**For research article**

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| Response to Reviewer 1 Comments | | |
| **1. Summary** |  |  |
| Thank you very much for taking the time to review this manuscript. Please find the detailed responses below and the corresponding revisions/corrections highlighted/in track changes in the re-submitted files*.* | | |

**Comment 1:**  Line 32: “Turkey hemorrhagic enteritis virus (THEV) belongs to genus *Siadenovirus*, family …”

*There is a “the” missing between “to” and “genus”.*

**Response 1:** We agree with this comment, and we have, accordingly incorporated the suggested missing word (Page 1; Line 32).

**Comment 2:**  Line 60: “These cytokines may further contribute to apoptosis and necrosis in bystander splenocytes, culminating in IMS (8, 11) (Figure 1).”

*I looked multiple times, but I can’t find figure 1 anywhere. Was this figure not submitted?*

**Response 2:** We thank the reviewer for pointing this out. Figure 1 must have been accidentally excluded during editing. We have therefore, included Figure 1 and its figure legend on page 3; Lines 66-70.

**Comment 3:**

Line 84: “We specifically focus on cellular processes related to cell survivability that can elucidate THEV-induced IMS.”

*I have admittedly never worked with turkey cells, but considering that the authors do have a hunch what could be the link to IMS, wouldn’t there have been an easier and more precise way other than RNAseq to study apoptosis in these cells?*

**Response 3:** The pathogenesis of the virus is woefully understudied, so the only thing that is known from previous studies is that infection induces apoptosis. No further detail such as which branch of the apoptotic pathway or which pro-apoptotic genes are involved were known. Therefore, it was necessary that a broad survey of all the genes differentially expressed during infection be performed with RNA-seq before any targeted experiments could be done. RNA-seq could point out possible biological processes mediating apoptosis which could then be investigated further with targeted experiments in follow-up studies. Therefore, we believe that RNA-seq was the best option available.

**Comment 4:**  Line 99: “..(MOI) of 100 GCN/cell,..”

*What were the infection levels achieved with this MOI? Where all the cells infected?*

**Response 4:** In a previous study (<https://doi.org/10.1016/j.virusres.2019.01.005>), the researchers used a qPCR-based titration method for THEV similar to our method. Their method is modified to titer the infectious virus particles (IVP). In their experiment, they used 20 IVP/cell as the MOI. Since our method is similar but measures the total genome copies, we believe that 100 GCN/cell should be a sufficiently high MOI to infect all the cells. We performed a one-step growth curve using qPCR and our results were consistent with infection of all cells.

**Comment 5:**  Line 101:” … and duplicate (mock-infected)..”

Are duplicate biological repeats sufficient to achieve statistically significant results or are additional biological repeats needed?

**Response 5:** We agree with the reviewer that using more biological replicates would yield more statistically robust results. However, it has been shown (<https://rnajournal.cshlp.org/content/22/6/839.long#F1>) that using the appropriate differential gene expression analysis tool can yield robust data even with low replicate experiments. DESeq2 (used in our analysis) is one of the best tools for experiments with few replicates. The researchers found that: “The best performing tools, *DESeq, DESeq2, EBSeq, edgeR*, and *limma*, successfully control their FPR, maintaining it consistently close to or below 5% irrespective of fold-change threshold or number of replicates, highlighting again that the primary effect of increasing replicate number is to increase the sensitivity of these tools, converting false negatives to true positives.” Thus, while we may have missed some differentially expressed genes (DEGs) as false negatives, we believe that the genes called as differentially expressed in our results are reliable. Furthermore, our qPCR validation was based on a separate infection; therefore, constitutes an independent experiment.

**Comment 6:**

Line 124: “Trimmed reads were mapped the reference turkey”

There is the word “to” missing in between “mapped” and “ to”.

**Response 6:** We thank the reviewer for pointing this out. We have inserted the suggested missing word on Page 4; Line 129.

**Comment 7:**   Line 172: ”… harvested at total RNA at 4-, 12-, 24-, and 72-hours post infection (hpi).

*The first “at” has to be removed. Secondly, how did the cells look at those time points, especially the 72-hour time point?*

**Response 7:** We thank the reviewer for pointing this out. We have removed “at” from the sentence on page 5; line 177.

THEV infection shows only one cytopathic effect (CPE), which is cell swelling but the CPE can be very subtle. We have included the following statement in the manuscript on page 5; line 177: “In the first 12 hours, there was no discernible CPE. At 24 hours, the CPE was very subtle but observable and at 72 hours, almost every cell was clearly swollen with numerous cytoplasmic vacuolation and granulation. Some cells were more than double the size of the mock-infected cells.”

**Comment 8:**  Line 182: “DEGs identified at 4- and 72-hpi did not yield any statistically significant results …”

*The 4-hour time point makes sense as the virus has just begun to replicate, but why would there be nor data at the 72-hour time point? Where all the cells already dead?*

**Response 8:** We have also asked ourselves the same question about the 72-hour time point, and these are some possible reasons. 1) Many cells were dead: the 72-hour time point is when the CPE of the virus was most clearly observable; therefore, the cell death may have been at its peak here. 2) Too few sequencing reads mapping to the host genome: from Table 1, we see that not only is there less sequenced reads for the 72-hour time point samples, but there are also far less percentage of mapped reads to the host genome. While we can only make speculations about the reasons for this such as an effect from the library preparation of the 72-hour samples or even contamination at this time point, the fewer mapping of the sequenced reads to genome would explain why only a handful of differentially expressed genes were detected. 3) It is also possible that a combination the reasons given in 1) and 2) account for this. This uncertainty is the reason why all the analysis and results were based on the 12- and 24-hour time points.

**Comment 9:**  *Line 187: Table 1. Summary of sequencing, quality control, and mapping processes.*

*The raw data presented in this stable is too unwieldy and should either be moved supplemental or should be presented in a more succinct and reader friendly way. The very same id true for all other tables presented results from the various pathway analyses.*

**Response 9:** We thank the reviewer for bringing this to our attention. The tables are consistent with tables in papers of this type. We would welcome editorial advise on how to simplify or make them more presentable.

**Comment 10:**   Line 192: Figure 2. (A)

*Why are there only two data point for the infected cells? Shouldn’t there be three as there were three biological repeats?*

**Response 10:**  One of the biological replicates at 12-hour time point did not pass the RNA integrity QC because it had degraded, so it was excluded from sequencing. We have included this sentence “One biological replicate from the 12-hour time point did not pass the RNA integrity quality control and was not sequenced” in the manuscript (page 6; line 197) for clarification.

**Comment 11:**   Line 327: “(*BNIP2*; interacts directly 327 with adenovirus E1B-19K protein),”

*It appears as if the authors are describing the interaction of a human adenovirus with human cells, not the turkey virus with turkey cells. This happens multiple times in the manuscript which makes it hard to understand what has been demonstrated for this adenovirus compared to other adenoviruses. It would be advisable if the authors could specify what virus interaction in what host they are referring to. While some of these interactions are surely very conserved across virus species in different hosts, I am not too sure if this observation can generally be extrapolated across species lines without having to specifically established them in the model of choice first.*

**Response 11:** We thank the reviewer for pointing this out. We have changed the indicated sentence to “(*BNIP2*; interacts directly with human adenovirus E1B-19K protein)” on page 64; line 341.

We have also modified line 349 (page 64) to “Previous studies of human adenoviruses have shown that forcibly transitioning the…” to clarify the host organism.

We have also modified line 351 (page 64) to “For human adenoviruses, interaction of the viral E1A…” to clarify the host organism.

We have also modified line 502 (page 68) to “The human adenovirus E1A proteins…” to clarify the host organism.

We have also modified line 505 (page 68) to “However, human adenoviruses have developed…” to clarify the host organism.

**Comment 12:**  Line 399: Figure 5. Upregulation of ER Unfolded Protein Response (UPR).

*Has that figure been previously published? Could there a copywrite issue here?*

**Response 12:** We thank the reviewer for pointing this out. The figure is a standard output when data is analyzed in the KEGG database for enriched pathways. We have obtained copyright permission from KEGG, so there should be no copyright concerns.

**Comment 13:**  Line 424: 3.8. Validation of DEGs by Reverse Transcriptase Quantitative PCR (RT-qPCR)

*I am fully aware that reagents for turkey cells are probably hard to come by, but would it be possible to show that these proteins are effect by the virus by looking at their protein levels? Are there any antibodies available that could be used for immunoblots?*

**Response 13:** There, no available reagents for our genes of interest in turkeys. Furthermore, our qPCR validation was based on a separate infection; therefore, constitutes an independent experiment. Moreover, our results are consistent with the findings of apoptosis in previous studies. Therefore, we believe the results are dependable. Also, most papers of this type typically validate the RNA-seq results with qPCR, not protein quantification

**Comment 14:**

Line 438: Figure 6.

*This might go being the scope of the presented experiments, but a 2-3-fold change in mRNA expression levels does not seems reflect a huge effect of the virus. The authors need to demonstrate that the protein levels are affected but such a minor change in mRNA levels or even better, should establish a functional assay demonstrating that the virus infection alters the pathways the authors have identified as significantly changed.*

**Response 14:** We have modified Figure 6 to show actual fold changes (ranging from 2.4-6.3), which may not have been apparent because the plot was on a log2 scale. Since we link the biological processes and genes discussed in our manuscript to apoptosis, a functional assay would only indicate that the infection induces apoptosis (which is already established). More specific assays are not validated in turkeys. Also, most papers of this type typically validate the RNA-seq results with qPCR, not protein quantification.

**Comment 15:**  Line 441: “… are statistically differentially expressed …”

*If this is the case, then the authors need to show p-values to demonstrate statistical significance.*

**Response 15:** We are grateful to the reviewer for pointing this out. We have, accordingly, modified the Figure 6 to show the p-values (page 67; line 448).

We have added the following sentence to the figure legend of Figure 6 for clarification on page 67; line 459: “The p-values are indicated on top of each bar and the fold changes for each gene are indicated inside its corresponding bar.”

Since we show the p-values from only the Student’s T-test, we have also removed all mention of the Mann-Whitney U test (lines 171 [page 5], 451 and 459 [page 67]).

**Comment 16:**  Line 468: “Thus, the obtained results are likely sub-optimal in amount of detail relative to results from well annotated and curated genomes of model organisms.”

*Not only that, but there will also be significant evolutionary difference in the pathways identified here between distantly related species like humans and turkeys. Thus, it would be conceivable that proteins essential in humans are not found in this analysis in turkey cells, while proteins identified here might not have a connection to apoptosis or any other identified pathway in humans and have thus not been clustered properly by the software in the here presented work.*

**Response 16:** We disagree with the reviewer here. The databases we used for the functional enrichment contain curated data for other organisms including turkeys. The results were based directly on the turkey specific pathways and GO terms. The mention of “sub-optimal” was only meant to denote that there is more curated information on the better-studied organisms such as humans than less-studied organisms such as turkeys, and not that our obtained results are inferior in quality.

**Comment 17:**

Line 585: “Authors should discuss the results and how they can be interpreted from the perspective of previous studies and of the working hypotheses. The findings and their implications should be discussed in the broadest context possible. Future research directions may also be highlighted.

*I don’t think this paragraph is supposed to be in the manuscript.*

**Response 17:** We are grateful to the reviewer for pointing this out. We agree with this comment; therefore, the said paragraph has been removed from the manuscript (page 70).