of bound antibodies
    - Coronavirus ELISAs or immunochromatographic tests comprise viral antigen bound to membranes across which test sera are washed and bound antibodies detected using a secondary labelled antibody
    - There is marked antibody cross-reactivity between closely related coronavirus species, as detected by IFATs based upon TGEV and FCoV (serotypes 1 & 2) [8, 9]
        - This property has been exploited by IFATs used to investigate the serological antibody response during the development of FIP: feline cells infected with either serotype 1 or serotype 2 FCoV can be used, or, alternatively, porcine cells infected with TGEV [50]
        - Although, it is likely that seropositive cats will have been exposed to FCoV cf. another coronavirus, this cannot be assumed; seropositive cats are often described as being coronavirus-positive rather than FCoV-positive
- Seroconversion occurs 2-3 weeks following exposure to FCoV [51]

- A high antibody titer (>1:1600) is a non-specific finding of limited value in the diagnosis of FIP, especially in cats from multicat households where the likelihood of seropositivity is high, in young cats where MDA may persist (up to 12-14 weeks) or in cats with recent exposure to others with known FCoV infection (e.g. another cat with FIP in the household) [52], since most of these cats will not go on to develop FIP
- A high antibody titer in association with compatible clinical signs, history etc. can be supportive of FIP particularly when coming from a household where the likelihood of seropositivity is low (e.g. few cats resident), in that it indicates the necessary exposure to FCoV
- Approximately 10% of cats with fulminant FIP may have negative serology due to peracute disease (seroconversion takes 2-3 weeks), immune-complex formation or immunosuppression [53]
- A positive antibody titer in a healthy cat does not indicate whether or not they are shedding FCoV in their feces [15, 54]. During an 8-month observation period, of 24 clinically normal cats with high FCoV antibody titers (≥1:1600) one frequently (>75% of samples) shed FCoV, 20 occasionally shed FCoV and 3 did not shed [55]. Within five breeding catteries where FCoV was endemic, between 35% and 70% of cats were shedding at any one time [55]

- Fecal reverse transcriptase-(quantitative) polymerase chain reaction (RT-(q)PCR) (see also **Box 1**)
  - RT-PCR may be used to detect, and in some cases quantify (i.e. RT-qPCR), FCoV shedding in feces
  - Intermittent fecal shedding of FCoV or laboratory error (e.g. due to carry-over of PCR inhibitors found in feces) can result in negative results [56]
  - Repeated testing is required to identify persistent or recurrent FCoV shedders in multi-cat households, or whether they have stopped shedding. The optimum frequency of sample collection is unknown
  - A positive RT-PCR result does not indicate whether a cat has, or will go on to develop FIP

Diagnosis of FIP

See **Figure 3** for a suggested approach cats suspected of having FIP

- Definitive diagnosis of FIP ante-mortem can be challenging, and:
    - Some consider histological co-localization of pyogranulomatous inflammation with presence of FCoV (demonstrated by immunostaining for FCoV antigen) within monocytes / macrophages necessary to make a definitive diagnosis of FIP, and this is frequently considered the reference standard in studies evaluating diagnostic techniques [7, 57]; however, this necessitates performance of procedures, of variable degrees of invasiveness, to obtain diagnostic samples
    - In contrast, for many clinical trials and some trials of diagnostic techniques, diagnosis has been made based upon a combination of signalment, clinical history, physical examination and clinicopathological findings (sometimes, but not always including RT-(q)PCR or immunostaining of tissue or effusions) [58-60]
- Ante-mortem a clinical diagnosis of FIP is more often based on the combination of compatible signalment, history, clinical signs, typical clinical pathology changes (see *Clinical pathological changes of FIP*), analysis of effusions (if present; see *Effusion analysis*), and analysis of other cytological samples (see *Analysis of cytological samples other than effusions*)
    - Identification of FCoV within effusions, tissue aspirates, cerebrospinal fluid (CSF) etc., either by immunostaining for FCoV antigen (see *Immunostaining for FCoV antigen*) or by RT-(q)PCR (see *Molecular diagnostics in the diagnosis of FIP* and **Box 1**) for genetic sequences of FCoV can be strongly supportive
    - In some cats, tissue biopsy (see *Tissue biopsy analysis*) may be required to provide sufficient support for a clinical diagnosis of FIP
    - The use of machine-learning techniques to enhance interpretation of combinations of data and indicate likelihood of disease are likely to be developed over the coming years [61]
- Imaging modalities (e.g. thoracic or abdominal ultrasound; radiography; computed tomography; magnetic resonance imaging) can reveal evidence of fluid accumulations, mass lesions, and vasculitis / inflammation

- No imaging sign is pathognomonic for FIP, but imaging can facilitate exclusion of other differential diagnoses
- Imaging may facilitate needle sampling for further diagnostics, e.g. cytology
- Fluid accumulations may progress over time, particularly following correction of dehydration, such that repeated imaging may be required

Clinical pathological changes of FIP [20, 45]

- Hematology – changes, if present, are non-specific, but could include:
  - Mild, non-regenerative anemia (common)
  - Severe, regenerative anemia due to immune-mediated hemolytic anemia or hemorrhage (uncommon)
  - Microcytosis in the absence of anemia (common)
  - Mild neutrophilia, with or left shift or toxic changes (common); neutropenia (uncommon)
  - Lymphopenia (common); lymphocytosis (uncommon)
  - Eosinopenia (common); eosinophilia (uncommon)
  - Monocytosis (common)
  - Thrombocytopenia, due to consumptive or immune-mediated processes (common); thrombocytosis (uncommon)
  - Increased coagulation test parameters (e.g. activated partial thromboplastin time, aPTT; prothrombin time, PT) may develop due to consumptive processes in fulminant FIP (e.g. disseminated intravascular coagulation)
- Serum biochemistry – changes, if present, are non-specific, but could include:
  - Mild hyperbilirubinemia (common), attributed to systemic inflammation or vasculitis affecting hepatic parenchyma; mild increases in hepatic enzyme activities (relatively uncommon)
  - Mild to severe hyperglobulinemia (common)
  - Serum protein electrophoresis typically shows a polyclonal gammopathy and hypoalbuminemia; less frequently decreased beta-1 globulins (negative acute-phase

proteins) or increased alpha-2 globulins (positive acute-phase proteins) are seen; rarely a 'monoclonal' or restricted oligoclonal gammopathy is noted [62]

- The frequency of electrophoretic changes appears to be decreasing over time, possibly as clinicians suspect / investigate FIP at an earlier stage in the disease, with increased numbers of cats reported with increased alpha-2 globulins without a gammopathy, and decreased numbers of cats with solely a gammopathy [63]

- Mild hypoalbuminemia is common, attributed to a combination of a negative acute-phase inflammatory response, compensation for hyperosmolarity, protein-losing enteropathy or nephropathy, and third spacing (if effusive)
- Albumin to globulin ratio is usually low (<0.4 likely FIP; >0.8 FIP is unlikely) [64]
- Acute-phase proteins measurements
  - Alpha1-acid glycoprotein (AGP), a positive acute-phase protein, is often elevated in cats with FIP [65-67]
    - AGP >1.5-2 mg/mL is considered supportive of FIP in cases where FIP is suspected [65]; however, elevations are not specific to FIP, but the greater the magnitude of the increase, the more helpful it may be for cases in which there is a lower suspicion of FIP [66-69]
    - In one study, 85% of cats with FIP (41 of the 48) had AGP >1.5 mg/mL, whilst all cats with effusions that were subsequently demonstrated *not* to have FIP (total of 21; 8 with cardiomyopathy, 6 with neoplasia, 5 with inflammatory / fibrotic disease, and 2 for which a definitive diagnosis was not achieved) had AGP <1.5 mg/mL, suggesting a specificity of 100% at this cut-off [65]; however, in the same study four of six cats with terminal FIV had AGP >1.5 mg/mL
    - In a second study, over 50% of cats with inflammatory disease had AGP >1.5 mg/mL [68]; they also found that AGP >1.5–2 mg/mL was supportive of FIP where pretest probability (defined as the probability of the presence of the condition before a diagnostic test) of FIP was high (i.e.

signalment, clinical signs, and other clinicopathological changes were suggestive of FIP), whilst in cats with a low pretest probability of FIP (i.e. few clinical signs nor a signalment suggestive of FIP), only AGP >3 mg/mL could support a diagnosis of FIP and even then the probability of FIP remained <50%

- In a third study, an optimal cut-off of 2.26 mg/mL achieved a sensitivity of 85% and specificity of 90% [66]; however, a definitive diagnosis (as confirmed histologically) was not made for the majority of FIP and non-FIP cats
- Cats with non-effusive FIP appear to have similar AGP values as those with effusive FIP [67]
- Some authors have found AGP to be particularly useful to support a diagnosis of FIP in cases where there was a strong suspicion of FIP, but where histology was equivocal [69]

- The utility of other positive acute-phase proteins (haptoglobin; serum amyloid A) in supporting a diagnosis of FIP has been evaluated [65, 66]; although both were significantly elevated in cats with FIP, as compared to healthy cats or those with cardiac disease, neither was as accurate as AGP in differentiating cats with FIP from those with inflammatory diseases (septic processes; retroviral infection; neoplasia)

Effusion analysis

- Analysis of FIP-associated effusions (if present) can provide strong support for a diagnosis of FIP
- Basic analysis – often FIP-associated effusions appear clear (i.e. of low cellularity), straw-yellow in color (reflecting the hyperbilirubinemia present), and viscous (i.e. highly proteinaceous)

- Total nucleated cell counts often <5 x10^9/L nucleated cells, comprising predominately non-degenerate neutrophils and macrophages, with some lymphocytes
- Protein often >35 g/L (but can be <30 g/L; esp. following repeated drainage)

- Similar protein changes to serum [66, 70, 71]: often low albumin to globulin ratio
- Cloudy fluid is sometimes noted
    - The Rivalta test – a simple and inexpensive point-of-care test on effusions
- Method: mix 8ml distilled water with 1 drop 98% acetic acid (or 2-3 drops white vinegar); place 1 drop of effusion onto surface. A positive result is indicated by the effusion drop holding its shape. A negative result is indicated by the effusion drop dissipating into solution.
- Positive results may also result from other inflammatory exudates, such as those found in septic peritonitis, cholangiohepatitis and neoplastic effusions
- The sensitivity of the Rivalta test in correctly identifying cats with FIP varies from 91.3% to 98% and the specificity from 65.5% to 80% [71, 72]
    - In one study, where there was a 57% prevalence of FIP, negative and positive predictive values were 97% and 86% respectively [71]. In a larger more recent study, where there was 34.6% prevalence of FIP, negative and positive predictive values were 93.4% and 58.4% respectively [72]
    - A recent study noted that when the Rivalta test was combined with fluid cytology, to identify and exclude cases of lymphoma and bacterial peritonitis / pleuritis, both specificity and positive predictive values improved (73.0% and 73.4% respectively) [72]
    - These data suggest that the Rivalta test is most useful as a screening test to rule out FIP

- Measurement of CoV antibodies in effusions – since both false positive (specificity of 86%) and false negative (sensitivity of 85%) results occur when used to predict the presence of FIP [71], this test is not recommended (i.e. more accurate tests are available)
- Measurement of acute phase proteins [66] in effusions – using a cut-off of 1.55 mg/ml for AGP had a sensitivity and specificity of 93% in the diagnosis of FIP, based upon results from 14 cats with and 53 cats without FIP; false-positive results included three cases of septic peritonitis and one retroviral positive cat with metastatic abdominal neoplasia. Measurement of haptoglobin and serum amyloid A were both less sensitive and less specific.

- For discussion of immunostaining and molecular diagnostics of effusions see respective sections below

Analysis of cytological samples other than effusions

- Other bodily fluids (e.g. CSF, aqueous humor) and tissue aspirates (e.g. lymph nodes; mass-lesions) can be useful in the diagnosis of FIP
- Cytology may provide evidence of pyogranulomatous to granulomatous inflammation: consistent with, but not diagnostic for, FIP
    - In CSF, non-septic pyogranulomatous (or mixed cellular, but including macrophages) inflammation compatible with FIP was present in 76% of the cats with FIP and 30% control cats [73]. NB: 14 of the 41 cats included in the study had samples collected immediately post-mortem, whilst the rest were collected during diagnostic investigations. The influence that this would have had on results, if any, is unknown
    - In aqueous humor, non-septic pyogranulomatous (or mixed cellular, but including macrophages) inflammation compatible with FIP was present in 69% of the 26 cats with FIP, but in only one of the 12 control cats [74]. NB: All samples were collected post-mortem using a larger gauge needle (22G) than would typically be used antemortem (27-29G), which might have increased cellular yield
    - On liver and kidney fine-needle aspirates (collected blind from cats with FIP at post-mortem examination), cytological sensitivity for non-septic pyogranulomatous inflammation was 82% for liver and 42% for kidney aspirates comparable to simultaneously collected needle-core biopsies [75]; however, eight of the 50 cytological samples were considered ‘not of diagnostic quality’ and therefore excluded from calculations. Concurrent samples processed using cytocentrifugation of aspirate material suspended in saline were even more likely to be considered non-diagnostic (21/32 samples) and of the remainder, all six of the liver aspirates but only three of the kidney aspirates revealed pyogranulomatous inflammation
- For discussion of immunostaining and molecular diagnostics of cytological samples other than effusions see respective sections below

Tissue biopsy analysis (histology)

- The primary disadvantage of biopsy analysis is that it requires invasive tissue collection to obtain the biopsy
- In some cats, both with and without FIP, histological examination is equivocal or misleading [45, 69, 76]
    - A small number of cats with idiopathic sterile pyogranulomatous inflammation (involving the head, neck, or mesenteric lymph-nodes) have also been described, where FIP has been excluded, some of which appeared to respond to corticosteroids [77]
- The sensitivity of histology for the diagnosis of FIP in clinical cases is unknown
    - For most studies that evaluate different diagnostic techniques for FIP, inclusion criteria use a combination of histology and immunostaining to either confirm FIP or to diagnose an alternate pathology on samples collected at post-mortem examination; equivocal cases are therefore either not recruited (and not mentioned) or are excluded from further analysis [7]
    - In one large study, 14 of 127 recruited cats (11%) were ultimately excluded based upon lack of a definitive diagnosis (including histological examination), a further five cats (4%) had not had histological examination performed and were also excluded [7]
    - In experimental FIP, of 19 cats with effusive disease examined post-mortem all had histological lesions (histocytic, neutrophilic, and fibrinous peritonitis) involving the omentum, mesentery, and serosal surfaces of the liver, spleen, mesenteric lymph nodes and intestines [78]; however, not all cats had pyogranulomatous lymphadenitis or hepatitis, none had lesions within the pulmonary or cardiac tissue (excluding the pericardium), and, in the absence of clinical signs or gross evidence of disease, ocular / nervous tissue was not evaluated. Restriction of lesions to serosal surfaces would have limited biopsy were these clinical cases, despite them all presenting in a similar manner (i.e. all had ascites)
    - Where blind needle-core biopsy of liver and kidneys has been evaluated in cats with FIP, possibly a better representation of what would happen clinically (cf. post-mortem derived samples), sensitivity has been limited [75]. Although all liver biopsies (n=25) were considered of diagnostic quality, only 16 (64%) had histological changes consistent with FIP, six were

equivocal for FIP, and three contained no lesions supportive of FIP; whilst 7/25 kidney biopsies were considered non-diagnostic, and of the ones that were diagnostic only seven had histological changes consistent with FIP (28% of total biopsies; 39%), two were equivocal for FIP, and nine contained no lesions supportive of FIP

- For discussion of immunostaining and molecular diagnostics of tissue see respective sections below

Immunostaining for FCoV antigen

- Immunostaining to assess for the presence of FCoV antigen within infected macrophages
- These assays include immunocytochemistry (ICC)[57] or immunofluorescence [71, 79] of cytological preparations (e.g. centrifuge-concentrated cell preparation) or immunohistochemistry (IHC) of formalin-fixed cell pellets and tissue [46]; monoclonal or polyclonal antibody preparations directed against FCoV antigens are used as reagents in these tests
- Sensitivity of these assays is impacted by both the cellularity of the samples being tested and percentage of virus-infected monocytes/macrophages present, since a positive test result depends on the detection of FCoV antigen within these cells
    - There appears to be variable geographical availability of immunostaining of cytological samples (both effusions and non-effusion samples) as well as differences in techniques (particularly the reagents used), sensitivities and specificities between laboratories
- Immunostaining for FCoV applied to effusions
    - The sensitivity for diagnosis of FIP on immunostaining varies from 57% [57, 71] to 95% [80]
    - The specificity for diagnosis of FIP on immunostaining varies from to 71% to 100%
    - False positive results were reported for cats with neoplasia (lymphoma, adenocarcinoma) and cardiac disease [57, 71, 81]
    - One author described IHC on formalin-fixed cell pellets to be more sensitive than ICC [46]
- ICC for FCoV antigen on cytological samples (both effusions and non-effusion samples), as a marker for FIP: a positive result provides support for a diagnosis of FIP, but a negative result does not rule out FIP; and as false-positives occur this should not be solely relied upon to make a diagnosis
- ICC for FCoV applied to cytological samples other than effusions

- On CSF, sensitivity for FIP diagnosis was 85% and specificity was 83% [73]; however, some samples were acellular and therefore excluded from calculations (1/21 of the FIP group and 2/20 of the control group). The three false-positive results were from a cat with lymphoma, a cat with lymphocytic meningoencephalitis, and a cat with hypertensive angiopathy, brain hemorrhage. There was no statistical difference between the sensitivities and specificities when the cats were further divided into those with or without neurological signs.
- On aqueous humor, sensitivity was 64% and specificity was 82% [74]; however, some samples were acellular and therefore excluded from calculations (1/26 of the FIP group and 1/12 of the control group). The two false-positive results were from a cat with lymphoma and a cat with pulmonary adenocarcinoma.
- On mesenteric lymph node aspirates (collected under direct visualization at post-mortem examination) sensitivity was 53% (16 of 30 cats with FIP were positive) and specificity was 91% (1 of 11 control cats were positive) [82], with all samples considered to be of diagnostic quality. Results of cytological analysis alone were not reported. The one false-positive result was from a cat with lymphoma
- On liver and kidney aspirates only five of the 16 (31%) liver aspirates, and two of the 19 (11%) kidney aspirates were positive for FCoV antigen [75]. No control cats were tested for comparison
- Unfortunately, the number of cases recruited into these studies (for both FIP and non-FIP categories) are small, and most are based upon post-mortem collected samples; this increases the confidence intervals for both sensitivity and specificity calculations and limits evaluation of diagnostic utility
    - Ideally large prospective studies would evaluate the utility of immunostaining (*and* molecular diagnostics) on the ante-mortem diagnosis of FIP in cats suspected of having FIP

- IHC for FCoV antigen as a marker for FIP on tissue samples

- Many consider the histopathological demonstration of FCoV antigen within macrophages associated with (pyo)granulomatous lesions the reference standard for the diagnosis of FIP [46]
- Distribution of FCoV within lesions can be variable [83, 84]
- In a large study, 62% of post-mortem collected tissue samples from cats with FIP were positive for FCoV within lesions [7]; however, due to collection methods not all of these tissues would have contained gross lesions
- The sensitivity of IHC on needle-core biopsy tissue samples was poor in the one study that has evaluated this: only six of 25 (24%) liver samples were positive and only three of 18 (17%) diagnostic kidney samples were FCoV antigen positive [75]

Molecular diagnostics in the diagnosis of FIP

- The utility of RT-(q)PCR for FCoV (see **Box 1**) as a marker for FIP has been investigated for blood, effusions, other cytological samples and tissue samples
  - The majority of, but not all, RT-PCR assays utilized in recent studies (and available clinically) are quantitative; despite this, only qualitative (i.e. positive or negative) results have been used to calculate diagnostic utility and, in some studies, only the qualitative data are reported
    - Quantitative results (e.g. copy number boundaries indicating degree of support) have not been evaluated for the diagnosis of FIP, although copy numbers are occasionally described for different samples and populations
    - RT-qPCR for FCoV are preferable to RT-PCR for a variety of reasons primarily related to quality control and initial assay optimization (see **Box 1**)
  - Multiple studies (reviewed elsewhere [47]) have shown that use of FCoV RT-qPCR of blood (or blood components) for the diagnosis of FIP is often of low sensitivity, even in cats with experimental FIP [78], and that false-positives occur in cats without FIP [85]
  - RT-PCR for FCoV on effusions
    - Sensitivity for diagnosis of FIP varies from 72% to 100% [7, 86, 87]

- Specificity for diagnosis of FIP varies from 83% to 100% [7, 86, 87]; although, numbers of samples tested in individual studies were often small
- Samples included in these studies were collected both ante-mortem, as part of the routine clinical investigation, and at post-mortem examination; the numbers in either category were not reported

- RT-PCR for FCoV on cytological samples other than effusions
  - On CSF sensitivity for diagnosis of FIP ranges from 42 to 63% for all cats [7, 88, 89] (combined total of 25 positive results from 49 cats); where differentiated, cats with neurological/ocular manifestations of FIP were more likely to have a positive result (86% cf. 17%) than cats without these manifestations [88]; specificity was 100% in all studies where control cats were tested. In all studies, samples were collected post-mortem
  - On aqueous humor sensitivity for diagnosis of FIP was 25% (4/16 samples; all collected post-mortem) [89]. No control cats were tested for comparison
  - On mesenteric lymph nodes aspirates (collected under direct visualization post mortem) sensitivity for diagnosis of FIP ranges from 85% to 90% (17 of 20 cats with effusive and non-effusive FIP, and 18 of 20 cats with non-effusive FIP, respectively) [60, 89] and specificity was 96% (1 out of 26 control cats was positive) [60]
  - On liver, spleen and popliteal lymph node aspirates (20 of each from cats with either effusive and non-effusive FIP; all collected post mortem) sensitivities for diagnosis of FIP were 85, 80 and 65% respectively [89]. No control cats were tested for comparison
  - Unfortunately, the number of cases recruited into these studies (for both FIP and non-FIP categories) are small, and most are based upon post-mortem collected samples; this increases the confidence intervals for both sensitivity and specificity calculations and limits evaluation of diagnostic utility
    - Ideally large prospective studies would evaluate the utility of molecular diagnostics (and immunostaining) on the ante-mortem diagnosis of FIP in cats suspected of having FIP

- RT-PCR for FCoV on tissue samples
    - All of these studies comprised samples collected at post-mortem examination
        - Larger volumes of tissue are often collected under these circumstances, which may increase the likelihood of achieving a definitive diagnosis and consequently the diagnostic sensitivity in cats with FIP
        - Tissues may have been sampled that would not necessarily have been collected clinically, potentially reducing diagnostic sensitivity in cats with FIP; e.g. liver and spleen biopsy in a cat with solely neurological signs
        - Conversely, samples may be collected from cats with more advanced clinical disease and pathological change, potentially increasing diagnostic sensitivity in cats with FIP
    - In studies comprising more than 20 cats with FIP, sensitivity per cat (i.e. where one or more samples were analyzed per cat, and a single positive result considered to be diagnostic for FIP) for diagnosis of FIP varied from 94% to 96% [7, 90], whereas when samples from individual tissues were considered sensitivity ranged from 88% to 90% [7, 91]; the tissues collected from individual cats (both FIP and non-FIP populations) varied widely
    - In studies comprising samples from more than 20 cats without FIP, specificity per cat (i.e. where one or more samples were analyzed per cat, and a single positive result considered to be diagnostic for FIP) ranged from 39% to 90% [7, 90, 92], whereas when samples from individual tissues were considered specificity was 92% [7]
    - Viral copy numbers were generally higher in cats with FIP as compared to those found in cats without FIP [7, 90]; viral copy numbers were also generally higher in tissue samples that were positive for FCoV antigen than for those that were negative for FCoV antigen [7]
- Overall, a positive RT-(q)PCR result on effusions, other cytological samples and tissue, but not blood (or its constituents), can provide strong support for a diagnosis of FIP; however:

        - Similar to immunostaining testing for FCoV antigen, sensitivity of RT-(q)PCR will be affected by the number of FCoV-infected cells present in the sample under test; for cytological samples this is influenced by both cellularity and pathology, whereas for tissue samples this appears to be a function of pathology distribution
        - On effusions and other cytological samples, a negative result does not rule out FIP, particularly where cellularity was low; whereas on tissue samples where there is supportive pathology a false negative result is rare [7]
        - Since false-positives occur, RT-(q)PCR should not be solely relied upon to make a diagnosis of FIP, particularly if multiple tissue samples are tested (as this can increase the likelihood of obtaining a single false-positive result). Fewer false-positives are documented for cytological samples, likely reflecting their lower cellularity as well as expected distribution of potentially infected macrophages; however, caution should be used when interpreting specificities for cytological samples due to the small sample sizes
- FCoV mutation analysis has been applied to samples previously determined to be positive by RT-qPCR (see **Box 1**)
    - The aim of mutation analysis is to differentiate FCoV pathotypes (i.e. 'FECV' from 'FIPV'), based upon differences in the viral genomic sequence, in the hope that this can be used to differentiate cats with FIP from those without [39]
    - Presence of mutations M1058L and S1060A within the fragment of *Spike* gene encoding the putative fusion peptide of the serotype 1 Spike glycoprotein has been most frequently studied for the diagnosis of FIP, albeit using different techniques, different sample types and with different conclusions (see **Table 2**)
    - Inclusion of *Spike* gene analysis alongside RT-qPCR does not appear to substantially improve specificity; further, a consequence of considering only results with *Spike* gene mutation as being diagnostic for FIP significantly reduces test sensitivity [7]
    - Detection of *Spike* gene mutations in cats without FIP was not unexpected, as it is estimated that 90% of cats that experience systemic FIPV infection do not go on to develop FIP [36]

- Some authors remain strongly supportive of the use of *Spike* gene analysis using allelic discrimination in the diagnosis of FIP where minimizing false-positive results is paramount [47]

Box 1: *Use of PCR in the detection of FCoV*

Polymerase chain reaction (PCR), is the method by which DNA is exponentially amplified using primers to target a specific sequence, enabling sensitive detection down to a very low starting DNA copy number. Post-PCR amplification processing (e.g. sequencing) can be applied as well if needed. PCR only amplifies DNA so because FCoV is an RNA virus a pre-PCR step using a viral enzyme, reverse transcriptase, is required to generate a strand of complementary DNA (cDNA) using the original FCoV RNA template, in a process known as reverse transcription. A combination of this process and PCR is known as Reverse Transcription-Polymerase Chain Reaction (RT-PCR).

Given that only a very small volume of diagnostic sample is ultimately added to each PCR reaction, this does result in a limit to PCR sensitivity, although it remains a highly sensitivity modality when compared to other tests for the presence of a pathogen.

Due to the high frequency of transcriptional errors during replication of RNA viruses and inherently increased variation between viral strains (as compared to replication of DNA viruses), primers designed for the detection of FCoV (see **Figure 4**) predominantly target sections of the genome that are considered to be highly conserved (e.g. non-structural protein 7b; the membrane glycoprotein-nucleocapsid protein border; 3' untranslated region), as determined by known sequence comparisons. Due to the conserved nature of these sections of the genome, other members of the *Alphacoronavirus* genus (i.e. canine coronavirus, transmissible gastroenteritis virus) may also result in a positive result using assays for FCoV [93]. In contrast, one study described a PCR using primers designed on the more variable envelope protein gene on the suspicion that FECV could be differentiated from FIPV based on limited sequence data [94], although this is not supported by more recent data [39]. The shorter the amplified fragment in PCR, the more efficient the assay which contributes to increased sensitivity; however, this does limit the length of amplified fragment subsequently available for sequencing if required. Regardless of how good primer

design is infrequently genomic variation, even in conserved regions, can result in the failure to detect FCoV (i.e. false negative results) even when likely present at high level [7].

Quantitative (sometimes known as real-time) RT-PCR (RT-qPCR) assays, that use either DNA-intercalation dyes (e.g. SYBR Green) or (TaqMan) hydrolysis probes to quantify the DNA within the reaction mixture after every amplification cycle, have been applied to the detection of FCoV [56, 95]. If the signal from the reaction exceeds a defined threshold it is taken to be a positive result and the cycle number at which the sample became positive is usually reported as either a CT (cycle exceeding threshold) or CP (crossing point). It should be noted that the lower the CT/CP value the higher the starting copy number, such that a CT/CP value of around 20 corresponds to around 10^6 copies per reaction, whereas a CT/CP value of around 40 corresponds to around 10 copies per reaction. Quantitative assays are more easily optimized and may result in them being more sensitive than conventional PCRs (which rely on detection of DNA at the end of the PCR process). Quantitative assays are also subject to less risk of laboratory contamination (a potential cause of false positive results) than conventional PCRs as the reaction wells containing amplified DNA remain sealed and do not require opening for completion of detection (using a gel for example) as for conventional PCR. In addition, hydrolysis probes have the potential to increase assay specificity (cf. use of the PCR amplification primers alone), by providing additional nucleotide sequence against which the target sequence must match to obtain a positive result. Hydrolysis probes also permit the duplexing of a FCoV assay with another PCR assay such as one for the detection of host DNA as an internal control.

As there is a reverse transcription step in the detection of FCoV by PCR, most assays will detect both genomic RNA contained within virions and messenger RNA. Produced during active transcription and translation of the virus, messenger RNA may be full length or subgenomic-length due to discontinuous transcription [96]. Relative abundance of individual fragments of the genome may therefore vary within a sample dependent upon the nature of the virus within that sample (e.g. cell-free virions vs. cell-associated viral replication) [91]. This may account for differences in sensitivity between assays targeting different sections of the genome. Differences between the structure of subgenomic mRNA and genomic RNA (see

Figure 4) have been exploited by some assays [91, 97], with the premise that detection of active viral transcription would only be present in cats with FIP; however, positive results were obtained from the blood of cats in a small number of cats without FIP [97].

PCR amplified DNA fragments may be sequenced, either by Sanger sequencing or by pyrosequencing. This has been applied to the sequence of the FCoV *Spike* gene associated with a switch in cell tropism, (see *FCoV mutation analysis*) [7, 86, 87]. Limitations of Sanger sequencing include lack of data from approximately the first 30-50 bases of the fragment, time taken to perform, and need for specialist equipment; however, sequencing of relatively large fragments (e.g. up to 1000+ bases) is possible and the target sequence does not need to be known. Bench-top pyrosequencing is typically used to rapidly sequence short sections (~10-20 bases) on much smaller fragments; this is often facilitated by knowledge of the sequence possibilities of this section of the genome. Sanger sequencing has also been applied to fragments amplified from different regions of the FCoV genome for phylogenetic comparisons of isolates collected from an epizootic outbreak of FIP [98]. An alternative method of *FCoV mutation analysis*, which has been applied to FCoV RT-qPCR positive samples, is allelic discrimination [89, 90, 99]. This is where two probes, each containing a different fluorescent dye, corresponding to the alternative FCoV genomic sequence (i.e. one mutated, one not) being targeted are included in an assay, with the ratio of one probe to another measured by the relative production of fluorescence during the thermal cycling.

Loop-mediated isothermal amplification (LAMP) is a similar technology to PCR, whereby targeted (c)DNA sequences are amplified; however, as amplification is performed at a constant temperature there is no-longer a requirement for a thermal cycler and is therefore potentially considerably cheaper and more robust in the field. DNA amplification is detected by an increase in turbidity often facilitated by the use of dyes, and post-amplification processing is limited (i.e. sequencing is not possible). This technology has been applied to the detection of FCoV (i.e. RT-LAMP), and although specific (i.e. only samples positive for FCoV gave positive results with RT-LAMP) its sensitivity was around half of that of PCR [100, 101].

1 ***Table 1*** *Overview of diagnostic tests for FIP*

Test	Sample	Target	False negatives	False positives	Comments
Rivalta's test [71, 72]	Effusion	Inflammatory proteins		Other causes of exudate e.g. bacterial peritonitis, lymphocytic cholangitis	Cheap, rapid point-of-care test Non-specific; little advantage over fluid cytology and protein analysis
Histopathology [7, 83]	Tissue	Inflammatory response to FIP	Tissue sampled not involved	Other causes of pyogranulomatous inflammation (consider tissue culture and IHC)	Systemic perivascular granulomatous or pyogranulomatous lesions strongly supportive of FIP in conjunction with compatible history, clinical signs etc. Most pathologists recommend IHC to confirm
FCoV RT-(q)PCR [7, 99]	Effusion; CSF; aqueous humor; tissue aspirates or biopsy; (blood = very poor sens.)	FCoV RNA	Low cellularity or sample degradation; lab error (e.g. strain not detected by PCR assay)	Lab error (contamination)	Non-specific: should not be used as a sole diagnostic test. Positive RT-(q)PCR on tissue, CSF, aqueous humor and effusions is strongly supportive of FIP in conjunction with compatible history, clinical signs, cytology etc. Sens. RT-(q)PCR > IHC

					In general, samples from cats with FIP have higher viral loads than samples from cats without FIP that are also infected with FCoV.
FCoV RT-LAMP [100]	Effusion, tissue, blood	FCoV RNA	Low cellularity or sample degradation; lab error (e.g. strain not detected by PCR assay)	Lab error (e.g. contamination)	Poor sensitivity cf. RT-qPCR; does not require expensive equipment to perform
Immunohistochemistry (IHC) [7, 83] / Immunocytochemistry (ICC) [57, 73, 74, 82]	Tissue, CSF, effusion	FCoV antigen within macrophages	Low cellularity effusion; non-representative tissue biopsy; antigen masked by patient's own FCoV antibody	Lab error (methodology dependent)	IHC considered reference standard for confirmation ICC of more limited specificity (lab dependent) can be interpreted as strongly supportive of FIP in conjunction with compatible history, clinical signs etc.

Table 2 Sensitivity (sens.) and specificity (spec.) of different modalities applied to *Spike* gene mutation analysis. Where specificity is not reported either no cats without FIP were included in those studies, or the relevant samples from cats without FIP were negative by FCoV RT-qPCR and therefore *Spike* gene mutation analysis could not be performed. *NA = not available*

Methodology	Sample type (corresponding sensitivity +/- specificity *cf. to FCoV RT-PCR alone*)					Notes
	Tissue	Effusions	Needle aspirates (tissues; lymph nodes)	CSF	Aqueous humor	
Pyrosequencing	Sens. = 81% Spec. = 95% (*cf. 90% and 93% respectively*) 14/19 samples from cats without FIP positive for FCoV were also positive for the *Spike* gene mutation [7]	Sens. = 74% Spec. = 96% (*cf. 91% and 96% respectively*) The one sample from a cat without FIP that was positive by FCoV RT-qPCR (of 28 tested) was also positive for the *Spike* gene mutation [7]	*NA*	*NA*	*NA*	*Spike* gene mutations were detected in FCoV-positive tissue from cats without FIP at the same frequency as in cats with FIP Able to obtain results at very low viral loads (down to 1.8 $x10^3$ viral RNA equivalents/mL effusion)

Sanger sequencing	Sens. = 70% Spec. = 88% (*cf. 91% and 50% respectively*) One cat, of the four without FIP positive for FCoV (of 8 tested), was positive for the *Spike* gene mutation [86]	Sens. = 40-64% Spec. = 83% (*cf. 72-100% and 83% respectively*) [86, 87] The one sample from a cat without FIP that was positive by FCoV RT-qPCR (of 6 tested) was also positive for the Spike gene mutation [86]	*NA*	*NA*	*NA*	
Allelic discrimination	Sens. = 30-71% Spec. = 100% (*cf. 65-95% and 90% respectively*) [89, 90]	Sens. = 64-69% Spec. = 96% (*cf. 86-97% and 88% respectively*) [89, 99]	Sens. = 15-45% (*cf. 65-85%*) [89]	Sens. = 44% (*cf. 63%*) [89]	Sens. = 10% (*cf. 25%*) [89]	The copy number below which allelic discrimination is not possible is reported to be 1.5 x10^6 viral RNA equivalents/mL effusion [99]; samples that are below the limit of detection are considered negative

7

Treatment

- Until recently, FIP was considered to be a progressive and ultimately fatal disease in the overwhelming majority of cases; however, with the advent of novel antiviral medication (i.e. protease inhibitors and nucleoside analogs), there is an argument to consider FIP as a potentially curable disease
- A handful of cats are suspected to have been able to confine the disease locally, at least for some time (months to years) [45, 102]
- A paucity of placebo- or 'current best-treatment'-controlled clinical trials of cats with definitively diagnosed FIP, along with a lack of licensed drugs with proven efficacy in curing FIP, limits treatment recommendations
- Supportive care – appetite stimulants (e.g. mirtazapine, up to 2mg/cat/day), vitamin B12 supplementation (0.02mg/kg by weekly subcutaneous injection; or 0.25mg/cat orally once daily), anti-oxidants, fluid therapy
- Benefit of draining effusions is debated
    - Thoracocentesis is indicated where dyspnea is present
    - Therapeutic abdominocentesis is controversial and may be detrimental due to exacerbation of dehydration if large volumes are removed (which often reform rapidly)
    - Some authors have described fluid drainage followed by intracavitary steroid administration (dexamethasone 1mg/kg once daily, until resolution of effusion or up to seven days); in one study where this was administered, in addition to other medications, effusions temporarily resolved in six of 36 cats, and although all succumbed to FIP (one within 7 days of diagnosis, four 21 days to 3 months of diagnosis, and one at 200 days post-diagnosis), this compared favorably with the median survival time of 8-9 days for all cats treated [103]
- Prednisolone – is frequently administered to ameliorate some of the clinical signs associated with the chronic inflammatory process; however, there have been no clinical trials to support its use. A starting dose of 0.5mg/kg twice daily orally is suggested (some texts suggest up to 1mg/kg twice daily), then tapered if possible

    - One study found that survival times of cats with non-effusive FIP were significantly shorter in cats who were treated with corticosteroids (by any route) concurrently with polyprenyl immunostimulant (median survival time 21.5 days cf. 73.5 days) [59]; however, authors could not rule out administration of corticosteroids as an indirect marker of disease severity
- Feline interferon-omega – often used but lacked convincing evidence of effect in a placebo-controlled trial [103]
- Many other drugs have been suggested but currently lack a robust evidence base for use including: pentoxifylline, propentofylline [104], polyprenyl immunostimulant (20% dry FIP cats in recent study had greater survival than expected, gaining more clinical interest)[59], ozagrel hydrochloride [105], cyclophosphamide, ciclosporin A, anti-TNF-α antibodies [106], itraconazole [107], mefloquine [108], turmeric-based compounds [109] and herbal medication
- Protease inhibitor GC376
    - The function of the FCoV protease is to cleave the viral polymerase from polyprotein 1, and is essential for viral replication; GC376 is a reversible, competitive inhibitor of the FCoV protease [110]
    - Administered by subcutaneous injection twice daily, GC376 produced remarkable responses in both experimental and naturally-occurring FIP: six of eight cats with experimentally-induced FIP were alive at 8-months post-treatment [29]; and 19 of the 20 cats with naturally-occurring FIP had a positive response (including, where present: rapid resolution of pyrexia; resolution of effusions and associated clinical signs; resolution of icterus; resolution of uveitis; resolution of mass lesions; weight gain) (sustained in seven) [58]
    - Based upon evidence of relapse of clinical signs following withdrawal of short courses of treatment, followed by a sustained response to re-institution of treatment, in cats with naturally-occurring FIP, the minimum duration of treatment was increased and is now recommended as 12 weeks
    - Reported side effects of GC376 administration included: injection reactions (transient pain upon administration; occasional foci of subcutaneous fibrosis; hair loss); and interruption of

normal dental development in cats aged <4 months (delayed development; abnormal eruption of permanent teeth) [58]

- Eight of the 13 cats that succumbed to naturally-occurring FIP did so due to severe neurological signs, and although some of these cats had experienced remission of clinical signs following an increase in dose of GC376 administered, ultimately they relapsed [58]. Cats that had initially presented with neurologic FIP had been excluded from this treatment trial based upon unpublished experimental studies; presumably poor response to treatment or high frequency of relapse

- Adenosine nucleoside analog GS-441524
  - GS-441524 acts as an alternative substrate and RNA-chain terminator of the viral RNA polymerase, thereby interfering with viral replication
  - Administered by daily subcutaneous injection, GS-441524 produced remarkable responses in both experimentally-induced and naturally-occurring FIP: all ten cats with experimental-induced FIP were alive at 8 months post-treatment [111]; and 26 of 31 cats with naturally-occurring FIP had a positive response (including, where present: rapid resolution of pyrexia; resolution of effusions and associated clinical signs; resolution of icterus; resolution of uveitis; resolution of mass lesions; weight gain) (sustained in 25) [112]
  - Based on evidence of relapse of clinical signs following withdrawal of short courses of treatment in cats with naturally-occurring FIP treated with GC376 [58] and in cats with experimentally-induced FIP treated with GS-441524 [111] (where treatment courses were of 2 weeks, with a repeated course in the two cats that experienced relapses), the minimum treatment duration for cats with naturally-occurring was set at 12 weeks
  - Cats with neurological FIP were associated with a poorer outcome and, where successful, required increased doses of GS-441524 (continued for a minimum of 12 weeks) to achieve clinical remission [112, 113]
  - Reported side effects of GS-441524 administration included [112]: injection reactions (transient pain upon administration, lasting 30-60s; ulcerations, progressing to open sores in some cats; scar formation); and development of transient azotemia in one cat

    - A rapid, transient rise in serum globulins was also found, associated with resolution of effusions
- Neither protease inhibitor GC376 nor nucleoside analog GS-441524 are commercially available; however, there are reports that some owners have sourced “black-market medication” via the internet (personal communications)
- Mutian® X, an adenosine nucleoside analogue, reported to be different to GS-441524, has been marketed for the treatment of FIP. Mutian® X is available as both oral and injectable formulations. Although no evidence has been published to support the use of Mutian® X to date, there is limited research describing its use to stop fecal shedding of virus [114]
- Functional changes to the FCoV genome that resulted in *in vitro* changes in susceptibility to GC376 have been demonstrated following chronic administration of GC376 to a cat with naturally-occurring FIP [115]; however, this was not accompanied by clinical evidence of drug resistance. This has raised concerns regarding the potential for emergence of resistance to anti-viral agents, particularly following chronic administration of treatment or when used in the treatment of enteric FCoV infection (i.e. to stop fecal shedding) which may ultimately result in the transmission of resistant strains to other cats

Prognosis

In the absence of GC376 or GS-441524 prognosis associated with FIP is grave (median survival time 9 days; range 3-200 days [103]; majority of the cats in that study had effusive disease)

Prevention

- Vaccination
    - Early immunization studies documented ADE [27]; whereby cats experimentally sensitized to one strain of FCoV subsequently developed more acute and severe disease than expected following exposure to an alternative strain
    - An intra-nasal vaccine (FELOCELL FIP, Zoetis), containing a temperature-sensitive, live-attenuated strain of FCoV is available in the USA and continental Europe

- According to manufacturer's guidelines, cats should be seronegative prior to vaccination, and ≥16 weeks at 1st dose, with 2nd dose 3 weeks later
- In situations in where FIP is a concern (e.g. catteries were FCoV is endemic), and therefore vaccination considered, exposure to FCoV will likely have occurred prior to the earliest recommended age of administration (i.e. 16 weeks) [116]
- Variable efficacy has been reported; in one study, although vaccination reduced the risk of developing FIP in those cats that had low or negative FCoV antibody titers at time of administration (from 10.7% to 3.3%), it did not eliminate the risk [116]
- ADE has not been reported for the intra-nasal vaccine when administered under field conditions [116, 117], but was reported under experimental conditions [118]
- Its use is controversial; and routine use is not recommended even where available (i.e. it is non-core) [119]
- It is not possible to differentiate vaccination-induced antibodies from those acquired following natural exposure, potentially limiting interpretation of serological antibody testing in the future

- In households or establishments where FIP has been confirmed, efforts should be made to:
  - Reduce transmission of FCoV – good litter tray hygiene, provision of adequate numbers of litter trays, food and water bowls placed away from litter trays (outdoor access for toileting is preferred)
  - Isolation of breeding queens 2 weeks before parturition and separating kittens from the queens (at 5-6 weeks) before MDA declines to prevent kitten exposure to FCoV has been described, but is controversial:
    - Often practically difficult for the breeder to maintain strict biosecurity conditions
    - Concerns regarding kitten welfare, socialization and development
  - Reduce stress – consider stocking density (i.e. keep as low as possible) such as rehoming non-breeding queens / neuters in breeding environment; maintain stable groups of cats; consider environmental provisions for each cat and environmental enrichment

- In domestic households (e.g. <4 cats), it has been suggested not to introduce any new cats for at least 2-3 months after a cat has died from FIP (to allow time for any residual virus to become inactive, and to possibly reduce shedding from remaining cats) [120]
- In breeding catteries, it is suggested to avoid breeding from cats repeatedly producing kittens that go on to develop FIP (especially stud males, as these have greater capacity to pass on their genetic material to future generations); often breeders are unaware (or reluctant to admit) of having endemic FCoV within their cattery, since FIP typically only manifests after kittens have been rehomed
- Use of serial fecal PCR to identify chronic FCoV shedders may enable segregation of cats but intermittent shedding and re-infection with FCoV can occur

Present relevance and future avenues to consider or to investigate

Questions remain regarding the pathogenesis, diagnosis, treatment and prevention of FIP. Many of the papers assessing the utility of specific tests to support the diagnosis of FIP (e.g. immunostaining; RT-PCR) have a number of significant limitations such that interpretation of results might not reflect the reality of clinical practice:

- Many do so in isolation of other supportive results such as clinical history, physical examination findings, routine clinic-pathological results, and sample cytology or histology
- Samples for testing are frequently obtained at post-mortem examination
- Control populations (i.e. non-FIP cats) might not necessarily represent cats in which FIP was a significant differential diagnosis (e.g. a middle-aged cat with heart failure and thoracic effusion)
- Numbers of cats enrolled in both FIP and non-FIP populations in many studies that utilize cytological samples (e.g. fine needle aspirate samples) are small, such that confidence intervals are wide and strong conclusions difficult to make
- As different papers perform different assays on subtly different populations, comparison of assay utility on limited numbers of cats remains complicated

Future studies would ideally compare a number of different test modalities and assess their utility in the diagnosis of FIP, possibly in combination, as part of a diagnostic algorithm applicable to clinical practice,

where less invasive techniques (e.g. fine-needle aspirates; needle-core biopsy) are preferred. In recent years there have also been dramatic leaps forward in the treatment of FIP with novel antiviral agents; more studies are required to determine if these can be curative. Looking forward, advances in knowledge across all areas, including prevention through vaccination, may occur as a consequence of the SARS-2-CoV outbreak in humans.

Summary/Discussion

Molecular diagnostics (primarily RT-qPCR) are providing increased support for the diagnosis of FIP, albeit not a reference standard for diagnosis. Samples suitable for RT-qPCR analysis are more amenable to minimally invasive diagnostic techniques, as compared to biopsy for histology and confirmatory IHC. In the advent of effective antiviral medication for the treatment of FIP, the focus of FIP diagnosis will likely switch to those modalities that maximize sensitivity, from those that maximize specificity.

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Conflict of interest

ST is a member of the World Forum for Companion Animal Vector Borne diseases, supported by Bayer Animal Health, and of the European Advisory Board on Cat Diseases (supported by Merial/ Boehringer

Ingelheim, the founding sponsor of the ABCD, and from November 2018 additionally by Virbac). She is also Chief Medical Officer for the Linnaeus Group.

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Figures

Figure 1 Drawing of a feline coronavirus virion with relative position of structural proteins and genomic single-stranded RNA (ssRNA) indicated. Modified from Barker & Tasker (*Accepted*), *In Practice*.

Figure 2 Schematic diagram of the feline coronavirus genome with component genes and nucleotide scale. UTR = untranslated region; nsp = non-structural protein. Modified from Phylogenetic Analysis of Feline Coronavirus Strains in an Epizootic Outbreak of Feline Infectious Peritonitis by Barker et al. *Journal of Veterinary Internal Medicine* **27**(3) pp. 445-550. Copyright © 2013 by the American College of Veterinary Internal Medicine, Wiley-Blackwell. DOI: 10.1111/jvim.12058.

Figure 3 Suggested diagnostic approach to cats with suspected FIP. Modified from Barker & Tasker (*Accepted*), *In Practice*.

Figure 4 Primer binding sites of selected RT-PCRs: A – amplifying a 295 base pair (bp) fragment of subgenomic mRNA of the ***M****embrane* gene [97]; B – amplifying a 688 bp fragment of the ***S****pike glycoprotein* gene [92]; C – amplifying a 170 bp fragment of the ***E****nvelope protein* gene [94]; D – amplifying a 171 bp fragment of the ***M****embrane glycoprotein-**N**ucleocapsid protein* gene border [56]; E –amplifying a 102 bp fragment of the *non-structural protein 7b* gene [95]; and F – amplifying a 223 bp fragment within the 3' untranslated region [93]. Modified from Phylogenetic Analysis of Feline Coronavirus Strains in an Epizootic Outbreak of Feline Infectious Peritonitis by Barker et al. *Journal of Veterinary Internal Medicine* **27**(3) pp. 445-550. Copyright © 2013 by the American College of Veterinary Internal Medicine, Wiley-Blackwell. DOI: 10.1111/jvim.12058.

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