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Research Article

RNA In Situ Hybridization for Pathology-Based Diagnosis of Feline Infectious Peritonitis (FIP): Current Diagnostics for FIP and Comparison to the Current Gold Standard

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Feline infectious peritonitis (FIP) is a systemic disease of cats caused by a highly pathogenic variant of feline coronavirus, or FCoV. Two distinct genotypes of FCoV exist (also referred to as serotypes): Type 1 viruses constitute the vast majority of FIP cases, while type 2 viruses are responsible for the remaining infections. Immunohistochemistry (IHC) currently serves as the gold standard for the diagnosis of FIP; however, IHC is limited by variations in sensitivity. RNA in situ hybridization (RNA ISH) has an established foothold in infectious disease diagnostics and presents a potentially improved method for the detection of FIP. This proof-of-concept study evaluated the efficacy of RNA ISH probes targeted to FCoV, as compared to IHC using the monoclonal antibody FIP 3-70. Formalin-fixed paraffin-embedded tissues from FIP-positive cats were used for ISH, with the presence of RNA determined chromogenically. ISH tissue slides were then compared to their IHC counterparts, with efficacy determined based on metrics including staining intensity and abundance. Positive ISH staining on tissue was found to be both more intense and more abundant than that for IHC, suggesting that ISH serves as a highly sensitive method for the detection of FCoV/FIP in comparison to IHC - a finding that awaits further validation.

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Introduction

Feline infectious peritonitis (FIP) is a leading cause of mortality in young cats. The clinical nature of the disease was characterized in the 1960s, and the etiologic agent was subsequently identified as a coronavirus, specifically feline coronavirus (FCoV)[1][2]. FCoV can be classified in several ways, with conventional terminology categorizing the virus into two distinct biotypes. The

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less virulent biotype, feline enteric coronavirus (FECV), is characterized as a self-limiting gastrointestinal disease with either an asymptomatic presentation or mild signs such as diarrhea. FECV is highly prevalent in multi-cat environments, and many domestic cats demonstrate evidence of prior infection through serology[3]. The more virulent biotype, feline infectious peritonitis virus (FIPV), arises in a small subset of FECV-infected cats through internal mutation, a process that is mediated by a combination of host and viral factors. The biotype switch from FECV to FIPV is characterized by productive infection of macrophages, leading to systemic infection, inflammation, and ultimately death[4]. It is possible that FECV and FIPV biotypes exist on a spectrum and that long-term persistence occurs in some cases. Two distinct genotypes of FCoV exist (also referred to as serotypes): Type 1 viruses constitute the vast majority of FIP cases, while type 2 viruses are responsible for the remaining infections. Type 1 FCoV is feline in origin, while type 2 FCoV appears to have resulted from recombination events between FCoV type 1 and canine coronavirus (CCoV)[5]. Importantly, both type 1 and 2 genotypes are expressed as FECV and FIPV biotypes.

The presentation of each individual case of FIP exists along a spectrum of clinical signs. The effusive/wet form of the disease is the most well-recognized and is characterized by rapid disease progression with the accumulation of a proteinaceous exudate in the abdominal and thoracic cavities, as well as less specific clinical signs including fever, malaise, anorexia, weakness, and shortness of breath[1]. The non-effusive/dry form of the disease is less common and generally has a more protracted disease course characterized by neurological and ocular signs, as well as granulomatous lesions in the abdominal organs [6]. Given the spectral nature of the disease, mixed forms of FIP also exist, with an overlap of both wet and dry presentations[4]. FIP is often fatal without treatment, and while experimental therapies now exist, they are not widely approved and can be hard to access. Consequently, FIP remains a leading infectious cause of mortality in cats.

Despite the well-characterized signs of FIP, diagnosis remains a considerable challenge because FECV and FIPV biotypes cannot be reliably distinguished on the basis of genetic or antigenic testing. Definitive diagnosis involves identifying infected tissue macrophages and/or the classic vasculitis and granulomatous lesions that accompany the more virulent FIP biotype. Due to the need for a surgical biopsy to obtain such tissue samples, FIP is generally considered a diagnosis of exclusion. Given the fatal nature of the disease and a lack of licensed treatments that extend beyond supportive care, euthanasia is common. Ideally, a definitive diagnosis is reached before opting for euthanasia or treatment, but this is rarely achieved, and most definitive diagnoses are made using post-mortem tissue testing[7]. Several diagnostic methods have been used in clinical and laboratory settings and involve limitations in the ante-mortem application as well as an overall trade-off in sensitivity and specificity.

Review of diagnostic options

Clinical evaluation

The presentation of FIP is diverse, with effusive, non-effusive, and mixed forms of the disease present, and transition between the wet and dry forms is possible[6]. Preliminary diagnosis involves obtaining a high index of suspicion

for FIP while ruling out other conditions. Signs that are common to both effusive and non-effusive forms (fever, anorexia, and lethargy) are non-specific to FIP. The hallmark presentation of wet FIP is effusion, which is also not entirely specific to FIP[8]. Similarly, in dry FIP, the neurologic and ophthalmologic signs seen in affected cats are not exclusive to FIP. Although signs may be suggestive of FIP—and other conditions may be ruled out to support an FIP diagnosis—the available tests are of limited utility to arrive at the diagnosis on their own and instead are considered in light of signalment, history, and all other available testing.

Serum biochemistry

Attempts at interpreting acute phase protein levels in the diagnosis of FIP have been associated with low specificity. Alpha-1-acid glycoprotein (AGP) is an acute phase protein whose levels are upregulated in the serum of cats with FIP and has the potential to be used as a diagnostic indicator of FIP. Importantly, AGP can also be upregulated in other conditions, thereby limiting its diagnostic specificity[9]. In a similar fashion, serum amyloid A (SAA) is another acute phase protein whose levels are upregulated in FIP. SAA is able to distinguish between infection of the two FCoV biotypes (FECV vs FIPV); however, as is the case with AGP, SAA can be upregulated in other disease processes, including other effusive conditions[10][11]. In tandem with other clinical signs, the use of SAA in the diagnosis of FIP may not reach the needed positive predictive value to arrive at a definitive conclusion. In summary, the use of acute phase proteins in the diagnosis of FIP, while advantageous in allowing for an antemortem evaluation, is limited by the upregulation of such proteins in other disease states—some of which also involve effusion and/or systemic inflammation.

Other serum biochemistry abnormalities have also been associated with FIP. Reports of jaundice in cats with effusive FIP led to the investigation of bilirubin levels in the clinical evaluation of FIP. Bilirubin as an indicator of FIP is most powerful when high serum levels are not accompanied by substantially increased liver enzymes, as a marked elevation of both may be reflective of liver disease as opposed to FIP. Hyperbilirubinemia is prevalent in FIP cases, and its prevalence can increase throughout disease progression, with the first assessment of cats with FIP in one study showing 36.1% prevalence, which later increased to 89.3% before death[12]. While elevated bilirubin levels may raise suspicion of FIP, its use as a diagnostic indicator is limited by several factors: first, the development of FIP is not always accompanied by hyperbilirubinemia, so its sensitivity is limited. While hyperbilirubinemia can be demonstrated in both effusive and non-effusive forms of the disease, it is more frequent in effusive FIP, somewhat limiting its utility as a marker for dry forms of the disease. While the suspicion of FIP is heightened with elevated serum bilirubin without an accompanying increase in liver enzymes, other conditions such as sepsis and pancreatitis can present similarly, thereby limiting specificity[8]. Lastly, hyperbilirubinemia often occurs late in the progression of the disease, limiting its utility in early diagnosis and treatment.

Hyperglobulinemia, alongside hypoalbuminemia, is frequently observed in FIP. The use of the albumin to globulin (A:G) ratio has been used as a diagnostic marker for FIP, with a lower ratio (<0.6) more indicative of the disease[13]. Importantly, A:G ratios do not significantly differ between cats presenting with or without effusion. The positive predictive value—the probability that an animal with a positive test has the disease of interest—is highly dependent on the

prevalence of the disease in the population. The low prevalence of FIP limits the ability of a low A:G ratio to confirm the disease. Conversely, the negative predictive value—the probability that a negative test result is truly indicative of an individual not having the disease—is more favorable. Therefore, while an A:G ratio that exceeds the threshold of suspicion for FIP is beneficial for ruling out FIP, a low A:G ratio is not always indicative of the disease[14].

Rivalta's test.

The presence of a proteinaceous exudate, characteristic of the effusive forms of the disease, has been used to develop an easily accessible, non-invasive diagnostic test for FIP. This test, Rivalta's test, is an inexpensive crude assay in which a small portion of the effusion is added to an aliquot of acetic acid. With a high protein content sample, as is the case with FIP effusion, a precipitate is produced from the addition of the clinical sample. Such a simple, cost-effective diagnostic test presents advantages for private practices and animal shelters, where financial resources may be limited and a clinician may not have access to the latest diagnostic technology. In some studies, Rivalta's test has demonstrated moderately encouraging results when it is used for the diagnosis of FIP, with a sensitivity and specificity of 91.3% and 65.5%, respectively, and a positive predictive value of 58.4% [15]. Importantly, while a positive Rivalta's test may increase the suspicion for FIP, it does not definitively support a diagnosis. Rivalta's test relies on the high protein content of the effusion of cats, rather than a specific detection of FIPV. Consequently, a positive Rivalta's test only demonstrates that the effusion is an exudate containing large plasma proteins, as opposed to a transudate that does not contain as much protein. As such, it has only limited diagnostic value.

Serology

While it seems intuitive that the use of FCoV serology may be of diagnostic utility for FIPV, a fundamental problem lies in the pathogenesis of the disease. FIP results from a prior infection of the FECV biotype that, through a combination of viral and host factors, mutates into the FIPV biotype. The close relationship between the two biotypes leads to considerable limitations in the diagnostic value of FCoV serology, as the two biotypes are virtually identical to each other antigenically—resulting in the production of highly similar antibody responses. Given the extensive prevalence of current or prior FECV infection among domestic cat populations, as well as the comparatively low prevalence of FIP, the return of a positive serology result does little to confirm the diagnosis of FIP. Rather, serology can only be used to determine if a cat has evidence of prior FCoV infection. Negative serology results are not always predictive, as high levels of complement-antibody complexes in affected cats may result in low or negative titers.

While the detection of FCoV antibodies does not offer much specificity in differentiating between the two biotypes, antibody titers have been investigated as a diagnostic marker between FECV and FIPV infection. Many healthy cats with FECV infection have antibody titers ranging from 1:100 to 1:400[16]. While these ranges do not necessarily exclude FIP, as cats with FIP can have titers within this range or even be seronegative, the upper extremes of FCoV antibody titers have some diagnostic value. Among cats with titers of 1:1600, 94% had FIP in one study[17]. The clinical suspicion of FIP increases as titers further increase to 1:3200[18]. Importantly, cats with high antibody titers constitute the minority

of FIP cases. Despite the strong positive predictive value of high FCoV antibody titers, the inconsistent prevalence of these high titer values among cats with FIP limits their utility in the clinical setting.

Molecular Diagnostic Tests

Achieving a timely clinical suspicion or diagnosis is crucial with FIP, given the severe, rapid progression of the disease and the frequent decision to perform euthanasia in response. Several diagnostic methods have been applied to the clinical setting with the aim of achieving a definitive diagnosis. Here, these molecular methods, along with their limitations, are discussed.

RT-PCR

Polymerase chain reaction (PCR) has been investigated for the diagnosis of FIP, given the technique's well-established foothold in infectious disease diagnostics[18]. One early study using such a method on the ascites of FIP-suspected cats, using a targeted approach that focused on sequence differences in the E2 gene (spike protein) between FECV and FIPV, showed a sensitivity and specificity of 91.6% and 94%, respectively[19]. However, the authors do stipulate that since cross-reactivity was only tested against one FECV strain, other FECVs with greater genetic similarity to the targeted FIPV strains may limit the specificity of the technique.

One of the major considerations with the application of PCR to the diagnosis of FIP is the collection site of the sample. FECV infection is generally considered to be self-limiting to the intestines, while FIPV, resulting from efficient monocyte/macrophage tropism, is systemic. The tropism of FECV for enterocytes does not limit the detection of viral RNA to the intestine. A large portion of FCoV-infected cats demonstrate viremia[20] while only a small subset go on to develop FIP. The detection of FCoV RNA systemically in both biotypes undermines the ability to distinguish FIP from another similarly presenting disease in a cat that also has FECV, although it has been suggested that cats with FIP demonstrate a higher viral load in haemolymphatic tissues compared to FCoV-infected cats without severe disease[21]. As is the case with PCR tests for the diagnosis of other infectious diseases, a negative result does not necessarily preclude that a cat is infected with FCoV or has FIP.

The design of current PCR tests, in and of themselves, frequently falls short of distinguishing between the two FCoV biotypes due to the close genetic relationship. Current PCR tests, in order to achieve the necessary sensitivity to detect all FIPV variants, target conserved portions of the viral genome such as the membrane (M) gene or the nucleocapsid (N) gene [22][23]. Attempting to design a PCR test that exploits the genetic differences between FECV and FIPV would allow for greater diagnostic specificity. Initially, it was believed that a truncated ORF3c gene might be responsible for the biotype switch from FECV to FIPV—presenting as an area for differentiation for PCR-based tests. However, this truncation is not present across all FIPV variants, thereby limiting sensitivity[24]. Additional investigation was also conducted with ORF7b as a possible differentiating genetic element between the two biotypes, with FIPVs showing an intact ORF7b, which was believed to be necessary for the macrophage tropism of the virus. However, with some FECVs also demonstrating an intact ORF7b, this genetic difference does not distinguish between the two biotypes and cannot be used to definitively diagnose FIP[25].

The interest in finding distinct genetic and antigenic differences between the two FCoV biotypes has led to consideration of the viral spike glycoprotein—the major protein involved in infectivity and cell tropism—to distinguish between FECV and FIPV given their different tropism. While key genetic differences do exist within the spike glycoprotein between the two biotypes, specifically in the S1/S2 cleavage site, which is cleaved by host proteases to facilitate viral entry and membrane fusion, these mutations are not constant across all FIPV variants[26][27][28]. Furthermore, the S1/S2 cleavage site is only present in serotype 1 FCoVs, which effectively limits its use as a suitable target for molecular-based diagnostics by excluding type 2 FCoVs. Other amino acid substitutions, M1058L and S1060A, have also been explored as potential distinguishers but, again, are not present across all FIPV variants[27][29][30]. In summary, current PCR tests fall short of differentiating between FECV and FIPV. The hope is that more extensive genomic, next-generation sequencing of the virus variants will overcome the shortcomings of the more traditional and restrictive PCR-based techniques.

Immunostaining

Immunostaining presents one of the most specific methods of FIP diagnosis and is where the current diagnostic gold standard lies. Immunostaining uses monoclonal or polyclonal antibodies that target FCoV antigen to deliver a colored or fluorescent label to the infected tissue. Importantly, given the close antigenic relationship between FECV and FIPV, detection of FCoV antigen itself does not necessarily indicate the presence of the FIPV biotype. Instead, the specificity of the technique comes through the detection of significant antigen in monocytes and macrophages.

Immunostaining as a diagnostic technique can be broadly classified into two methods. Immunocytochemistry (ICC) uses a fluorescent secondary antibody targeted against the FCoV-specific primary antibody to allow for evaluation of effusion and/or fine needle aspirates via fluorescent microscopy. ICC presents a relatively non-invasive option for a specific diagnosis. A study examining the use of ICC for FIP diagnosis placed its sensitivity at 53% and specificity at 91%, indicating that while a positive result often indicates an accurate FIP diagnosis, there is ample opportunity for false-negative results[31]. Importantly, this study was performed with fine needle aspirates of mesenteric lymph nodes, which tend to have a high viral load. For effusion samples, the ability to achieve a positive result may be further limited by the poor cellularity of the fluid, which is a characteristic of FIP-related effusions.

The other method of immunostaining, immunohistochemistry (IHC), utilizes the detection of FCoV antigen in fixed tissue, typically in macrophages. IHC is considered the current diagnostic gold standard and has demonstrated 100% specificity, meaning a positive result is always indicative of the cat having FIP[31]. IHC is performed by the sampling of tissue which, due to its invasive nature, generally limits the technique to the post-mortem setting. Therefore, rather than using the technique to guide clinical decision-making, IHC is mainly performed post-mortem to offer closure to clinicians and the family of the pet.

Despite the high specificity of IHC, the major drawback is sensitivity (as with ICC). FCoV immunostaining typically employs a monoclonal antibody (mAb) that recognizes the viral nucleocapsid. Sensitivity and specificity can vary greatly by mAb. A 2008 study examined the performance of mAb FIPV 3-70 (a commonly employed mAb for FIP IHC) compared to CCV2-2, both of which are directed

against canine coronavirus (CCoV) and cross-react with FCoV. FIP 3-70 and CCV2-2 bind different epitopes on the viral nucleocapsid. Double immunostaining of tissue from FIP-positive cats revealed that the CCV-2 epitope was more conserved in the sample population, with 46/49 (94%) samples reacting with CCV-2 and only 18/49 (37%) of samples reacting with FIPV 3-70. These findings imply antigenic heterogeneity in the coronavirus nucleocapsid protein, leading to wide variations in sensitivity depending on which mAb is employed – and present a considerable drawback for a test considered to be the gold standard.

Investigation of RNA ISH

Instead of investigating a new antigenic target for IHC, we reasoned that RNA in situ hybridization (ISH) presents an excellent alternative for the diagnosis of FIP. RNA ISH can be particularly useful when there is no known or available antibody epitope with adequate sensitivity and specificity to allow for consistent detection of a pathogen via immunohistochemical methods. As opposed to the use of an antibody to detect a specific antigen, RNA ISH functions through the detection of specific RNA sequences through oligonucleotide probes - effectively allowing for the detection and microscopic visualization of pathogen-specific RNA sequences in tissue. In the case of FIPV, the technique may provide a key advantage. By targeting RNA, as opposed to protein with IHC, the aforementioned antigenic heterogeneity is bypassed—as there is no direct need for the presence of a specific epitope. Rather, there must just be sufficient viral RNA to allow for visualization. Should a given tissue be suitable for productive infection, viral RNA should be present in sufficient quantities to allow for detection.

Traditional RNA ISH techniques have lacked sensitivity, requiring significant expression of the desired transcripts, and also required a considerable amount of individual labor—thereby limiting their utility in the clinical setting[32][33]. However, in recent years, in situ technology that offers greater diagnostic sensitivity has emerged. One such option is RNAScope™, which uses custom-designed probes that are targeted to 18-25 base pair regions of the desired RNA sequence and a series of amplifiers and chromogenic labeling to allow for proper visualization. Here, we investigate the performance of this RNA ISH technique in the histological diagnosis of FIP, evaluating it in comparison to the current diagnostic gold standard of IHC.

Methods

Selection of probe target region

A preliminary evaluation of target RNA was performed using a review of the existing literature. A previous study successfully developed a pancoronavirus RT-PCR assay for the detection of all known coronaviruses based on a conserved portion of the ORF1ab gene that encodes the viral RNA polymerase[34]. Therefore, in order to capture all FIPV variants and limit the decline in sensitivity that would be observed by choosing a less conserved region, ORF1ab presented an optimal target site for the RNA ISH probes. A set of thirteen ORF1ab FIPV sequences (accession numbers: FJ938052.1, FJ938059.1, FJ938055.1, KX722529.1, KY566211.1, KY566210.1, KY566209.1, KY292377.1, MG893511.1, KF530123.1, KX722531.1, KX722530.1, EU186072.1) was shared with Advanced Cell Diagnostics (Hayward, CA) for the development of a custom probe. A probe based on type 2 FIPV ORF1ab sequences was identified in preliminary investigation to have the greatest sensitivity across both genotypes. The probe had a target area from base

pairs 12380 to 13396 with 84.4% pairwise identity (average percent of exact matches over the aligned sequences) and a Tm of 83.5°C.

IHC and selection of FIP samples for ISH

Immunohistochemistry was performed by the Section of Anatomic Pathology, Department of Biomedical Sciences at the Cornell University College of Veterinary Medicine Animal Health Diagnostic Center (AHDC). Unstained slides containing sections of tissue 5 μm thick were baked in an oven at 80°C for thirty minutes. On a Lecia BOND-MAX Stainer, slides were de-waxed with Bond Dewax Solution (Leica-cat#AR9222). Epitope retrieval was then performed using Bond Epitope Retrieval Solution 1 (Leica-cat#AR9961), with the solution applied for thirty minutes. Monoclonal antibody FIPV 3-70 (Custom Monoclonals-Cat#MCA2194) was diluted at 1:1000 and applied to the slide for sixty minutes, after which PV-AP-Anti-Mouse IgG Reagent (Leica-cat#PV6110) was applied to the slide for thirty minutes. Lecia Bond Polymer Red Detection (Lecia-cat#DS9390) was applied for fifteen minutes and then followed by hematoxylin for five minutes (Lecia-cat#DS9390). Slides were then removed from the stainer and allowed to air dry. When dry, slides were dipped in xylene and cover-slipped. The 1:1000 dilution of mAb FIPV 3-70 was chosen for this study based on the best signal and lack of background staining on the automated equipment used at the Cornell AHDC.

A collection of immunohistochemical slides of feline tissues with suspected FIP collected prior to the COVID-19 pandemic was obtained from a previous study and evaluated by light microscopy for the presence of FIPV antigen. Tissues included brain, liver, kidney, lung, spleen, intestine, and mesenteric lymph node. Slides that demonstrated immunoreactivity were further assessed for the abundance of FIPV-containing lesions. Slides that demonstrated larger, more intensely stained lesions were cross-referenced to formalin-fixed paraffin-embedded (FFPE) tissue blocks from which the slides originated and selected for examination with RNA ISH. Tissue samples from a unique thirty cats were selected for evaluation with RNA ISH.

RNA in situ hybridization

FFPE blocks corresponding to the selected IHC slides were cut into 5 μm sections and placed onto slides by the Section of Anatomic Pathology. RNA ISH was performed using the RNAscope™ 2.5 HD Reagent Kit RED provided by Advanced Cell Diagnostics (Hayward, CA). After obtaining the slides from the diagnostic laboratory, slides were first deparaffinized on a slide warmer at 60°C for 20 min, followed by two changes in xylene for 5 min and two changes in 100% ethanol for 3 min each. After drying, slides were treated with hydrogen peroxide for 10 min at room temperature, washed twice with water, and boiled in a target retrieval solution for 15 min. After two additional washes in water followed by an additional wash in ethanol and air drying, slides were then treated with the kit-included protease (Cat. #322330) and incubated at 40°C for 30 min.

The target probe (Cat. #462091) was applied to each of the target slides except one select duplicate, to which a negative control probe was applied. Probes were allowed to hybridize and were incubated at 40°C for two hours, followed by two washes in wash buffer (Cat. #310091) for 2 min each. A series of six amplification steps, each followed by two 2 min washes in wash buffer, were performed. Amplifiers one through four were incubated at 40°C, alternating between 30 min and 15 min durations. Amplifiers five and six were incubated at room

temperature for 30 min and 15 min. Slides were then treated with RED chromogenic reagent for 10 min and then washed with water. Slides were then counterstained in hematoxylin, after which they were rinsed under tap water for 5 min. After dehydrating, slides were cover-slipped and examined by light microscopy.

Microscopy

All slides were evaluated by light microscopy using a histology microscope. Pictures for proof-of-concept research purposes were taken using an ECHO Revolve microscope (San Diego, CA) using the brightfield configuration. Microscope settings were: brightness; 32, contrast; 75, color balance; 75, LED; 67%. Magnification for pictures was set at 4x.

Results

Comparison between IHC and RNA ISH

All thirty RNA ISH slides returned positive results that were either comparable to or exceeded the gold standard of IHC. Tissues stained with RNA ISH showed staining to be more robust than their IHC counterparts and were more diffuse throughout the examined organ sections. RNA ISH staining was not limited by tissue type and was well corroborated by the IHC slides, with staining across both slides being associated with well-defined lesions. Negative controls were adequate, upholding the specificity of the technique compared to the IHC gold standard.

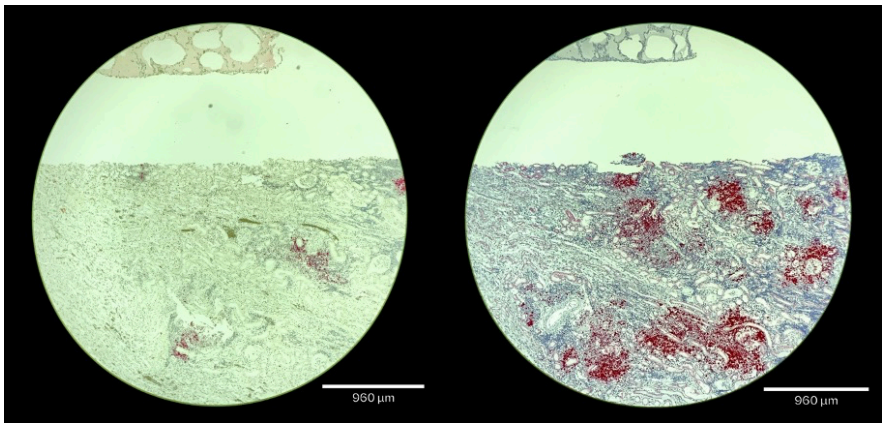

Figure 1. Cat A. Comparison of FIPV 3-70 IHC and RNA ISH using the FIPV ORF1ab probe. Images were taken with matching areas of tissue sections under a 4x magnification lens. Immunohistochemistry is shown on the left, while RNA ISH is shown on the right. Tissues shown are kidney (large tissue) and lung (small tissue).

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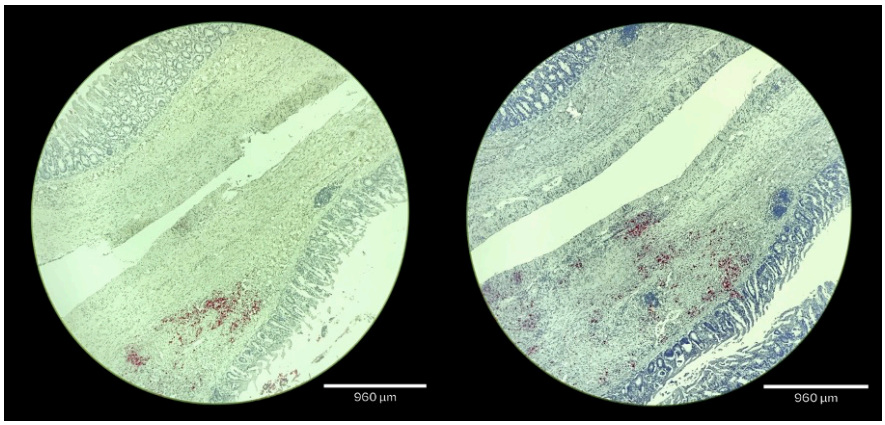

Figure 2. Cat B. Comparison of FIPV 3-70 IHC and RNA ISH using the FIPV ORF1ab probe. Images were taken with matching areas of tissue sections under a 4x magnification lens. Immunohistochemistry is shown on the left, while RNA ISH is shown on the right. Tissues shown are intestine.

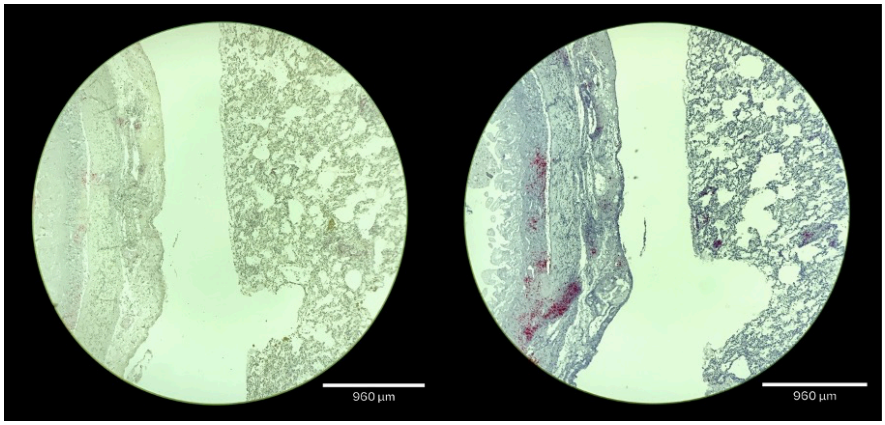

Figure 3. Cat C. Comparison of FIPV 3-70 IHC and RNA ISH using the FIPV ORF1ab probe. Images were taken with matching areas of tissue sections under a 4x magnification lens. Immunohistochemistry is shown on the left, while RNA ISH is shown on the right. Tissues shown are intestine and lung (left and right tissues of the image, respectively).

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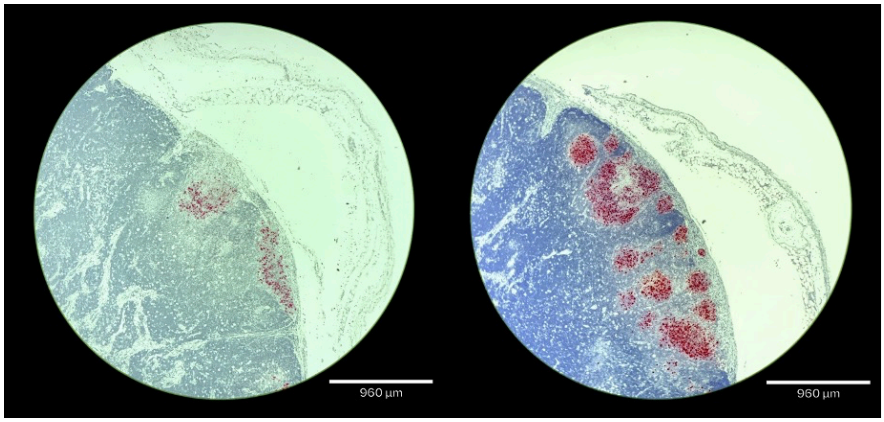

Figure 4. Cat D. Comparison of FIPV 3-70 IHC and RNA ISH using the FIPV ORF1ab probe. Images were taken with matching areas of tissue sections under a 4x magnification lens. Immunohistochemistry is shown on the left, while RNA ISH is shown on the right. Tissues shown are mesenteric lymph node.

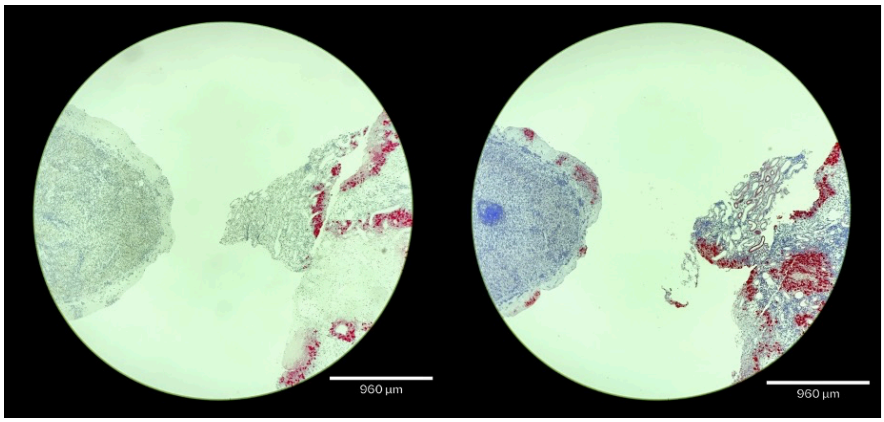

Figure 5. Cat E. Comparison of FIPV 3-70 IHC and RNA ISH using the FIPV ORF1ab probe. Images were taken with matching areas of tissue sections under a 4x magnification lens. Immunohistochemistry is shown on the left, while RNA ISH is shown on the right. Tissues shown are spleen (left tissue) and kidney (right).

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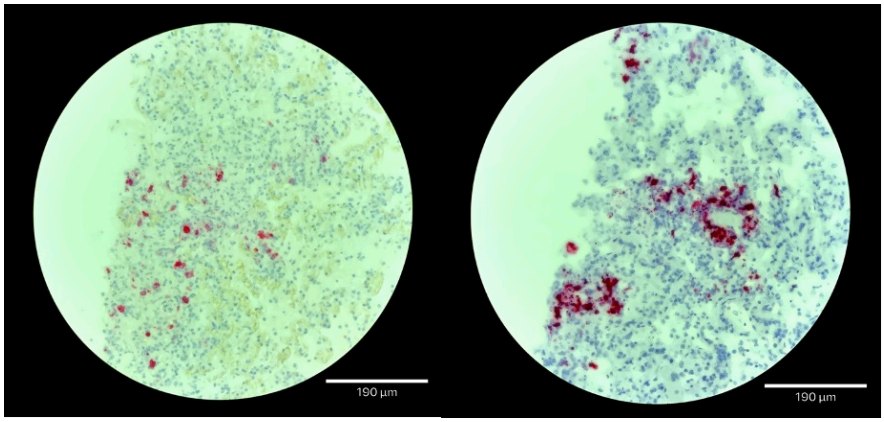

Figure 6. Cat C. Higher magnification comparison of FIPV 3-70 IHC and RNA ISH using the FIPV ORF1ab probe. Images were taken with matching areas of tissue sections under a 20x magnification lens. Immunohistochemistry is shown on the left, while RNA ISH is shown on the right. Tissues shown here are intestine. Red dots represent detection of FIPV nucleocapsid (for IHC) or detection of FIPV ORF1ab gene (for RNA ISH).

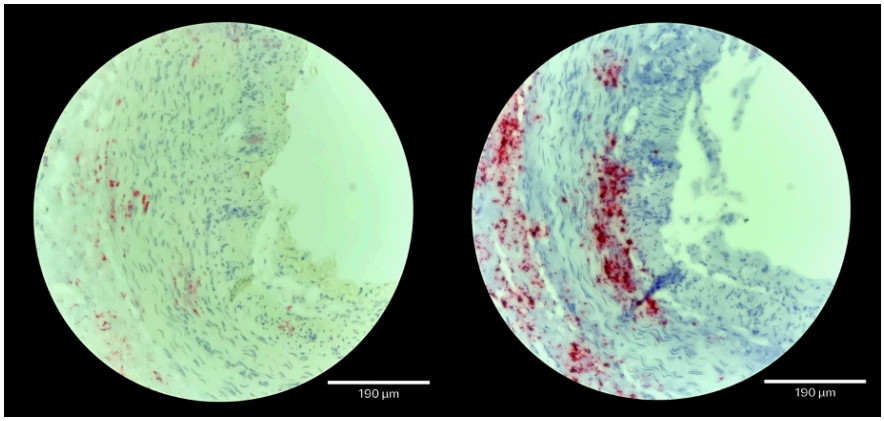

Figure 7. Cat F. Higher magnification comparison of FIPV 3-70 IHC and RNA ISH using the FIPV ORF1ab probe. Images were taken with matching areas of tissue sections under a 20x magnification lens. Immunohistochemistry is shown on the left, while RNA ISH is shown on the right. Tissues shown here are lung. Red dots represent detection of FIPV nucleocapsid (for IHC) or detection of FIPV ORF1ab gene (for RNA ISH).

Discussion

The present study serves as a preliminary investigation of an FIPV RNA ISH probe for the diagnosis of FIP by performing a side-by-side comparison of the RNA ISH probe with the current gold standard of immunohistochemistry. The comparison between the current gold standard of IHC and our RNA ISH probe demonstrates evidence that the use of RNA ISH, with the conserved genetic target of ORF1ab, allows for improved performance in FIPV detection compared to IHC with FIPV mAb 3-70. While only tissues from cats with IHC-confirmed FIP were used in this study, several samples showing RNA ISH positivity were much

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stronger in their positive staining and not solely limited to a few individual cells, as they were in IHC, but present in large lesions. FIPV mAb detection was occasionally very faint in these cases, where it would be limited to a few individual cells—leaving considerable possibility for these cases to be incorrectly classified as a negative result.

A high sensitivity value presents as a critical component of a diagnostic technique. As this study did not investigate tissues from FIPV-negative cats, it was not possible to ascertain the specificity of the technique. Given that FIPV staining by RNA ISH tended to match a similar distribution to that of IHC when there was abundant staining for both techniques, it supports RNA ISH closely following the specificity of the gold standard and not leading to aberrant positive staining. Again, as specificity is calculated using the proportion of test-negative cases over the sum of test-negative and false positive individuals, the use of only true positive cases in this study prevents a true validation of specificity with the samples we have examined here. Furthermore, in using a third-party RNA ISH assay, the technology and its detailed workings remain largely proprietary information. Not being directly involved in probe design hinders the ability to refine the probes and their targeted genetic areas that may have important implications for the sensitivity and specificity of the technique. The corroboration of this RNA ISH technique by immunohistochemical staining, however, allows for confidence in the technique and the suitability of the genetic target.

We consider that RNA ISH offers significant benefits to support its further investigation and adoption in the clinical setting. The improvements in sensitivity of FIP diagnosis that RNA ISH provides can support those working with FIP in both the clinical and research spheres. While further validation studies must be carried out to ascertain the true sensitivity and specificity of the technique, the preliminary investigation conducted here supports its use for the confirmation of FIP. The current study was performed manually for proof-of-concept research purposes. With the advent of automated machinery, the methodology described here can be readily adapted and streamlined—and made more cost-effective—for routine use.

An improved ability to detect FIPV presents benefits for research purposes, allowing for retrospective investigation of cats that were suspected to have FIP but whose tissues have yielded negative IHC results. In having a broader scope of detection among these cases, examining FIP cases that return positive RNA ISH results but negative IHC results presents exciting research questions, particularly when combined with qPCR techniques to investigate the possibility of low-level persistent FCoV infections in cats and determining antigenic or genomic differences that may exist between viruses that can be detected by IHC versus those that are detected by RNA ISH. We believe that studying the virological properties of these IHC-undetected FIPV variants and any potential differences with the detectable population may uncover insights that allow for a better understanding and characterization of FIPV and its circulating variants, and may uncover important viral clues and characteristics that allow for a more complete understanding of the disease syndrome recognized as FIP.

Statements and Declarations

Ethics

Ethical review and approval were not required for the animal study because the study utilized archived diagnostic tissue samples obtained from the Cornell University Animal Health Diagnostic Center prior to the initiation of this specific research project. Samples were anonymized.

Data Availability

The raw data supporting the conclusions of this article will be made available by the authors upon reasonable request. Inquiries can be directed to the corresponding author.

Author Contributions

Conceptualization, G.W.; methodology, A.S., N.A., B.N.L., G.W.; validation, A.S., N.A.; formal analysis, A.S., N.A.; investigation, A.S., N.A.; resources, G.W.; data curation, A.S., N.A.; writing—original draft preparation, A.S., N.A.; writing—review and editing, B.N.L., G.W.; visualization, A.S., N.A.; supervision, G.W.; project administration, G.W. All authors contributed to the article.

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Declarations

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