Review of: "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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Potential competing interests: The author(s) declared that no potential competing interests exist.

The study deals with the diagnosis of FIP. As such, the topic is always interesting and disputed. Despite the title, the manuscript reports the findings of a very preliminary and still incomplete validation protocol. The study is not blind. Case selection was strongly biased by the selection of cases itself. It could only be considered a proof-of-concept study. RNA ISH vs compared on only positive cases, selected for their strong immunoreactivity confirmed by RNA ISH. This is only the preliminary step of a validation.

Honestly, I do not share the approach. It relies on the draconian and too simplistic assumption that " two distinct biotype exist …FECV..and FIPV….FECV cause no or mild disease…mainly enteric but also systemic. In the 15% of cases, it may persist longlife with chronic shedding in feces. Genomic factors (i.e. mutation in the S proteins among others) may clusters FCoV in biotypes but they are not exhaustive (i.e mutated strains may not invariably cause disease as well as also the mutation of the FECV should be interpreted as a host immune system driven event). In the reviewer opinion there is a spectrum of FECV infections with a very low letality (i.e. only a minority of cases are fatal). Claiming that FIP forms are invariably fatal is as claiming that fatal cases of cancer are always fatal.

Having said that, the manuscript is very well written and easy to read with the distinctive clarity and simplicity of the Anglo-American authors. I would suggest to shorten the introduction section.

Below some suggestions are provided to eventually improve the manuscript.

Please, specify the FCoV prevalence and reference it.

"small subsets": please specify and reference.

"..systemic infections": as specified later, also FCoV infections may be systemic.

Pag 2, half-page: "from" should read form, I suppose.

"The progression of FIP is always fatal without treatment" please, reference this statement.

"entirely specific" and "exclusive": so obvious that it wouldn't be reminded

Page 3, first paragraphs: although I also consider AGP as an overstated marker, this paragraph tends to underestimate its relevance. Some more info should be provided here as the thresholds and relative sensitivity/specificity performances of AGP.

"routinely performed". This is not true. We did perform them routinely. Many commercial labs perform them routinely.

Reference #7 is not an evidence based reference. That transaminase elevations are indicative if lymphoplasmocytic

cholangitis and may be used to rule out FIP is anecdotal and never demonstrated.

The Rivalta Test should be discouraged. The whole paragraph should be deleted. The reference 15 should be deleted since evident limits in study design may have biased that study.

Please, citing sensitivity and specificity without referring the study design may be misleading for unexperienced readers. Any diagnostic performance may be achieved by inaccurate study design. Diagnostic accuracy between studies different in their design may induce the readers to errors. Please, be consistent throughout the manuscript in dealing with this issue. In my opinion the best way is to also provide the design drawbacks together with the diagnostic performances.

Page 3, last rows: not just the prevalence but also the study design (i.e. proportion of subjects with high viral titers, inclusion criteria, groups composition ond so on)

Page 5, first paragraph. The dot at the end is lacking.

Reverse transcription….PCR product": this sentence is misleading. The RT is used since the virus is an RNA virus. The RT does not solve the problem of the PCR contaminations. However, I would also suggest that contamination was a problem of the past. Nowadays, close-tube PCR and fluorescent dyes are widely diffused, and the problem of the contamination is much less a concern than in the past. Also nested PCR is indeed a RT-nested PCR. The step of reverse transcribing RNA is mandatory in coronavirus PCR testing.

I would suggest the following reading to address the limit of molecular testing in FIP:

Barker et al. Vet Res. 2017 Oct 5;48(1):60. doi: 10.1186/s13567-017-0467-9. I would suggest you to concentrate the focus of this chapter on the target quantification which could have an impact to differentiate FECV from FIPV forms.

Pag 5: "as false negatives are a known shortcoming of diagnostic PCR tests." This sentence should be deleted since it is soundly out of context and overstated. In general, PCR test are the diagnostic tests with the highest sensitivity.

"development of a more specific PCR … risk of false negative results" also this statement is overstated and unsubstantiated nor referenced. Please, delete.

Overall, the section of molecular test should be improved.

Page 7: the advantages of RNA ISH versus IHC or ICC against antigen are weak. Since the antigenic heterogeneity is caused by heterogeneity of the viral genome and hence of the subgenomic RNA, this is not an inherently better method while I understand that RNA ISH has the advantage to overcome the availability of antibodies. Conversely, antigens are much more conserved in formalin-fixed tissue than RNA.

Page8: Selection of target region: the criteria used are not the best. Since most of the viral RNA are subgeneomic transcribed RNA, the orientation of the probe should be specified (against + or – RNA). Also, it is well known that the transcription proceed from 3' to 5'. Thus 3' gene products are much more represented than 5' (as the one targeted by the probes). To seek for sensitivity, I would have chosen targets more towards the 3' end. This limit should be mentioned in the manuscript.

"collected prior to the COVID-19 pandemic". Is it important to mention?

"previous study". The study should be mentioned.

Page 9 Negative controls were not specified. Nothing could be drawn about specificity. Please. Delete the sentence.

Page 13. "Preliminary" term should be added in the title

A side-by-side comparison" is not a validation method. Clearly, in a preliminary way, one is oriented to establish a method

and to evaluate that its performance is at least technically equivalent in a “not problematic” case series.
Much of the discussion deals with vague or unquantifiable impressions. Generic terms are used “few” “more” without any quantification. This approach is justifiable in a preliminary step, but it should be kept to a minimum.
“The specificity value of 100% for IHC presents as a critical component of the diagnostic technique”. It’s just an opinion. As a general rule, sensitivity, specificity, PPV and NPV should be balanced. It does not make any sense to establish a 100% specificity. Also, 100% in general does not make any sense.

Page13-14: While I have appreciated the frank discussion about the limitations (also considering the that the details are proprietary and not fully disclosed), Likewise, I consider too soon to include this assay in clinical settings without a fully validation.
“Current clinical advice….developing FIP”. Please, this statement should be referenced. FECV are widespread. This advice may have an evidence-based basis only in case of household outbreaks.
It has not been mentioned that RNA is strongly degraded in formalin-fixed tissue. This evidence usually hampers the reliability of its detection. It would be interesting to assess RNA ISH in real world diagnostics.