**Peer Review File**

**Manuscript Title: Enteric viruses replicate in salivary glands and infect through saliva**

## Reviewer Comments & Author Rebuttals

**Reviewer Reports on the Initial Version:**

Referees' comments:

Referee #1 (Remarks to the Author):

This study by Ghosh et al. explores the potential transmission of enteric viruses through saliva. The manuscript is clearly written, and the authors provide intriguing data indicating retro-ductal transmission of murine rotavirus and norovirus to maternal mammary glands from pup saliva, suggest cellular tropism for ductal and acinar cells of the salivary glands, and demonstrate that virus can be propagated in salispheres. An additional interesting observation reported is that human norovirus can be propagated in a ductal cell line. While these findings are very enticing and would be an important advance in our understanding of enteric viral pathogenesis, there are some additional pieces of information needed to clarify this phenomenon.

Major:
1) There is no clear information provided in the figure legends or methods regarding the amount of virus administered to mice (either pfu, genome copies, or ID/DD50), making these infections very difficult to compare to the published literature or to clarify what may have been genuine infection versus passive transfer of genome copies.
2) What are limits of detection of qPCR assays used in Figure 1? Is this assay truly sensitive enough to reliably detect a range from 4e5 (1d) to 0.1 copies (1j)?
3) Was enteric viral replication assessed in dams from Figure 1f? Similarly, was enteric sIgA assessed in dams from Figure S1b and c?
4) Do persistent strains of MNV persist in the salivary gland?
5) Because CD300lf is the known MNV receptor, it would be valuable to assess salivary gland infection in CD300lf-/- mice, which are commercially available. Similarly, how do CD300lf-/- salispheres perform?
6) Can salivary gland infection be inhibited with antiviral treatments, such as IFN-lambda or 2'-C-methylcytidine or others?
7) For human NV passaging, is virus becoming adapted to ductal cell line? How substantial are genetic alterations over this time?
8) Have the authors tested human salispheres, as an opportunity to better define in vivo relevance and cellular tropism?

Minor:
1) Please reorder panels in Fig. S1 to match how these are reported in the text. Further labeling of S1b versus S1c would also make this more accessible.

Referee #2 (Remarks to the Author):

In the manuscript by Ghosh et al, the authors test an interesting hypothesis that salivary glands represent a productive target tissue and source of gastrointestinal viruses including mouse rotavirus, norovirus, and astrovirus. Given that rotavirus, norovirus, and astrovirus have different receptors, known target cells, and mechanisms of pathogenesis, this finding would be both particularly surprising and interesting to the field. The authors demonstrate virus can be transmitted between pups and dams by nursing and that virus RNA can be recovered from saliva. They also show virus antigen positive cells in the mammary glands and salivary glands. However, enthusiasm for the manuscript is limited by modest effect sizes, insufficient controls, and small sample sizes which lead to overly broad conclusions given the existing data. Extensive further experimentation including leveraging of available tools are needed to make the broad and generalizable conclusions the authors try to make.

Major

• A number of the figures represent only a single experiment. For example, figure 1J, 1K, 3M, 4A, B etc. Biological replicates are needed for all experiments to demonstrate the phenotypes are true across time, litters, etc.

• The data presentation limits interpretation of the magnitude of effect. Specifically, what do the y-axis copies/mock refer to in the figures (ex. Fig 1B-C, 4A,D,E etc)? The mock is 0 so how do the authors normalize to 0? Do the authors mean Viral RNA copies/GAPDH RNA copies? It is difficult to interpret the figure without understanding what the authors are measuring and normalizing to.

• Assays assessing viral replication in vivo and ex vivo are confusing as described, technically limited, and of modest magnitude. Measuring infectious virus by plaque assay rather than viral RNA is important for key experiments. While this is not possible for EDIM or astrovirus, the methods are straightforward for MNV. SARS-CoV-2 highlights this as an important example because although SARS-CoV-2 viral RNA can be isolated from saliva, infectious virus is not detected in saliva (with possible rare exception). Figure 2 B-D, 3B-D, S3, 4A should be quantified by plaque assay for MNV

• Critical controls are missing

o What's the negative control in Fig 3M? How do we know the qPCR assay is specific?

o Is CD300lf protein detected as claimed in Lines 146-148 (this data is not shown)? Negative control mice such as CD300lf KO mice would be incredibly informative here to distinguish productive from non-productive infection. Two independent CD300lf KO mouse lines are commercially available at Jax and use of these mice would strengthen the conclusions and facilitate interpretation.

o Figure 3A, does anti-CD300lf or anti-MNV VP1 neutralize viral genome replication?

o Figure 4GH, co-staining with a nonstructural protein (or anti-dsRNA) is necessary. There is abundant VP1 in the inoculum and the polyclonal antibodies can be non-specific. VP1 staining is not sufficient to define a cell as infected. For example, HNoV VLPs can bind and be taken up resulting in positive staining without productive infection. Additional controls demonstrating HNoV replication is real are needed such as anti-VP1 sera.

• It is shocking that MNV can infect a rat cell line (A5) given rats are resistant to MNV and there are significant differences between mouse and rat CD300 proteins. If true, this is very interesting. The co-crystal structure of MNV VP1 and mouse CD300lf revealed 11 VP1 contact residue (PMID 30194229). 5 of these 11 residues differ between mouse and rat CD300lf making an interaction between MNV and rat CD300lf unlikely. Extensive controls (which are available) are needed here to demonstrate MNV replication in rat cells is real and robust. The read out should be viral replication

by plaque assay (or TCID50) and not qPCR. Second, controls such as anti-CD300lf, 2CMC, or anti-VP1 would be very helpful. Third, rat CD300lf should be transfected into a non-susceptible cell line such as 293T or HeLas to show rat CD300lf can support MNV entry as suggested here. Fourth, the species identity and purity of the rat cells needs to be further confirmed (ie PCR for a mouse specific transcript) beyond karyotyping which is not sensitive for low level contamination of MNV-susceptible murine cells (<1 in 50 cells) in the rat cultures which could explain low level viral replication.

• The magnitude of differences observed are modest at best for many of the assays (ie. often only several fold) and not logarithmic within the kinetics expected for these viruses as would be expected if the salivary glands were major targets and sources of infectious virus. Graphs of viral load would be best presented as log and not linear scales¬

• Can infected cells be quantified in salivary glands and sorted to confirm they are productively infected? Demonstrating that salivary gland cells can be isolated, sorted, and infectious virus recovered by plaque assay or at least qPCR would be very convincing as done by Lee et al (PMID 28966054)

• Can salivary glands be surgically removed in mice as they can in humans? If so, this would be a useful experiment to prove salivary glands are essential for infection in young mice.

Minor

• Figure 3I only shows that at least a single infectious virus was present. This is not particularly informative and could readily be explained by contamination from mice being housed in a cage/environment with MNV. Quantification of infectious virus by plaque assay is the definitive experiment.

• Salivary glands are in the oral cavity and thus the MS title is not precise.

• Line 1 and 39 herpes etc are the disease not the virus. The viruses are HSV1/2, etc.

• Line 40-1: unclear if SARS-CoV-2 shed into the saliva is infectious. This reference identifies viral RNA and not infectious virus in saliva

• Line 41 needs a ref regarding saliva testing (at least for SARS-CoV-2)

• There is not good evidence that SARS-CoV-1 or SARS-CoV-2 infect or replicate in the salivary glands, only that viral RNA is detectable in saliva. The origin and nature of this RNA is unclear. This should be clarified.

• Line 111: The authors should remove “highly” since they did not quantify infectious virus. They only assessed the presence or absence of infectious virus.

• Line 606: How do the authors determine a multiplicity of infection for HNoV when it can’t be plaqued?

• Line 613: How do the authors infect mice with 10^6 TCID50 of EDIM and murine astrovirus when they don’t replicate in culture and thus a TCID50 can’t be determined?

Referee #3 (Remarks to the Author):

Review of Kumar etc al.

The authors describe a series of experiment that uncover a potential new route of transmission of enteric pathogens and a new site of replication, namely in salivary glands.

Their first observation is that infected pups can transmit virus back to the mother and cause an infection in the mammary gland. This is a powerful observation, but I have a minor concern regarding the way in which the observations are implied to hold true for all enteric viruses, where experiments have been performed primarily with rotavirus (see minor comments below).

The authors go on to demonstrate that both norovirus and rotavirus RNA, as well as protein, can be found in saliva, and that the virus in the saliva can transmit infection from adult mice to pups.

Direct replication of noro, rota and astrovirus in salivary glands was observed, but as noted below, the manner in which the RNA copy number data is presented is somewhat non-standard. They demonstrate a modest increase in replication following inoculation – as noted below there is a need to control for residual RNA from the input. The infection of salivary glands is very convincing as multiple approaches have been used, but the overall amount of infectious virus present in the samples appear to be very low (See minor comment below). Furthermore, replication in salivary organoids appears confirmatory but again the levels of replication appear modest and no attempt to quantify actual infectious virus present in these organoid infections is presented.

The growth of human norovirus in the ductal cell line is intriguing and could be a major step forward for the field, if it was seen to be robust and applicable to a wide range of isolates. The conclusion that they provide a robust replication system, isn't yet supported by the data, but it could be with a few limited additional experiments.

Overall, I feel this is great piece of work that would be of interest to the readership of the journal. It uncovers new modes of transmission for enteric pathogens that would be of interest to anyone working in the areas of infectious diseases. However, there is a need for some changes in text and the way in which the data are presented to ensure the data and the interpretation of it, are truly objective and comparable to studies in the field. Additional experiments around the robustness of the observations and the reproducibility of the data in vitro culture of human norovirus would greatly improve the manuscript and solidify the conclusions, that are only partly supported by the current data.

Major comments:
- The level of replication seen in salivary glands and organoids is modest – a 4 fold increase in viral RNA from day 1 to day 3 is typical. It will be important to control for the residual RNA present in the input sample. This could be achieved by infecting animals/organoids side by side with UV-treated inoclum or by the addition replication inhibitor such as 2CMC (see https://pubmed.ncbi.nlm.nih.gov/23986582/)

- The levels of replication of human norovirus in the ductal cells appear modest, but admittedly comparable to work published on BJAB cells and some HIE models. Notably others have seen much more robust replication in the absence of a robust interferon response. Have the authors examined whether the system can be improved using inhibitors of the pathway? Equally the robustness of the culture system and observations is difficult to assess given only a single viral isolate was used. The use of replication inhibitors, such as 2CMC in such a model, along with more isolates of human norovirus would be required to support their conclusion (ln 200) that these line can provide a robust replication system.

- Ln 196: the authors conclude that salivary glands are a “major site of replication for enteric viruses” – however the use of the term “major” appears somewhat subjective. I agree that their data indicate replication occurs, but I do not see how this can then be sold as a “major site of replication” based on the levels of replication observed. Taking on board the rather non-standard way in which the replication data is presented, I don't agree with their conclusion here.

Minor comments:
- Ln 69 and throughout the text: Seems to be some experiments performed with MNV and rotavirus and some with just one. A key such experiment is the demonstration that 10-day post-partum dams (pup free) do not show any replication in the mammary glands – indicative of transfer of the infection from the pups to the dams mammary gland. It is implied that the observation holds true for both model organisms (norovirus and rotavirus), when in fact this conclusion cannot be made against. The authors need to be more restrained in drawing sweeping conclusions about “enteric viruses” when not all experiments were performed with both Noro and Rota. Multiple example of this exist in the manuscript (e.g. ln 102)

- The way in which the RNA copy number is being presented throughout the manuscript is not standard. It is presented as RNA copies/mock – why aren’t absolute viral RNA copies being shown? Mock infected animals don't have any viral RNA, how can this be used as a reference? The Y axis on the graphs appear to be linear with splits at various point making the interpretation a bit of a challenge e.g. Fig 2d Y axis is 0 to 8 x 10^3. This approach makes direct comparisons with data from others in the field a challenge. Absolute viral RNA levels would allow a much better comparison with published data using mouse models of these diseases.

- Fig 2: Given the amount of viral protein present in the saliva samples, I was surprised that the authors didn't assess viral infectivity in these samples directly. Clearly these samples contain infectious virus as it transmits but the way in which the infection data in figure 2d is presented, it difficult to gauge the degree of replication and to compare it to published work.

- Figure 3I: Again this is a non-standard presentation of viral infection of RAW 264.7 cells. Why isn’t pfu or TCID50 data presented?

**Author Rebuttals to Initial Comments:**

**We would like to thank all the reviewers for their enthusiasm and comments regarding our manuscript. We have responded to all their comments/queries below with additional new experiments which are now included in the revised manuscript. Reviewers comments are in blue and all our responses are in *italics.***

**Referees' comments:**

**Referee #1 (Remarks to the Author):**

This study by Ghosh et al. explores the potential transmission of enteric viruses through saliva. The manuscript is clearly written, and the authors provide intriguing data indicating retro-ductal transmission of murine rotavirus and norovirus to maternal mammary glands from pup saliva, suggest cellular tropism for ductal and acinar cells of the salivary glands, and demonstrate that virus can be propagated in salispheres. An additional interesting observation reported is that human norovirus can be propagated in a ductal cell line. While these findings are very enticing and would be an important advance in our understanding of enteric viral pathogenesis, there are some additional pieces of information needed to clarify this phenomenon.

Major:

1) There is no clear information provided in the figure legends or methods regarding the amount of virus administered to mice (either pfu, genome copies, or ID/DD50), making these infections very difficult to compare to the published literature or to clarify what may have been genuine infection versus passive transfer of genome copies.

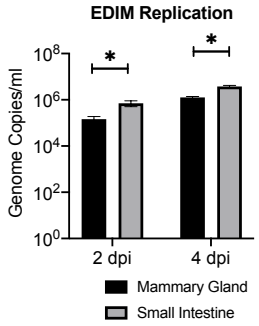
*Response: The Materials section has been updated with the information about the amount of virus being administered to mice.*

2) What are limits of detection of qPCR assays used in Figure 1? Is this assay truly sensitive enough to reliably detect a range from 4e5 (1d) to 0.1 copies (1j)?

*Response: In the revised manuscript, we developed standard curves for the amplicons for the corresponding primers used for detection of the viruses. Our qPCR analysis only considers replication cycles lower than 30 as standard as higher cycle number may not be reliably reflecting replication of the viral genome as the standard curve generated higher standard deviation values signifying higher probability of false positives owing to primer-dimer formations. As per the standard curve 30 cycles corresponds to ~$10^5$ genome copies/ml (for MNV-1 and EDIM) and $10^2$ for CR-6. The revised data sets are re-calculated according to the standard curves for each primer sets and Y-axis is now represented in terms of genome copies/ml. A detailed description has been updated in Methods section.*

3) Was enteric viral replication assessed in dams from Figure 1f? Similarly, was enteric sIgA assessed in dams from Figure S1b and c?

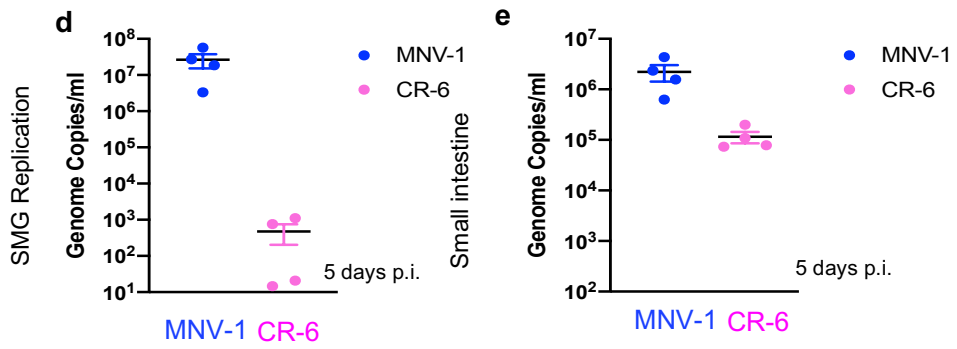
*Response: Enteric viral replication had been assessed in dams. Viral replication was observed in the intestine as these dams stay in the same cage as the infected pups and are licking them. This is likely the cause of their intestinal infection (see figure below for EDIM replication). For this reason, we performed the control experiment study in Fig. 1 k, l & m, where we showed that by oral inoculation of the adult mice EDIM infected the intestinal cells but not the mammary glands (which only got infected by the dams directly suckling their infected pups).*




## 4) Do persistent strains of MNV persist in the salivary gland?

*Response: We found that CR-6, a persistent MNV, could replicate in the salispheres (Fig. 3g, Fig. S3b), which are the organoid model of salivary glands. We also report here that salivary gland epithelial and leukocytes, along with salispheres all express CD300lf receptors (Fig. 3c, Fig S3c). However, when we inoculated mice with CR-6 we did not observe significant replication in the salivary glands whereas we did find replication in the intestines (see below). As mentioned before $10^5$ copy numbers/ml for MNV-1 and $10^2$ copy number/ml for CR-6 corresponds to Ct= 30 (Ct values higher are prone to false positives) (a detailed description available in Methods section). Previous studies have also found some differences in tissue tropism between MNV-1 and CR-6, for instance unlike MNV-1, CR-6 does not replicate in the spleen (Nice et al.,2013). It has been proposed that these differences are likely due to additional viral entry factors such as glycans (Taube, S., J.Virol, 2012) and host microbiome (Grau, K., Nature Microbiology, 2019). These factors may also be the reason for why CR-6 did not replicate in vivo in the salivary gland but did in the ex vivo organoid model.*

Fig. S3



5) Because CD300lf is the known MNV receptor, it would be valuable to assess salivary gland infection in CD300lf-/- mice, which are commercially available. Similarly, how do CD300lf-/- salispheres perform?

*Response: We have performed MNV-1 infection in CD300lf-/- mice and observed that replication in salivary glands is inhibited (Fig. 3d). Since these CD300lf-/- mice are in C57BL/6J background, we also performed a control experiment to see whether MNV-1 is similarly infecting this strain of mice. Similar to our Balb/c results, the C57BL/6J mice also showed replication in salivary glands (Fig 3d & S2g). This validates the requirement of CD300lf receptors to infect salivary glands.*

6) Can salivary gland infection be inhibited with antiviral treatments, such as IFN-lambda or 2'-C-methylcytidine or others?

*Response: We carried out these experiments and found that salivary gland infection by MNV-1 can be inhibited with 2'-methylcytidine both in-vivo and ex-vivo (salispheres) (Figs. 2o and 3i). A detailed description has been added to the Methods section regarding the dosage.*

7) For human NV passaging, is virus becoming adapted to ductal cell line? How substantial are genetic alterations over this time?

*Response: This is an important extension of the current study. Unfortunately, the restrictions due to the current circumstances limited our ability to carry out this experiment in a timely manner. We are currently propagating the virus in the ductal and acinar cell lines beyond passage 4. Any findings from these ongoing studies will be the backbone of a future manuscript.*

8) Have the authors tested human salispheres, as an opportunity to better define in vivo relevance and cellular tropism?

*Response: Due to the COVID pandemic, we have not been able to obtain patient explants.*

Minor:

1) Please reorder panels in Fig. S1 to match how these are reported in the text. Further labeling of S1b versus S1c would also make this more accessible.

*Response: We have presently introduced previously Fig. S1(sIgA from pup intestine and dam's milk) in Fig. 1 for more accessibility and better presentation of the data.*

Referee #2 (Remarks to the Author):

In the manuscript by Ghosh et al, the authors test an interesting hypothesis that salivary glands represent a productive target tissue and source of gastrointestinal viruses including mouse rotavirus, norovirus, and astrovirus. Given that rotavirus, norovirus, and astrovirus have different receptors,

known target cells, and mechanisms of pathogenesis, this finding would be both particularly surprising and interesting to the field. The authors demonstrate virus can be transmitted between pups and dams by nursing and that virus RNA can be recovered from saliva. They also show virus antigen positive cells in the mammary glands and salivary glands. However, enthusiasm for the manuscript is limited by modest effect sizes, insufficient controls, and small sample sizes which lead to overly broad conclusions given the existing data. Extensive further experimentation including leveraging of available tools are needed to make the broad and generalizable conclusions the authors try to make.

Major

• A number of the figures represent only a single experiment. For example, figure 1J, 1K, 3M, 4A, B etc. Biological replicates are needed for all experiments to demonstrate the phenotypes are true across time, litters, etc.

*Response: In the revised manuscript we have incorporated more data points for the experiments mentioned.*

• The data presentation limits interpretation of the magnitude of effect. Specifically, what do the y-axis copies/mock refer to in the figures (ex. Fig 1B-C, 4A,D,E etc)? The mock is 0 so how do the authors normalize to 0? Do the authors mean Viral RNA copies/GAPDH RNA copies? It is difficult to interpret the figure without understanding what the authors are measuring and normalizing to.

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• Assays assessing viral replication in vivo and ex vivo are confusing as described, technically limited, and of modest magnitude. Measuring infectious virus by plaque assay rather than viral RNA is important for key experiments. While this is not possible for EDIM or astrovirus, the methods are straightforward for MNV. SARS-CoV-2 highlights this as an important example because although SARS-CoV-2 viral RNA can be isolated from saliva, infectious virus is not detected in saliva (with possible rare exception). Figure 2 B-D, 3B-D, S3, 4A should be quantified by plaque assay for MNV.

*Response: In the revised manuscript, we have performed the TCID50 for MNV-1 in RAW cells for salivary gland infection, infectious viral release in saliva, and for MNV-1 and CR-6 in salispheres. The data has now been incorporated in the revised version in Fig. 2d & n, Fig 3h.*

• Critical controls are missing

o What's the negative control in Fig 3M? How do we know the qPCR assay is specific?

*Response: We have addressed the issue by introducing HeLa cells as a negative control in the revised manuscript.*

o Is CD300lf protein detected as claimed in Lines 146-148 (this data is not shown)? Negative control mice such as CD300lf KO mice would be incredibly informative here to distinguish productive from non-productive infection. Two independent CD300lf KO mouse lines are commercially available at Jax and use of these mice would strengthen the conclusions and facilitate interpretation.

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o Figure 3A, does anti-CD300lf or anti-MNV VP1 neutralize viral genome replication?

*Response: As stated above, salivary gland viral replication is significantly inhibited in the animals lacking CD300lf. In addition, we now show that MNV-1 can be inhibited with antiviral treatments with 2'-methylcytidine both in-vivo and ex-vivo (salispheres) (Figs. 2o and 3i). A detailed description has been added to the Methods section regarding the dosage.*

o Figure 4GH, co-staining with a nonstructural protein (or anti-dsRNA) is necessary. There is abundant VP1 in the inoculum and the polyclonal antibodies can be non-specific. VP1 staining is not sufficient to define a cell as infected. For example, HNoV VLPs can bind and be taken up resulting in positive staining without productive infection. Additional controls demonstrating HNoV replication is real are needed such as anti-VP1 sera.

*Response: In the revised manuscript, we performed FISH staining for the negative strand (replicative) of human NV and also probed for anti-NS-7, anti-NS-6 non-structural proteins. Both confirm NV RNA replication and RNA translation in the cell lines (Fig. 4 c,f,g,h,i,j&k and Fig. S4e,f). Furthermore, we now show that replication in the cell lines is most robust with NV-loaded extracellular vesicles isolated from human stool samples whereas free NV in stool does not replicate (Fig. 4d-4g). This is consistent with recent studies from our group ( Santiana et al., Cell Host Microbe 2018; and Zhang et al., Environ Sci Tech 2021) where we show that free enteric viruses in stools are significantly less infectious and more susceptible to environmental damage then vesicle-cloaked viral clusters. This is due to a combination of high MOI afforded by being trafficked inside vesicles in viral multiples as well as having a membrane protective coat from proteases, nucleases and other environmental aggressors.*

• It is shocking that MNV can infect a rat cell line (A5) given rats are resistant to MNV and there are significant differences between mouse and rat CD300 proteins. If true, this is very interesting. The co-crystal structure of MNV VP1 and mouse CD300lf revealed 11 VP1 contact residue (PMID 30194229). 5 of these 11 residues differ between mouse and rat CD300lf making an interaction between MNV and rat CD300lf unlikely. Extensive controls (which are available) are needed here to demonstrate MNV replication in rat cells is real and robust. The read out should be viral replication by plaque assay (or TCID50) and not qPCR. Second, controls such as anti-CD300lf, 2CMC, or anti-VP1 would be very helpful. Third, rat CD300lf should be transfected into a non-susceptible cell line such as 293T or HeLas to show rat CD300lf can support MNV entry as suggested here. Fourth, the species identity and purity of the rat cells needs to be further confirmed (ie PCR for a mouse specific transcript)
beyond karyotyping which is not sensitive for low level contamination of MNV-susceptible murine cells (<1 in 50 cells) in the rat cultures which could explain low level viral replication.

*Response: In the original manuscript we showed that MNV-1 replicated in the rat A5 cells but compared to the murine salispheres, the replication was significantly lower. We have since assayed whether the A5 cells are productively infected and find that they do not release infectious virus (data not shown) and therefore removed the A5 data from the revised manuscript. In contrast the murine salispheres robustly replicated and released infectious virus, as determined by TCID50 (Figs. 3g-3i).*

• The magnitude of differences observed are modest at best for many of the assays (ie. often only several fold) and not logarithmic within the kinetics expected for these viruses as would be expected if the salivary glands were major targets and sources of infectious virus. Graphs of viral load would be best presented as log and not linear scales¬

*Response: The y-axis of all the graphs in the revised manuscript are now in Genome copies/ml and presented in* log scale and *validate that the salivary gland and saliva are major replication sites and transmission mediums respectively for enteric viruses.*

• Can infected cells be quantified in salivary glands and sorted to confirm they are productively infected? Demonstrating that salivary gland cells can be isolated, sorted, and infectious virus recovered by plaque assay or at least qPCR would be very convincing as done by Lee et al (PMID 28966054)

*Response: We performed the cell sorting as requested by the reviewer for both MNV-1 in pup and adult salivary glands and analyzed this by qPCR. MNV-1 replication was detected in both salivary epithelial and CD45+ cells but notably there was 5-fold higher replication in CD45+ cells than epithelial cells (Fig. 3a&b).*

• Can salivary glands be surgically removed in mice as they can in humans? If so, this would be a useful experiment to prove salivary glands are essential for infection in young mice.

*Response: We developed a surgical removal procedure of the main salivary glands from adult mice ( which also replicate MNV-1). Note we had to leave behind the smaller salivary glands which are spread throughout the oral cavity. Nevertheless, our experiments revealed that in animals lacking their main salivary glands, who we orally inoculated with MNV-1, the level of intestinal infection was 10-fold less than in animals with intact main salivary glands. In addition, the intestinal infection in the animals lacking the main salivary glands appeared to clear faster than in animals having their main salivary glands. These findings suggest that the salivary glands act as reservoir for MNV-1 and through either ingestion of MNV-1 in saliva and/or trafficking of salivary infected CD45+ immune cells to the gut tissues, re-infects the intestines and helps continue the infection (Figs. 3e & 3f).*

Minor

• Figure 3I only shows that at least a single infectious virus was present. This is not particularly informative and could readily be explained by contamination from mice being housed in a cage/environment with MNV. Quantification of infectious virus by plaque assay is the definitive experiment.

*Response: In the revised manuscript, we have performed the TCID50 for MNV-1 in RAW cells for salivary gland infection and infectious viral release in saliva, and for MNV-1 and CR-6 in salispheres. The data has been incorporated in the revised version of figures.*

• Salivary glands are in the oral cavity and thus the MS title is not precise.

*Response: We acknowledge the concern and have made amendments to the title.*

• Line 1 and 39 herpes etc are the disease not the virus. The viruses are HSV1/2, etc.

*Response: Changes have been made in the revised version.*

• Line 40-1: unclear if SARS-CoV-2 shed into the saliva is infectious. This reference identifies viral RNA and not infectious virus in saliva

*Response: When the first version of the manuscript was submitted there was another group, we have known was working on the aspect whether SARS-Cov2 in saliva is infectious and whether the virus is replicating in the salivary gland. Recently their publication has been accepted and has been cited by us in the revised version of the manuscript (Huang, N., Nature Medicine, 2021)*

• Line 41 needs a ref regarding saliva testing (at least for SARS-CoV-2)

*Response: We added the required reference for SARS-Cov2.*

• There is not good evidence that SARS-CoV-1 or SARS-CoV-2 infect or replicate in the salivary glands, only that viral RNA is detectable in saliva. The origin and nature of this RNA is unclear. This should be clarified.

*Response: Please refer to the previous response.*

• Line 111: The authors should remove "highly" since they did not quantify infectious virus. They only assessed the presence or absence of infectious virus.

*Response: This is clarified in the revised manuscript.*

• Line 606: How do the authors determine a multiplicity of infection for HNoV when it can't be plaqued?

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Their first observation is that infected pups can transmit virus back to the mother and cause an infection in the mammary gland. This is a powerful observation, but I have a minor concern regarding the way in which the observations are implied to hold true for all enteric viruses, where experiments have been performed primarily with rotavirus (see minor comments below).

The authors go on to demonstrate that both norovirus and rotavirus RNA, as well as protein, can be found in saliva, and that the virus in the saliva can transmit infection from adult mice to pups.

Direct replication of noro, rota and astrovirus in salivary glands was observed, but as noted below, the manner in which the RNA copy number data is presented is somewhat non-standard. They demonstrate a modest increase in replication following inoculation – as noted below there is a need to control for residual RNA from the input. The infection of salivary glands is very convincing as multiple approaches have been used, but the overall amount of infectious virus present in the samples appear to be very low (See minor comment below). Furthermore, replication in salivary organoids appears confirmatory but again the levels of replication appear modest and no attempt to quantify actual infectious virus present

in these organoid infections is presented.

The growth of human norovirus in the ductal cell line is intriguing and could be a major step forward for the field, if it was seen to be robust and applicable to a wide range of isolates. The conclusion that they provide a robust replication system, isn't yet supported by the data, but it could be with a few limited additional experiments.

Overall, I feel this is great piece of work that would be of interest to the readership of the journal. It uncovers new modes of transmission for enteric pathogens that would be of interest to anyone working in the areas of infectious diseases. However, there is a need for some changes in text and the way in which the data are presented to ensure the data and the interpretation of it, are truly objective and comparable to studies in the field. Additional experiments around the robustness of the observations and the reproducibility of the data in vitro culture of human norovirus would greatly improve the manuscript and solidify the conclusions, that are only partly supported by the current data.

Major comments:
- The level of replication seen in salivary glands and organoids is modest – a 4 fold increase in viral RNA from day 1 to day 3 is typical. It will be important to control for the residual RNA present in the input sample. This could be achieved by infecting animals/organoids side by side with UV-treated inoclum or by the addition replication inhibitor such as 2CMC (see https://pubmed.ncbi.nlm.nih.gov/23986582/)

*Response: We now show in the revised manuscript that salivary gland infection by MNV-1 can be inhibited with antiviral treatments with 2'-methylcytidine both in-vivo and ex-vivo (salispheres). A detailed description has been added to the Methods section regarding the dosage and figures added to Figs. 3a & 3i.*

- The levels of replication of human norovirus in the ductal cells appear modest, but admittedly comparable to work published on BJAB cells and some HIE models. Notably others have seen much more robust replication in the absence of a robust interferon response. Have the authors examined whether the system can be improved using inhibitors of the pathway? Equally the robustness of the culture system and observations is difficult to assess given only a single viral isolate was used. The use of replication inhibitors, such as 2CMC in such a model, along with more isolates of human norovirus would be required to support their conclusion (ln 200) that these line can provide a robust replication system.

*Response: We now show that replication in the cell lines is most robust with NV-loaded extracellular vesicles isolated from human stool samples whereas free NV in stool does not replicate (Fig. 4d-4g). This is consistent with recent studies from our group (Santiana et al., Cell Host Microbe 2018; and Zhang et al., Environ Sci Tech 2021) where we show that free enteric viruses in stools are significantly less infectious and more susceptible to environmental damage then vesicle-cloaked viral clusters. This is due*

*to a combination of high MOI afforded by being trafficked inside vesicles in viral multiples as well as having a membrane protective coat from proteases, nucleases and other environmental aggressors.*

*In the revised manuscript we also used multiple different isolates of human NV and showed that consistently only the vesicle contained NV populations (and not the free viruses) were able to infect and replicate in the cells (Figs. 4e-l, Fig. S4e,f)*

- Ln 196: the authors conclude that salivary glands are a "major site of replication for enteric viruses" – however the use of the term "major" appears somewhat subjective. I agree that their data indicate replication occurs, but I do not see how this can then be sold as a "major site of replication" based on the levels of replication observed. Taking on board the rather non-standard way in which the replication data is presented, I don't agree with their conclusion here.

*Response: In the revised manuscript we now represent all the replication levels in genome copies/ml, in log-scale. We find the replication in the glands to be not only comparable to that in intestines but also clearing slower (Figs. 1b and 1d, Fig. S1d versus Figs. 2g and 2h). Nevertheless, we have removed the word major and instead state that it is a significant replication site for enteric viruses.*

Minor comments:
- Ln 69 and throughout the text: Seems to be some experiments performed with MNV and rotavirus and some with just one. A key such experiment is the demonstration that 10-day post-partum dams (pup free) do not show any replication in the mammary glands – indicative of transfer of the infection from the pups to the dams mammary gland. It is implied that the observation holds true for both model organisms (norovirus and rotavirus), when in fact this conclusion cannot be made against. The authors need to be more restrained in drawing sweeping conclusions about "enteric viruses" when not all experiments were performed with both Noro and Rota. Multiple example of this exist in the manuscript (e.g. ln 102)

*Response: We acknowledge the reviewer's concern and have made changes as per the suggestion.*

- The way in which the RNA copy number is being presented throughout the manuscript is not standard. It is presented as RNA copies/mock – why aren't absolute viral RNA copies being shown? Mock infected animals don't have any viral RNA, how can this be used as a reference? The Y axis on the graphs appear to be linear with splits at various point making the interpretation a bit of a challenge e.g. Fig 2d Y axis is 0 to 8 x 10^3. This approach makes direct comparisons with data from others in the field a challenge. Absolute viral RNA levels would allow a much better comparison with published data using mouse models of these diseases.

*Response: In the revised manuscript, we developed standard curves for the amplicons for the corresponding primers used for detection of the viruses. Our qPCR analysis only considers replication cycles lower than 30 as standard as higher cycle number may not be reliably reflecting replication of the viral genome as the standard curve generated higher standard deviation values signifying higher probability of false positives owin to primer-dimer formations. As per the standard curve 30 cycles*

*corresponds to ~$10^5$ genome copies/ml (for MNV-1 and EDIM) and $10^2$ for C6R. The revised data sets are re-calculated according to the standard curves for each primer sets and Y-axis is now represented in terms of genome copies/ml. A detailed description has been updated in Methods section.*

- Fig 2: Given the amount of viral protein present in the saliva samples, I was surprised that the authors didn't assess viral infectivity in these samples directly. Clearly these samples contain infectious virus as it transmits but the way in which the infection data in figure 2d is presented, it difficult to gauge the degree of replication and to compare it to published work.

*Response: In the revised manuscript, we have performed the TCID50 for MNV-1 in RAW cells for salivary gland infection and infectious viral release in saliva, and for MNV-1 and CR-6 in salispheres. The data has been incorporated in the revised version in Fig. 2d & n, Fig. 3h.*

- Figure 3I: Again this is a non-standard presentation of viral infection of RAW 264.7 cells. Why isn't pfu or TCID50 data presented?

*Response: We have removed this panel from main figure as we understand this was a non-standard presentation. In the revised manuscript we are presenting the TCID50 instead Fig. 2n.*

**Reviewer Reports on the First Revision:**

Referees' comments:

Referee #1 (Remarks to the Author):

The authors have done a thorough job of addressing the majority of prior issues, and the data is increasingly convincing. The addition of antiviral treatment, CD300lf-/- mice, and measurements of infectious virus are critical additions that strengthen the paper. However, there is one major and several associated minor concerns that remain based upon these new reports.

Major
1. Thank you for performing the very important CD300lf-/- pup experiment. It is concerning that in these mice, there are 10^4 genome copies being detected without any increase, indicating a high level of "background"(?) genome copies in pup SMGs that are associated with presumably non-replicating virus. The authors do not incorporate this important observation into their interpretation of their other results, nor do they address whether there is a similar "background" level of viral genome copies in adult CD300lf-/- SMGs, nor do they address TCID50 measurements for the pup SMGs - what proportion of the infectious virus is "background"? This is key for addressing the prior concerns about which data can be interpreted as "true" infection.

Minor
2. Throughout, the axes are labeled "replication" when they are detecting genome copies. This is misleading, as discussed above, and should be corrected to just say "viral genome copies".

3. Please also add the limit of detection for these qPCR assays to each graph to make interpretation easier.

4. Wasn't a DD50 or ID50 (diarrheal or infectious dose determined for the EDIM stock used? This is routine for murine rotavirus studies and is normally how experimental inoculation dose is determined.

Referee #2 (Remarks to the Author):

The authors have made a number of key revisions that clarify and strengthen the manuscript from original submission; however, there are still over-generalizations, discrepancies amongst methods, missing controls, and unclear methods of normalization which diminish enthusiasm for this manuscript as highlighted below:

Major
The fact that MNV CR-6 doesn’t replicate in the salivary gland in mice argues against the authors central thesis which is that salivary glands are a general target site of infection for diverse enteric viruses. Also, arguing against this central hypothesis is the lack of significant human norovirus

replication (except amongst vesicle associated virus which is only 4-fold by protein levels at peak replication).

1. The new Figure 3D suggests the vast majority of MNV-1 detectable represents the viral inoculum rather than replicating virus. There is only a scant 0.5-1.5 log increase in viral genomes and only at two late time points (5 and 7dp)i. This is inconsistent with the premise of the manuscript that SGs represent a critical site of replication.
2. Figure 3H requires a negative control. How do the authors demonstrate that this is not viral input? A 1 hour post-infection or drug treated control is needed here with readout by TCID50 or plaque assay and not qPCR
3. Figure S4A requires a negative control.
4. For figure S4D-E how was fold increase compared to a mock infected cell? The authors state a 100 to 250-fold difference but it is not appropriate to compare a fold increase relative to zero. This data is presented unclearly and is not convincing of actual human norovirus replication as presented.
5. There is a discordance between the claims that ~60% of cells are infected with human norovirus and only a 4-fold increase in NS7 expression by western blot.
6. Figure 1I&J Fig 2D/E, etc viral genomes per ml of what? Is this genomes per ml of milk?

Minor
7. What is the limit of detection in 3C? Is the CD300lf in Epcam+ cells above background? The limit of detection for each assay should be clearly stated or shown. This is not immediately clear for a number of experiments.
8. The distribution and subcellular localization of Propol staining in Figure S2 is atypical as this antibody should primarily stain the viral replication complex which is punctatated and in the ER in contrast to the diffuse cytoplasmic staining shown here. Co-staining with NS4 and Propol or dsRNA and Propol would make this more convincing.
9. Of note, MNV-1 is the outlier strain amongst mouse noroviruses in terms of tissue and cell tropism so the fact that CW3 replicates in salispheres but not CR6 is not consistent with salivary glands being broadly important for norovirus as one would infer from the abstract.
10. The new figure S3 should be shown as a viral growth curve with the readout of PFU/ml (or TCID¬¬¬50/ml) and not fold-replication which obscures important data.
11. In the abstract, rabies is the disease. Rabies virus is the virus.
12. Enteric viral RNA (not enteric viruses as stated) have been detected in saliva- pg 1.
13. The statement that MNV-1 replicates in epithelial cells (e.g. tuft cells) is not correct see PMID 33177207 and 29650672. The authors need to determine whether the MNV-1 associated with SG epithelial cells is mediating productive infection or just sticky viral RNA from the virus replicating in the CD45+ cells.

Referee #3 (Remarks to the Author):

Overall I would like to congratulate the authors on their work and the revisions that now significantly improve their manuscript. The discovery of enteric virus replication in salivary glands is an important one which will undoubtedly add to the field and stimulate further research.

The work on human norovirus replication is the least complete component of the work and arguably one of the most challenging. What the authors have shown is really very interesting and will undoubtedly stimulate work in the area, but they seem to be pushing a narrative that this ductal line may solve the issue around norovirus culture – it likely won't, but it could help (as do all the other systems). It is really important the authors don't try to force this narrative as the field has been hindered by numerous other reports of cell lines that support replication, only for other to find that they are not robust. The net effect is that non-expert reviewers outside the field get the impression that the major challenges in the field have been resolved when in fact the levels of replication seen in these systems are very modest and arguably lower than many studies on other lines/organoids. Importantly however, the fact that they see ANY HuNoV replication in these ductal lines at all is really very interesting and that combined with the elegant in vivo models would make this worthy of publication.

Major:
- Pg 9 – Comment relating to fold increase in human norovirus viral RNA. Figure S4: Y Axis panels C-E appear to be copies per mock? There is no virus in mock infected samples so how can this be used as a reference? Comparing the data to a mock infected cell doesn't make any sense as there is none present in the sample. The fold increase should be compared to 6 h or an alternative control such as +/- 2CMC or UV inactivated virus. This would give an indication of the true level of replication, taking into consideration how much material will simply hang around in the culture.

- The closing statement "Furthermore, we have found that SMG cell lines and organoids can be robust replication systems for these viruses, especially when the inoculum is enriched in vesicle-cloaked enteric viruses" – is somewhat misleading. How do they define a robust culture system for HuNoV? The authors have generated a system that is comparable to others in terms of yield, arguably lower than some studies using intestinal organoids which see up to 10,000 fold increases in titre, yet by they state at the top of page 9 that "human norovirus lack robust in vitro models". They cannot then conclude that a system that produces a 250 fold at best is now robust. Therefore I cannot see from the data presented that their ductal line model changes that statement. The ductal lines provide another system with which to improve HuNoV in vitro culture so this statement should be clarified with respect to HuNoV. It's exciting, it's undoubtedly useful, but we still have a long way to go.

Minor comment:
- The Vp1 positivity levels (40%) and the negative strand positive levels (60%) are somewhat different from each other – this could be simply a reflection of the sensitivity of each assay. Equally – with a 60% -ve sense RNA positivity rate, one would expect much more than the observed increase in viral RNA seen. This is interesting and could suggest that there is likely an intracellular block to replication, fitting with other observations in the field i.e. entry alone is insufficient to allow robust

replication to occur. This might be worth a comment in their discussion.

**Author Rebuttals to First Revision:**

We thank all the reviewers for their valuable and constructive comments. We have now responded with additional experiments, including experiments with **multiple** persistent murine norovirus strains and demonstrate persistent high viral replication and virus production in salivary glands. We have also replaced qPCR measurements with TCID50 viral titers in salivary glands and saliva. The TCID50 measurements corroborate our prior findings demonstrating 3- to 5-log increases in viral titers over background levels. These data unambiguously support our conclusion that the salivary glands are a major site of enteric virus replication, production, and secretion, on par with the intestinal system. Furthermore, the high and persistent levels of enteric viruses in saliva make it a more likely and relevant transmission mechanism for public health in settings such as schools, nurseries, restaurants, cruise ships etc- through spit, kissing, licking- rather than oral-fecal transmission.

**Referee#1**

**The authors have done a thorough job of addressing the majority of prior issues, and the data is increasingly convincing. The addition of antiviral treatment, CD300lf-/- mice, and measurements of infectious virus are critical additions that strengthen the paper. However, there is one major and several associated minor concerns that remain based upon these new reports.**

**Major**

**1. Thank you for performing the very important CD300lf-/- pup experiment. It is concerning that in these mice, there are 10^4 genome copies being detected without any increase, indicating a high level of "background"(?) genome copies in pup SMGs that are associated with presumably non-replicating virus. The authors do not incorporate this important observation into their interpretation of their other results, nor do they address whether there is a similar "background" level of viral genome copies in adult CD300lf-/- SMGs,**

The earlier data referred the by the reviewer were qPCR measurements of MNV-1 genome copies per ml of reaction and this type of measurement may pick up non-replicating RNA. Nevertheless, we still had observed a 3-log increase by 5 dpi going from $10^4$ genome copies/ml to $10^7$ genome copies/ml in viral RNA.

But **in the updated manuscript we now present viral titers by TCID 50 per mg of salivary gland tissue** for both CD300lf +/+ and CD300lf-/- knockout pups inoculated with MNV-1 (**Figure 3i**).

This shows that at 1 dpi, the TCID50 /mg of salivary gland tissue is ~$10^1$ for both the CD300lf +/+ and CD300lf-/- knockout pups. This level was what we defined as the background post inoculation. This was also the limit of detection by TCID50 in this experiment.

**By 5 dpi**, the TCID50 for MNV-1 in CD300lf +/+ salivary glands increased **by more than 4-logs or 10,000-fold over the background TCID 50 measurements taken at 1 dpi.** In contrast, in CD300lf-/- knockout mice salivary glands, it remained the same as background (1 dpi). The MNV-1 titers in CD300lf-/- knockout salivary glands continued to remain at this background level throughout the course of the experiment (10 days).

These data unambiguously establish the requirement for CD300lf receptors for MNV-1 replication and infectious virus production in the salivary glands.

**nor do they address TCID50 measurements for the pup SMGs - what proportion of the infectious virus is "background"? This is key for addressing the prior concerns about which data can be interpreted as "true" infection.**

In addition to the above experiment with CD300lf+/+ and CD300lf -/-knockout mice, we now include virus titers by TCID50/mg or TCID50/ml for murine norovirus strains and genomic copy number measurements as copies/ml by qPCR for EDIM, taken as early as 6 hours post-inoculation (6 hpi) for all pup and adult salivary gland infections done in the BALB/c mice background. These starting levels (e.g., 6 hpi ) we explicitly now state as the starting background level post-inoculation (**Figures 1b, 1c,1h and Figures 2d, 2e, 2g-2n) and we normalize all measurements taken after that time point to that starting time point (**e.g., 6 hpi)**.** Note that time points earlier than 6 hpi were beyond the limit of our detection.

For all of the enteric viruses tested, including acute and persistent MNV strains, the levels of viral replication in salivary glands **increased by 4-5 logs compared to the background level taken at 6 hpi (Figures 2g-2j, Figure 2k-2n). Indeed, for the persistent MNV strains, the infectious virus levels per mg of salivary gland tissue were comparable to infectious virus levels per mg of proximal colon tissue and remained at that comparable level persistently over at least 3 weeks (Figure 2l-2n).**

Minor

2. Throughout, the axes are labeled "replication" when they are detecting genome copies. This is misleading, as discussed above, and should be corrected to just say "viral genome copies".

We have corrected this in the revised manuscript and now the axis label where appropriate states Genome copies/ml.

3. Please also add the limit of detection for these qPCR assays to each graph to make interpretation easier.

Both for the qPCR and for TCID50 measurements, the limit of detections are now indicated in each of the graphs by dashed lines.

4. Wasn't a DD50 or ID50 (diarrheal or infectious dose determined for the EDIM stock used? This is routine for murine rotavirus studies and is normally how experimental inoculation dose is determined.

DD50 or the 50% diarrheal dose for the EDIM stock we have is $10^5$ pfu and this has been indicated in the Methods section of the revised manuscript under the section of viral infection.

**Referee #2 (Remarks to the Author):**

**The authors have made a number of key revisions that clarify and strengthen the manuscript from original submission; however, there are still over-generalizations, discrepancies amongst methods, missing controls, and unclear methods of normalization which diminish enthusiasm for this manuscript as highlighted below:**

**Major**

**The fact that MNV CR-6 doesn't replicate in the salivary gland in mice argues against the authors central thesis which is that salivary glands are a general target site of infection for diverse enteric viruses.**

Our initial study focused on the MNV-1 acute strain of the mouse norovirus (MNV). Other strains (e.g. CR6, MNV-3, MNV-4 and WU23) have been extensively studied and reported upon, as intestinal MNV persistent strains (Nice and Virgin., 2013; Arias and Goodfellow; Wobus 2018; Grau and Karst 2019; Roth and Karst 2020). Importantly, CR6, MNV-3 and MNV-4 contain the amino acid residues changes in VP1 and NS1/2 known for persistence (Nice and Virgin., 2013; Baldridge 2020; Walker and Baldridge 2021) and all require the CD300lf receptor for entry into cells (Graziano and Wilen 2020). While for CR6, the intestinal Tuft cells have been shown to be the site of persistence (Wilen and Virgin 2018), it remains to be determined whether Tuft cells or another intestinal cell type is required for MNV-3, MNV-4 and WU23 persistence (Wobus 2018).

In the revised manuscript, we now show in **Figure 2e**, and **Figures 2k-2n** that MNV-3, MNV-4 and WU23 all replicate persistently in salivary glands at levels comparable to that in the proximal colon (whereas they are cleared from spleen or Peyer's Patches) (**Figures 2k-2n**). **Indeed, for these persistent MNV strains, the infectious virus levels per mg of salivary gland tissue were comparable to infectious virus levels per mg of proximal colon tissue and remained at that comparable level persistently (Figure 2l-2n).** Furthermore, we show that these strains are persistently secreted in saliva (**Figure 2e)**. These data demonstrate that salivary glands are critical sites for the replication and transmission of acute and persistent MNV strains, of rotavirus (EDIM) and of murine Astrovirus.

Unlike MNV-3, MNV-4 and WU23 we found that CR6 did not replicate in salivary glands even though salivary glands contain both epithelial and immune cells expressing the viral receptor CD300lf (**Figures 3a and 3b**). To probe further, we tested different routes of inoculation (oral, tail vein); inoculation into immunocompromised mouse backgrounds (STAT1 knockout, IFNAR knockout) and even germ-free mice (**Figure S2a-S2d**). In all cases CR6 did not replicate to any appreciable level in the salivary glands. What made this finding even more intriguing was that CR6 robustly replicated in murine salispheres, a salivary organoid model comprised of murine salivary epithelial cells (which also express CD300lf) **(Figure S3b and S3c)**. The robust replication in ex vivo salispheres yet lack of replication in wild type, STAT1/IFNAR knockout and even germ-free mice (not shown), suggests that its infectivity may be inhibited by factors secreted from salivary glands, but this is beyond the scope of this study. Nevertheless, most MNV strains tested in our study (MNV-3, MNV-4 and WU23) all persistently and robustly infected salivary glands and persistently secreted into saliva. We have uncovered crucial differences among persistent murine norovirus strains which until now were considered comparable because they were only studied for their replication and persistence in the colon as well as shedding and spreading to others via feces.

We are thankful to the reviewers and editors to inquire about the general relevance of our findings. Our extensive testing of multiple viral strains demonstrates how general the infection of salivary glands is for mouse norovirus. Additionally, we now leverage our new understanding to establish a universal system of propagation for norovirus, using salivary gland organoid and salivary cell lines.

**Also, arguing against this central hypothesis is the lack of significant human norovirus replication (except amongst vesicle associated virus which is only 4-fold by protein levels at peak replication).**

We show that in NS-SV-DC line, with straight stool filtrate we obtain **~2.5-log** increase in human norovirus (HuNoV) genomic RNA between 6 hpi and 96 hpi (**Figure 4g**). Furthermore, if we isolate the vesicles containing HuNoV from this filtrate and inoculate them into NS-SV-DC cultures, we obtain a **100-** to **1000-fold** increase in HuNoV genomic RNA between 6 hpi and 96 hpi, depending on the original stool isolate tested (**Figure 4j**). This demonstrates a significant level of replication of the HuNoV genome in a salivary human cell line. The high sensitivity and linearity of qPCR-based quantification enabled quantification of HuNoV replication in these cultures. Note that the levels of HuNoV replication we measured was comparable to those reported with human enteroid cultures (Ettayebi et al., Science 2016).

Our immunoblots did show a significant increase of NS-7 and NS-6 protein levels between 6 hpi and 96 hpi. However, it is not possible to quantitatively compare the change in viral protein levels observed by immunoblotting (4-fold) to the change in viral genomic RNA levels we measured by qPCR ( **100 to 1000-fold**) as the former has much lower sensitivity than qPCR and is a non-linear method dependent on antibody specificity and sensitivity. Indeed, even in the enteroid cultures of human norovirus (Etteyabi et al., Science 2016) or RAW cells infected with murine norovirus (Emmott et al., MCP 2017) the increase in the levels of viral replication and structural proteins are similar to what we see and most importantly orders of magnitude lower than viral genome levels that were reported in each culture system.

1. The new Figure 3D suggests the vast majority of MNV-1 detectable represents the viral inoculum rather than replicating virus. There is only a scant 0.5-1.5 log increase in viral genomes and only at two late time points (5 and 7dpi). This is inconsistent with the premise of the manuscript that SGs represent a critical site of replication.

The earlier data referred the by the reviewer were qPCR measurements of MNV-1 genome copies per ml of reaction and this type of measurement may pick up non-replicating RNA. Nevertheless, we still had observed a 3-log increase by 5 dpi going from $10^4$ genome copies/ml to $10^7$ genome copies/ml in viral RNA.

But **in the updated manuscript we now present viral titers by TCID 50 per mg of salivary gland tissue** for both CD300lf +/+ and CD300lf-/- knockout pups inoculated with MNV-1 (**Figure 3i**).

This shows that at 1 dpi, the TCID50 /mg of salivary gland tissue is ~$10^1$ for both the CD300lf +/+ and CD300lf-/- knockout pups. This level was what we defined as the background post inoculation. This was also the limit of detection by TCID50 in this experiment.

**By 5 dpi**, the TCID50 for MNV-1 in CD300lf +/+ salivary glands increased **by more than 4-logs or 10,000-fold over the background TCID 50 measurements taken at 1 dpi.** In contrast, in CD300lf-/- knockout mice salivary glands, it remained the same as background (1 dpi). The MNV-1 titers in CD300lf-/- knockout salivary glands continued to remain at this background level throughout the course of the experiment (10 days).

These data unambiguously establish the requirement for CD300lf receptors for MNV-1 replication and infectious virus production in the salivary glands.

2. Figure 3H requires a negative control. How do the authors demonstrate that this is not viral input? A 1 hour post-infection or drug treated control is needed here with readout by TCID50 or plaque assay and not qPCR

In our revised manuscript, these data are now reported in **Figures 4b and 4c**. In the revised figures we now present MNV-1 TCID50 titers at 6 hpi ('background' and our limit of detection) and show a **3-log increase** in titers between 6hpi to 48 hpi (**Figure 4b**) that is sensitive to inhibition by 2-CMC (**Figure 4c**).

3. Figure S4A requires a negative control.
In revised **Figure 4e-k**, we now use the 6 hpi time point (after the inoculum has been washed off) as the starting "background" level of human norovirus genomic RNA or viral proteins and we compare any change in genomic RNA or viral protein levels at later time points to those levels at 6 hpi.

4. For figure S4D-E how was fold increase compared to a mock infected cell? The authors state a 100 to 250-fold difference but it is not appropriate to compare a fold increase relative to zero. This data is presented unclearly and is not convincing of actual human norovirus replication as presented.
As described above, in after inoculum is washed, we now measure and present the genome or protein levels in cell lysates or supernatants at the 6 hpi time point and use this level as the limit of detection (LOD) upon which we normalize the levels of viral genome/protein at all time points after.

5. There is a discordance between the claims that ~60% of cells are infected with human norovirus and only a 4-fold increase in NS7 expression by western blot.

The sensitivities of each method (immunofluorescence, FISH, Western Blot) and what each method measure are different and cannot be *quantitatively* compared.

In the FISH experiment, we are scoring the cells that have a negative template RNA strand- an indicator of replication taking place in the cell and by this method we find that ~60% of the cells remaining on the coverglass are replicating human norovirus. Note that infected cells that have lysed and/or floated off from the coverglass cannot be analyzed by FISH and thus would not be included in the quantification. Furthermore, the presence of negative strand RNA does not reflect the quantity of replication taking place within that cell- that we can do only by qPCR-based quantification of the positive genomic RNA strands.

Regarding our immunoblots showing increase in NS-7 and NS6 protein levels between 6 hpi and 96 hpi. It is not possible to quantitatively compare the change in viral protein levels observed by immunoblotting (4-fold) to the change in viral genomic RNA levels we measured by qPCR (**100 to 1000-fold**) as the former has much lower sensitivity than qPCR and is a non-linear method dependent on antibody specificity and sensitivity. Indeed, even in the enteroid cultures of human norovirus (Etteyabi et al., Science 2016) or RAW cells infected with murine norovirus (Emmott et al., MCP 2017) the increase in the levels of viral replication and structural proteins are similar to what we see and most importantly orders of magnitude lower than viral genome copy replication levels reported for each culture system.

6. Figure 1I&J Fig 2D/E, etc viral genomes per ml of what? Is this genomes per ml of milk?
In the revised manuscript, we now present all quantifications of viral RNA as genome copies/mg of tissue assayed.

Minor
7. What is the limit of detection in 3C? Is the CD300lf in Epcam+ cells above background? The limit of detection for each assay should be clearly stated or shown. This is not immediately clear for a number of experiments.
These data now presented as **Figure 3h** show CD300lf RNA levels in Epcam+ and CD45+ cells as fold change in over CD300lf RNA levels in RAW 264.7 cells, the latter a mouse macrophage cell line routinely used to propagate murine norovirus strains.

8. The distribution and subcellular localization of Propol staining in Figure S2 is atypical as this antibody should primarily stain the viral replication complex which is punctatated and in the ER in contrast to the diffuse cytoplasmic staining shown here. Co-staining with NS4 and Propol or dsRNA and Propol would make this more convincing.

We now show in **Figures 3d-3g** single cell confocal high magnification images with **anti-dsRNA** (J2 antibody) and **anti-NS4** antibodies- both of which are reporters of viral replication, in uninoculated and inoculated animal salivary glands.

9. Of note, MNV-1 is the outlier strain amongst mouse noroviruses in terms of tissue and cell tropism so the fact that CW3 replicates in salispheres but not CR6 is not consistent with salivary glands being broadly important for norovirus as one would infer from the abstract.

In fact, we show that CR6 replicates robustly in salispheres, similarly to MNV-1 (**new Figures S3a-S3d**): this observation may prove useful for researchers studying CR6 as it provides another model system to prepare virus. Regarding the issue raised by the reviewer of the broad importance of salivary glands for norovirus: we performed new in vivo experiments with the persistent strains MNV-3, MNV-4 and WU23 **(Figure 2e**, **Figures 2k-2n)**, and demonstrated that these viruses do persistently replicate in the salivary glands and are persistently released into the saliva. Hence these additional experiments reemphasize that the salivary glands **are indeed an organ broadly important for enteric virus replication and transmission, on par with the small intestines and colon.**

10. The new figure S3 should be shown as a viral growth curve with the readout of PFU/ml (or TCID¬¬¬50/ml) and not fold-replication which obscures important data.

We have revised all the figures related to murine norovirus infection in **Figures 1b, 1c, 2g-2k, 3k** per the recommendation of the reviewers. We agree with the reviewers that measuring TCID50 is a better measure of the presence of the virus, rather than our previous viral genomic measurements (nucleic acid materials can be detected even after clearance of a virus). Hence, we are now systematically using TCID50 analysis represented /mg of tissue or TCID50/ml of saliva to quantify murine norovirus strain levels. For EDIM, as it is not possible to carry out TCID50 due to lack of cell lines that replicate EDIM, we used qPCR to measure viral genomic RNA/mg of tissue.

11. In the abstract, rabies is the disease. Rabies virus is the virus.
Thank you for pointing out this error we have now corrected it.

12. Enteric viral RNA (not enteric viruses as stated) have been detected in saliva- pg 1.
We have repeated all our saliva experiments with murine norovirus (acute and persistent) and now show TCID50 data which measures infectious virus titers

13. The statement that MNV-1 replicates in epithelial cells (e.g., tuft cells) is not correct see PMID 33177207 and 29650672. The authors need to determine whether the MNV-1 associated with SG epithelial cells is mediating productive infection or just sticky viral RNA from the virus replicating in the CD45+ cells.

We have removed that statement from the manuscript.

Regarding whether this is a productive infection of salivary glands by MNV-1 or for that matter any of the persistent strains:
We now include infectious murine norovirus (acute and persistent) titers for both pup and adult salivary glands, measured by TCID50/mg and by TCID50/ml and EDIM genomic RNA levels by Genome copies/ml at 6 hpi (which is at the limit of our detection) in **Figures 2d, 2e, 2g, 2h, 2k-2n.** As demonstrated by the data in these panels, for acute MNV-1 strains, the levels of viral replication in salivary glands **are 4-5 logs higher by 3-5 dpi above background levels (6 hpi) (Figures 2g and 2h).**

Consistent with this in **Figure 3a and 3b** of the revised manuscript, we show that at 3-5 dpi, the TCID50/ml titers of MNV-1 in salivary gland CD45+ and Epcam+ cells in both pups and adults, are 10,000 and 1000-fold respectfully above the background, and in **Figures 3d-3g** we **demonstrate replication** in both Epcam+ and CD45+ cells by **J2 (dsRNA) and NS4 immunostaining**.

Finally, for the persistent MNV strains, in the salivary glands by 2 weeks, we reach a **4-log** increase over the 6 hpi background level **(Figures 2k-2n).** Indeed, for the persistent MNV strains, the salivary glands levels are comparable to MNV in proximal colon and remain at that level persistently (**Figure 2l-2n**).

Collectively the data above demonstrate that enteric viruses, including acute and persistent MNV strains, productively infect salivary glands and release into saliva.

Referee #3 (Remarks to the Author):

Overall I would like to congratulate the authors on their work and the revisions that now significantly improve their manuscript. The discovery of enteric virus replication in salivary glands is an important one which will undoubtedly add to the field and stimulate further research.

The work on human norovirus replication is the least complete component of the work and arguably one of the most challenging. What the authors have shown is really very interesting and will undoubtedly stimulate work in the area, but they seem to be pushing a narrative that this ductal line may solve the issue around norovirus culture – it likely won't, but it could help (as do all the other systems). It is really important the authors don't try to force this narrative as the field has been hindered by numerous other reports of cell lines that support replication, only for other to find that they are not robust. The net effect is that non-expert reviewers outside the field get the impression that the major challenges in the field have been resolved when in fact the levels of replication seen in these systems are very modest and arguably lower than many studies

on other lines/organoids. Importantly however, the fact that they see ANY HuNoV replication in these ductal lines at all is really very interesting and that combined with the elegant in vivo models would make this worthy of publication.

We thank the reviewer for seeing the value of our work and in particular in the addition of the HuNoV/salivary cell line replication data to the in vivo data we present with acute and persistent murine norovirus, rotavirus and astrovirus strains replicating in salivary glands. In the revised manuscript we have removed jargon such as 'robust', and describe the salivary cell lines and organoids simply as useful additions to the existing culture model repertoire in which to replicate and propagate HuNoV.

Major:
- Pg 9 – Comment relating to fold increase in human norovirus viral RNA. Figure S4: Y Axis panels C-E appear to be copies per mock? There is no virus in mock infected samples so how can this be used as a reference? Comparing the data to a mock infected cell doesn't make any sense as there is none present in the sample. The fold increase should be compared to 6 h or an alternative control such as +/- 2CMC or UV inactivated virus. This would give an indication of the true level of replication, taking into consideration how much material will simply hang around in the culture.

The figures pertaining to salispheres and human salivary cell lines all now include the 6 hpi data-which is the starting background levels after the inoculum has been washed off. Viral genome and viral protein levels at all later time points are compared to those respective levels at 6 hpi **(Figures 4a-4k and Figure S3).**

- The closing statement "Furthermore, we have found that SMG cell lines and organoids can be robust replication systems for these viruses, especially when the inoculum is enriched in vesicle-cloaked enteric viruses" – is somewhat misleading. How do they define a robust culture system for HuNoV? The authors have generated a system that is comparable to others in terms of yield, arguably lower than some studies using intestinal organoids which see up to 10,000 fold increases in titre, yet by they state at the top of page 9 that "human norovirus lack robust in vitro models". They cannot then conclude that a system that produces a 250 fold at best is now robust. Therefore I cannot see from the data presented that their ductal line model changes that statement. The ductal lines provide another system with which to improve HuNoV in vitro culture so this statement should be clarified with respect to HuNoV. It's exciting, it's undoubtedly useful, but we still have a long way to go.

We show that in NS-SV-DC line, with straight stool filtrate we obtain **~2.5-log** increase in human norovirus (HuNoV) genomic RNA between 6 hpi and 96 hpi (**Figure 4g**). Furthermore, if we isolate the vesicles containing HuNoV from this filtrate and inoculate them into NS-SV-DC cultures, we obtain a **2-** to **3-log** increase in HuNoV between 6 hpi and 96 hpi, depending on the original stool isolate tested (**Figure 4j**). The high sensitivity and linearity of qPCR-based quantification enabled quantification of HuNoV replication in these cultures. Our findings demonstrate a significant level of replication for the HuNoV genome in a salivary cell line. Indeed, the levels we measure are comparable to those that have been eported with human enteroid cultures, as the latter typically is anywhere from 1-log to 4-logs increase in HuNoV genome copies over background, depending on the stool filtrates tested (Ettayebi et al., Science 2016). Given this, in the revised manuscript we have accordingly clarified our statements removing jargon such as "robust or more robust". Instead, we report the HuNoV replication

and egress levels and describe the salivary cell lines and organoids simply as useful additions to the existing culture model repertoire in which to replicate and propagate HuNoV.

We believe our finding that the vesicle-cloaked viruses are more infectious than free noroviruses may raise the efficacy of other existing human norovirus model systems. It is customary practice in this field to pass the stool solution through mesh (0.2-0.4 micron pore) to void the stool from harmful bacteria before using it as an inoculum in enteroids or other model systems. But this procedure also decreases the pool of norovirus-containing vesicles, specifically ones of size = or >200nm, thus depleting the inoculum from a very valuable source of infectious material (Santiana et al., ***Cell Host and Microbe 2018***). In the revised manuscript we offer this as a potential solution to increasing the level of infectivity in all ex-vivo human norovirus culture systems.

We have shown over the past 6 years (Chen et al., ***Cell 2015***; Santiana et al., ***Cell Host and Microbe 2018***; Altan-Bonnet N and Esteban Domingo ***Virus Research 2019***; Zhang et al., ***Environmental Sci Technology 2021***; and Kerviel and Altan-Bonnet ***Annual Review in Cell and Developmental Biology 2021***) that the vesicle-contained viruses, and not the free viruses, are *the* most infectious units for human norovirus, rotavirus, poliovirus and many others. In particular we have shown that human norovirus and rotavirus vesicles make up ≥50% of the total stool norovirus or rotavirus virus pools and they are more infectious than the stool free viruses (Santiana et al., ***Cell Host and Microbe 2018***). Increased infectivity of the vesicle-form over the free virus, with identical inoculum doses of each form of the virus, is due to the former increasing the multiplicity of infection enabling multiple viral particles *en masse* into the cell to overcome replication barriers that exist to single viruses (Chen et al., ***Cell 2015***; Santiana et al., ***Cell Host and Microbe 2018***; Altan-Bonnet N and Esteban Domingo ***Virus Research 2019***; Zhang et al., ***Environmental Sci Technology 2021***; and Kerviel and Altan-Bonnet ***Annual Review in Cell and Developmental Biology 2021)***. In addition, as we showed for stool, the vesicle membrane protects the viruses from stool proteases and nucleases (Santiana et al., ***Cell Host and Microbe 2018)***.

We believe that this data highlighting the remarkable difference in infectivity of the two pools, vesicle-cloaked and free, will motivate others to isolate and enrich for the human norovirus vesicles from stool and increase the robustness of their model culture systems (e.g., enteroids and B-cells)

Minor comment:
- The Vp1 positivity levels (40%) and the negative strand positive levels (60%) are somewhat different from each other – this could be simply a reflection of the sensitivity of each assay. Equally – with a 60% -ve sense RNA positivity rate, one would expect much more than the observed increase in viral RNA seen. This is interesting and could suggest that there is likely an intracellular block to replication, fitting with other observations in the field i.e. entry alone is insufficient to allow robust replication to occur. This might be worth a comment in their discussion.

The VP1 staining was done with the stool filtrates which are primarily free virus with reduced vesicle-cloaked norovirus population and thus lower infectivity (**Figure 4e-4h**). In contrast, the FISH negative strand experiment was done with vesicle inoculum isolated from the stool and thus is of higher infectivity (**Figure 4l and 4m**).

But the reviewer is correct, the sensitivities of each method (immunofluorescence, FISH, Western Blot) and what each method measure are different and thus difficult to *quantitatively* compare.

Regarding FISH: we are scoring the cells that have a negative template RNA strand- an indicator of replication in the cell but not necessarily the quantity of replication within the cell. By FISH we found that ~60% of the cells remaining on the coverglass are replicating human norovirus. But infected cells that had lysed or floated off from the coverglass were not analyzable by FISH.

Regarding immunoblots vs qPCR: our immunoblots show increase in NS-7 and NS6 protein levels between 6 hpi and 96 hpi. It is not possible to quantitatively compare the change in viral protein levels observed by immunoblotting (4-fold) to the changes in viral genomic RNA levels measured by qPCR (**100-1000 fold**) as the former has much lower sensitivity than qPCR, is a non-linear method, dependent on antibody specificity and sensitivity, and levels of viral RNA translation do not necessarily have to scale linearly with viral RNA synthesis [Boersma et al., 2020 Cell]).

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**Reviewer Reports on the Second Revision:**

Referees' comments:

Referee #1 (Remarks to the Author):

The authors have addressed my prior concerns and the data is substantially more convincing.

Referee #2 (Remarks to the Author):

The revised manuscript is significantly improved and the new data represents a tremendous amount of work and significantly clarifies the important findings described. I commend the authors on such a nice story which will be of broad interest. I have no remaining concerns requiring new experiments but do have a number of concerns that can be clarified with text changes, simple re-analysis, and deletion of non-robust data.

1. Line 117: The MuAstV data in Fig S1D is not robust with what appears to be only 2 mice in one group and 3 mice in the other group. In addition to the unacceptable sample size, there is no input control and the magnitude of difference is modest. This data should be repeated with more animals or astrovirus should be deleted throughout the paper.
2. Fig 4G-H. The y-axis can not be fold-change relative to mock as stated by two reviewers previously. It can be fold change relative to input or better just shown as copies relative to GAPDH. This can not be interpreted as presented and should be re-graphed and new statistics performed.
3. The authors graph data points below their LOD. This is not appropriate. A data point cannot be below the LOD by definition. See Fig 1B, 1F, 1H, 2G, 2H, 2L, 2M, 2N, 3I, 3J, 4C, 4J, etc. These graphs need to be corrected and re-analyzed.

Minor
4. Fig 4B Line 490. The authors should double check the t-test was performed on log-transformed data rather than untransformed data. The p-value at 24hpi seems much lower than expected based on the variance and overlap.
5. Line 102: The authors state Wu23 causes persistent infection. The persistence of Wu23 is not assessed in any of the three cited papers and if WU23 is persistent that is a novel finding to this paper which should be clarified.
6. Line 123; “were considered equivalent” should be referenced or deleted.
7. Line 124 and 131: “replication” should be “viral RNA” as detection of viral RNA in a tissue is merely suggestive of replication and the virus could come from another tissue source.
8. Line 120: NS1/2 but not VP1 is important for persistence. The VP1 is not a determinant of persistence but rather lethality in IFNAR mice and systemic infection.
9. Line 68: an 8 log increase is not observed as stated. It is 6-logs.
10. Line 85 is 6-logs and not 7-logs
11. Also, the authors should define persistent infection. The authors only looked to day 14 and many would argue that this is not persistence as seen at >21 days post infection when the adaptive immune system has had plenty of time to clear infection.

12. Line 38: Inappropriate to say “first line of defense for ALL invading…” Maybe many but this is not known for “ALL”
13. Line 41: “Enteric viruses such as norovirus, rotavirus and astrovirus have been frequently detected in saliva” should be clarified. Viral RNA was detected for these viruses but not necessarily infectious virus. If there are primary references (as opposed to the three reviews cited) that demonstrate infectious norovirus, rotavirus, and astrovirus from the saliva then these should be cited or the statement should be revised to reflect only viral RNA was detected.

Referee #3 (Remarks to the Author):

As indicated during the previous round of reviews, I feel strongly that this is an important study for the field and one that will be of interest to anyone working on gastrointestinal pathogens. It has wide-reaching implications when we think about how GI viruses transmit. It will undoubtedly stimulate further research to understand the biological relevance of the observations in human-human transmission of GI viruses. I am fully satisfied by the reviewers responses.

Finally, I would like to pass on my congratulations to the authors for their work and continued efforts to improve the quality and clarity of their manuscript, particularly given the challenges we are all facing at the present time. This is a great piece of work, which they should all be very proud of.

Minor comments:
- Fig S3: I appreciate the western blots for human norovirus infection of the salispheres are a technically challenging - however, the quality of the blots presented in this figure are not in line with the others in their manuscript. I don't believe there is a need to repeat these, simply improve their clarity. Panel f is spliced together - it should be made clear where the splices are by the inclusion of gaps or solid lines indicating a splice. Panel g -is also spliced and it's unclear which of the proteins shown in the 6/96 hpi lanes for vesicle HuNoV are being quantified - there appears to be a doublet of some sort - please indicate which protein band is being quantified or if both are used for quantification.

## Author Rebuttals to Second Revision:

**Response to Reviewers**
We thank all the reviewers for their positive and constructive comments and most importantly for their time in reviewing our manuscript. We have addressed all their remaining concerns below.

**Referees' comments:**

**Referee #1 (Remarks to the Author):**

The authors have addressed my prior concerns and the data is substantially more convincing.
We thank the reviewer.

**Referee #2 (Remarks to the Author):**

The revised manuscript is significantly improved and the new data represents a tremendous amount of work and significantly clarifies the important findings described. I commend the authors on such a nice story which will be of broad interest. I have no remaining concerns requiring new experiments but do have a number of concerns that can be clarified with text changes, simple re-analysis, and deletion of non-robust data.

1. Line 117: The MuAstV data in Fig S1D is not robust with what appears to be only 2 mice in one group and 3 mice in the other group. In addition to the unacceptable sample size, there is no input control and the magnitude of difference is modest. This data should be repeated with more animals or astrovirus should be deleted throughout the paper.

In the new revised Fig S1D we now:
- show data from 3 animals for each time point (6 hpi, 8 dpi, 12 dpi)- total 9 animals
- plot the data in log scale which shows a 100-fold increase from 6 hpi to 8 dpi; and a 1,000-fold increase from 6 hpi to 12 dpi for levels of murine astrovirus in the salivary glands.

These data indicate significant murine astrovirus replication in salivary glands and, given this, we would like to include these data in the supplementary figure.

2. Fig 4G-H. The y-axis cannot be fold-change relative to mock as stated by two reviewers previously. It can be fold change relative to input or better just shown as copies relative to GAPDH. This cannot be interpreted as presented and should be re-graphed and new statistics performed.

In the revised manuscript, Fig. 4g-h have been revised as suggested. In Fig. 4g, the measure of HuNoV genome content of lysates is now reported as number of genome copies relative to GAPDH. In Fig. 4h, the measure of HuNoV genome content of supernatant, is now reported as genome copies relative to the input inoculum.

3. The authors graph data points below their LOD. This is not appropriate. A data point cannot be below the LOD by definition. See Fig 1B, 1F, 1H, 2G, 2H, 2L, 2M, 2N, 3I, 3J, 4C, 4J, etc. These graphs need to be corrected and re-analyzed.

In the updated manuscript the TCID50 data have been re-plotted with the black dashed line marking the background signal based on the input or 6 hpi tissue sample.

As mentioned in the Method section of the updated manuscript, we determined the qPCR limit of detection (LOD) for the numbers of viral copies for each animal tissue, by measuring mock-infected (inoculated with PBS) animals. For increased accuracy, we reproduced this measurement in 10 replicates, and we estimated our LOD to be below ~100 copies per milligram of tissue (≥ 95% confidence). This LOD probably corresponded to signals from primer-dimer or false positive signals. So, across all analysis, we set $10^2$ copies/ mg of tissue as the limit of detection (LOD) and marked it alongside each figure measuring viral copy numbers.

Minor
4. Fig 4B Line 490. The authors should double check the t-test was performed on log-transformed data rather than untransformed data. The p-value at 24hpi seems much lower than expected based on the variance and overlap.

The plot was re-analyzed and updated with the correction.

5. Line 102: The authors state Wu23 causes persistent infection. The persistence of Wu23 is not assessed in any of the three cited papers and if WU23 is persistent that is a novel finding to this paper which should be clarified.
Walker and Baldridge have recently reported that WU23 establishes a persistent infection in the colon (Walker et al., 2021). We now cite this reference for WU23 but state that it is not yet a well-characterized persistent murine norovirus strain.

6. Line 123; "were considered equivalent" should be referenced or deleted.
We deleted this. It now reads:
"This points to crucial differences among persistent murine norovirus strains."

7. Line 124 and 131: "replication" should be "viral RNA" as detection of viral RNA in a tissue is merely suggestive of replication and the virus could come from another tissue source.
We have replaced "replication" with "viral RNA".

8. Line 120: NS1/2 but not VP1 is important for persistence. The VP1 is not a determinant of persistence but rather lethality in IFNAR mice and systemic infection.
We have corrected this and now state:
"Murine norovirus strain CR6, which shares with MNV-3, MNV-4 the critical sequence motifs in non-structural (NS1/2) and capsid (VP1) proteins that are needed for persistence and systemic infection respectively[17,23,24]…"

9. Line 68: an 8 log increase is not observed as stated. It is 6-logs.
We have now corrected this in the revised manuscript.

10. Line 85 is 6-logs and not 7-logs
We have now corrected this in the revised manuscript.

11. Also, the authors should define persistent infection. The authors only looked to day 14 and many would argue that this is not persistence as seen at >21 days post infection when the adaptive immune system has had plenty of time to clear infection.
We show in our manuscript that at **21 days post infection** (Figures 2e, 2k, 2l, 2m, 2n) the MNV-3, MNV-4 and WU23 strains continue to robustly replicate in the salivary glands at levels equivalent to or higher than that in the colon and continue to secrete into saliva. Measurements of replication at 21 days have also been used by others to assess the persistence of murine norovirus infections (Compton 2008; Shortland and Heeney 2014; van Winkle and Nice 2018; Wilen and Virgin 2018; Wobus 2018; Walker and Baldridge 2021). In contrast, acute MNV-1 infections are cleared within 7-10 dpi post inoculation (Figure 1b).

12. Line 38: Inappropriate to say "first line of defense for ALL invading…" Maybe many but this is not known for "ALL"
We have revised this, it is now: "Saliva forms the first line of defense for *many* pathogens entering through the oral route.."

13. Line 41: "Enteric viruses such as norovirus, rotavirus and astrovirus have been frequently detected in saliva" should be clarified. Viral RNA was detected for these viruses but not necessarily infectious virus. If there are primary references (as opposed to the three reviews cited) that demonstrate infectious norovirus, rotavirus, and astrovirus from the saliva then these should be cited or the statement should be revised to reflect only viral RNA was detected.

We have revised the sentence, it is now:
"Norovirus, rotavirus and astrovirus genomic RNA have been frequently detected in saliva in symptomatic and asymptomatic individuals…"

Referee #3 (Remarks to the Author):

As indicated during the previous round of reviews, I feel strongly that this is an important study for the field and one that will be of interest to anyone working on gastrointestinal pathogens. It has wide-reaching implications when we think about how GI viruses transmit. It will undoubtedly stimulate further research to understand the biological relevance of the observations in human-human transmission of GI viruses. I am fully satisfied by the reviewers responses.

Finally, I would like to pass on my congratulations to the authors for their work and continued efforts to improve the quality and clarity of their manuscript, particularly given the challenges we are all facing at the present time. This is a great piece of work, which they should all be very proud of.

We thank the reviewer very much for this appreciation.

Minor comments:
- Fig S3: I appreciate the western blots for human norovirus infection of the salispheres are a technically challenging - however, the quality of the blots presented in this figure are not in line with the others in their manuscript. I don't believe there is a need to repeat these, simply improve their clarity. Panel f is spliced together - it should be made clear where the splices are by the inclusion of gaps or solid lines indicating a splice. Panel g -is also spliced and it's unclear which of the proteins shown in the 6/96 hpi lanes for vesicle HuNoV are being quantified - there appears to be a doublet of some sort - please indicate which protein band is being quantified or if both are used for quantification.

We have now indicated in the revised figure explicitly where the splicing is and which protein bands were used for quantification.

**Reviewer Reports on the Third Revision:**

**Author Rebuttals to Third Revision:**

**Reviewer Reports on the Third Revision:**

Referees' comments:

Referee #2 (Remarks to the Author):

No major concerns remain and the authors should be commended on a superb and exciting study.

However, Figure 1B, 2G, 2H, 3L, 3M still have points below the LOD. This is impossible by definition as no values can be below the LOD. These values should be plotted at the LOD and re-analyzed as such.

Also, the LOD in the revised Fig 4G-H should be shown in the figure.

**Author Rebuttals to Third Revision:**

Response to Referee#2

We are most thankful to the referee for their thorough and constructive comments on our manuscript. We have responded to their requests below.

Referee #2 (Remarks to the Author):

No major concerns remain and the authors should be commended on a superb and exciting study. However, Figure 1B, 2G, 2H, 3L, 3M still have points below the LOD. This is impossible by definition as no values can be below the LOD. These values should be plotted at the LOD and re-analyzed as such.

In Figures 1B, 2G, 2H, 3L and 3M we have replotted and reanalyzed the TCID50 data after including the limit of detection (LOD), which is the lowest concentration of virus where at least 50% CPE was observed. The LOD is now explicitly shown on the figures by a dashed line and there are now no values that remain on the plots below it. The raw data and calculations for all the TCID50 are now provided in the source files corresponding to each figure.

Also, the LOD in the revised Fig 4G-H should be shown in the figure.
In Figs 4G-H, the qPCR data was reanalyzed and replotted with the LOD, which is now indicated in the figures and described in the methods.