nature portfolio

# Peer Review File

# SARS-CoV-2 Delta variant re-emerges in US farmed mink and free-ranging white-tailed deer in 2022–2023

Corresponding Author: Dr Andrew Bowman

**This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.**

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
I very much enjoyed reading this interesting manuscript about the re-emergence of Delta infections in farmed American mink, wit eventual spillover to deer. It is challenging to sample in mink farms, so the cooperative approach taken here was much appreciated, and provided a valuable dataset. Overall, I think the work is interesting and well executed. I have several comments which I hope are constructive.

On line 295, the manuscript indicates that there are mink-adapted mutations. I did not see any evidence supporting that these mutations are mink adapted. This is important of course, because such mutations would support the inference that the virus has been circulating among mink for some period of time (vs spillover from elsewhere). I expected to see analyses of mutation spectra or such to identify whether the mutations are mink adapted. Was this done? Otherwise, please clarify what you consider as evidence.

On line 330, the authors state that “To our knowledge, the only prior reported instance of interspecies transmission involving two non-human hosts is a report of SARS-CoV-2 transmitting from farmed mink to barn-dwelling feral cats in the Netherlands in 2020”. Note that the paper by Pickering et al. (2022; already cited in the manuscript) identifies that the MRCA of the divergent lineage of SARS-CoV-2 in Ontario deer (B.1.641) is a virus derived from mink farms in nearby Michigan. Pickering et al. infer that spillover of SARS-CoV-2 from Michigan mink farms into deer led to the evolution of B.1.641 in Ontario deer. This seems highly relevant to mention given the similar dynamic in the present manuscript of Delta infections spilling from mink farms into deer.

There were also mink farm workers with the same virus in Michigan as the mink (i.e., also the MRCA for B.1.641), so human involvement in the chain of transmission between mink and deer cannot be ruled out; however, there is a substantial probability that transmission was mink to deer. This entire Michigan-Ontario scenario is discussed in an interesting New York Times article:
https://www.nytimes.com/2022/05/22/health/coronavirus-mink-michigan-spillover.html

The Pickering paper also contains an analysis of mutation spectra that uses ordination to compare mutations from global SARS-CoV-2 databases among deer, mink, and humans, finding that B.1.641 clustered with other deer-derived viruses, leading to the inference that the mutations were deer-adapted, and that the virus had been circulating in deer for up to a year. It strikes me that a similar analysis would be useful in the present manuscript, to confirm that the mutations are mink adapted.

Minor comments

Throughout the manuscript, VOCs are written in lower case (e.g., delta, omicron, etc.). I believe the first letters of these should be capitalized.

Figure 3. I find this figure rather confusing. The caption refers to tips being shaded and mink isolates being highlighted and shaded, and these overlapping descriptions are somewhat confusing. I could not tell what the small colored circles were pointing out (shaded mink isolates?), and generally had a difficult time telling the mink- from the human-derived isolates. It might also be easier to interpret a rectangular phylogeny, though that is only a suggestion. At a minimum, the caption needs to be written more clearly.

Line 350. I think One Health is typically written with capital O and H.

Line 388. Worth specifying whether the Genscript assay used the wild-type or Omicron virus neutralization kit.

Reviewer #2

(Remarks to the Author)
This is a timely study integrating active surveillance, genomics and phylodynamics to investigate SARS-CoV-2 transmission in non-human hosts. The dataset is substantial, the analyses are generally rigorous, and Figures 4 and 5 are particularly effective at communicating the evolutionary narrative. The documentation of a delta (AY.39) lineage re-emerging in mink after apparent disappearance in humans is especially compelling. I recommend only minor revisions and hope that my comments below improve the manuscript.
- In the Methods (p.20), the study states that procedures were performed under an IACUC protocol. However, earlier in the manuscript the voluntary surveillance framework is noted. It would be helpful to explicitly explain whether WTD samples were collected under separate wildlife ethics approvals and the lack of animal ethics approvals generally.
- The study reports 760 rRT-PCR positives across three farms, yet only 293 genomes were sequenced. Later, 526 PCR-positive samples were sent for for sequencing, but only 293 passed QC. Were the 526 samples selected based on Ct threshold? If so, what cutoff? What proportion failed sequencing due to low viral load versus QC filtering? Were sequenced samples evenly distributed across PCR positive samples?
- Given the finding of elevated evolutionary rates in mink and deer, it would be highly informative to examine host-associated mutational signatures using mutational spectrum analysis (e.g., as in Nat Commun 2023; s41467-023-42916-w). Specifically, are there shifts in transition/transversion ratios in mink versus humans? Do mink-adapted mutations cluster in particular sequence contexts?
- The global phylogeny (Figure 3) includes sequences up to April 24, 2023. It might be worthwhile to incorporate publicly available sequences from ~12 months after the study period to assess whether any additional AY.39 genomes cluster near the Farm P mink clade. And this would determine whether mink-adapted mutations appear subsequently in humans or other animals.
- The manuscript argues that AY.39 was circulating undetected during the 4-8 month gap. It would be worth further discussion of the undersampling of low-prevalence human transmission chains and regional sampling bias of sequence data in GISAID.
- Figure 2: Currently, farm panels (N, O, P) are separated. It would be helpful if all farm epidemic curves were superimposed in a single comparative panel. Or, alternatively, include a normalised prevalence panel (percent positive per farm per week) to allow direct visual comparison. This would improve clarity when comparing outbreak dynamics between farms.
- Line 49: “some the country’s largest mink-producing states” → should read “some of the country’s largest mink-producing states.”
- Line 59: “led to led to widespread breakthrough infections” → duplicated phrase.
- Line 194: “sampled in in humans” → duplicated word.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have done a nice job of responding to my previous review comments. I have no additional suggestions.

Reviewer #2

(Remarks to the Author)
Thanks to the authors for including my suggestions and I'm satisfied that the manuscript is ready for publication

We thank both reviewers for their thoughtful comments and suggestions. We have addressed each comment below and made the indicated revisions. The reviews are reproduced in full below, with our indented responses in blue.

**REVIEWER COMMENTS**

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

**I very much enjoyed reading this interesting manuscript about the re-emergence of Delta infections in farmed American mink, wit eventual spillover to deer. It is challenging to sample in mink farms, so the cooperative approach taken here was much appreciated, and provided a valuable dataset. Overall, I think the work is interesting and well executed. I have several comments which I hope are constructive.**

On line 295, the manuscript indicates that there are mink-adapted mutations. I did not see any evidence supporting that these mutations are mink adapted. This is important of course, because such mutations would support the inference that the virus has been circulating among mink for some period of time (vs spillover from elsewhere). I expected to see analyses of mutation spectra or such to identify whether the mutations are mink adapted. Was this done? Otherwise, please clarify what you consider as evidence.

We thank the reviewer for this comment. The amino acid substitutions we refer to as mink-adaptive (NSP9:G37E and S:Y453F) have been previously identified in the literature based on evidence of convergent evolution following mink spillovers (refs. 5, 19, 21) and, for NSP9:G37E, GWAS analysis (ref. 20). We agree that this basis for classification was not sufficiently clear in the original text. We have therefore revised the manuscript to clarify that these mutations have been characterized as mink-associated in prior studies. The revised text now reads (Lines 203-206):

*Most viruses in Clade A contain two amino acid substitutions (NSP9:G37E and S:Y453F) that were characterized as mink adaptive in prior studies[15-18], which emerged early in the Clade A outbreak (August 28, 2022).*

On line 330, the authors state that "To our knowledge, the only prior reported instance of interspecies transmission involving two non-human hosts is a report of SARS-CoV-2 transmitting from farmed mink to barn-dwelling feral cats in the Netherlands in 2020". Note that

the paper by Pickering et al. (2022; already cited in the manuscript) identifies that the MRCA of the divergent lineage of SARS-CoV-2 in Ontario deer (B.1.641) is a virus derived from mink farms in nearby Michigan. Pickering et al. infer that spillover of SARS-CoV-2 from Michigan mink farms into deer led to the evolution of B.1.641 in Ontario deer. This seems highly relevant to mention given the similar dynamic in the present manuscript of Delta infections spilling from mink farms into deer.

There were also mink farm workers with the same virus in Michigan as the mink (i.e., also the MRCA for B.1.641), so human involvement in the chain of transmission between mink and deer cannot be ruled out; however, there is a substantial probability that transmission was mink to deer. This entire Michigan-Ontario scenario is discussed in an interesting New York Times article: https://www.nytimes.com/2022/05/22/health/coronavirus-mink-michigan-spillover.html

> We thank the reviewer for pointing out the relevance of Pickering et al. (2022) to our discussion of interspecies transmission and we have revised the Discussion accordingly (Lines 390-403):
>
> *Possible mink-to-deer SARS-CoV-2 transmission was hypothesized in a previous study by Pickering et al. (2022)[9], where a highly divergent B.1.641 lineage found in WTD near the US-Canadian border in southwest Ontario, Canada, shared a common ancestor with viruses collected from a mink farm outbreak (and farm workers) located across the border in northern Michigan. The long branch lengths and large gaps in sampling make it difficult to ascertain whether spillover occurred directly from the mink farm to WTD, or whether the divergent WTD virus was sourced from an unsampled virus population circulating in humans or another host species. In contrast to our study, the Ontario WTD sequences identified by Pickering et al. did not have any known mink adaptations. Nor were the Ontario WTD sequences nested phylogenetically within a discrete mink clade in the way the WTD sequences in our study were nested within the mink-only AY.39 clade. While the Michigan mink outbreak is less clearly connected to the subsequent detection of the virus in WTD in Ontario, both studies expose major sampling gaps in animals that render it difficult to finely reconstruct multiple interspecies transmission events involving humans, mink, deer, and potentially other species.*

The Pickering paper also contains an analysis of mutation spectra that uses ordination to compare mutations from global SARS-CoV-2 databases among deer, mink, and humans, finding that B.1.641 clustered with other deer-derived viruses, leading to the inference that the mutations were deer-adapted, and that the virus had been circulating in deer for up to a year. It strikes me that a similar analysis would be useful in the present manuscript, to confirm that the mutations are mink adapted.

> This is a good suggestion. We performed a mutation spectrum analysis in the revised manuscript (**Figures 6B-C**, **Figures S9-13**, **Supplemental file 3**) that reveals a

mutational profile for the AY.39 mink-derived sequences that is distinct from human- and deer-associated spectra. We revised the text as follows (Lines 265-283, Results):

***Mutation patterns in mink**. Differences in mutation spectra were examined across AY.39 sequences obtained from mink, humans, and WTD (**Figure S9** and **Supplemental File 3**). Mink sequences experienced a significant decline in C>T substitution frequency over time ($R^2$ = -0.40, p = $3\times10^{-21}$; **Figure 6B**), in contrast to stable patterns in humans ($R^2$ = 0.004, p = 0.216). An upward trend in WTD is consistent with previous reports[13,14], but was not statistically significant in our dataset, likely due to limited sample size. Other substitutions also increased over time in mink, with the strongest effects observed for C>G ($R^2$= -0.626) and T>A ($R^2$=0.578) (**Figure S10**; **Supplemental File 3**). Mutation spectra differed significantly among mink, human, and deer in a principal components analysis (**Figure S11**). The State 2 deer sequences diverged from the mink clade over time, with the earliest deer sequence (December 4, 2022) clustering near the mink group along PC1 and deer sequences collected one month later (January 5, 2023) shifting toward the deer cluster, consistent with evolution from mink-like to deer-like (**Figure S12**). Permutation-based analyses confirmed significant differences between host groups (p < 0.001, **Supplemental File 3**). As expected, AY.39 mink sequences associated with a single outbreak formed a more compact cluster (dispersion = 0.036) than AY.39 deer sequences sampled across multiple spillovers (dispersion = 0.066; **Figure S13**). Mutation spectrum divergence from the human baseline increased progressively over the course of the mink outbreak ($R^2$ = 0.504, p = $2.74\times10^{-28}$; **Figure 6C**), demonstrating that the mink-specific spectral signature accumulated during circulation.*

Minor comments

Throughout the manuscript, VOCs are written in lower case (e.g., delta, omicron, etc.). I believe the first letters of these should be capitalized.

The text was revised as suggested and all VOCs are capitalized in the text and figures.

Figure 3. I find this figure rather confusing. The caption refers to tips being shaded and mink isolates being highlighted and shaded, and these overlapping descriptions are somewhat confusing. I could not tell what the small colored circles were pointing out (shaded mink isolates?), and generally had a difficult time telling the mink- from the human-derived isolates. It might also be easier to interpret a rectangular phylogeny, though that is only a suggestion. At a minimum, the caption needs to be written more clearly.

We agree with the reviewer that the shading in Figure 3 was confusing. We have replaced the color blocks that were previously highlighting only mink viruses, shaded all tips by WHO variants of concern and now use labels in the revised figure.

Line 350. I think One Health is typically written with capital O and H.

Agreed. Now reads: "In conclusion, our study demonstrates the benefit of integrating multiple data streams from humans, domestic animals, and wildlife into a **One Health** framework…"

Line 388. Worth specifying whether the Genscript assay used the wild-type or Omicron virus neutralization kit.

The text was revised to address this suggestion. The text now reads (Lines 456-461):

"Potential SARS-CoV-2 exposure was investigated using the SARS-CoV-2 Surrogate Virus Neutralization Test (sVNT, GenScript L00847-A, Piscataway, New Jersey USA) according to the manufacturer's protocol. **The assay was modified by replacing the wild-type RBD-HRP with an Omicron variant RBD-HRP (GenScript Z03730-500) and the corresponding Omicron neutralizing antibody standard (GenScript A02161-100).** Serum samples from 12 farms with the presence of unvaccinated mink were tested for evidence of seroconversion."

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

**This is a timely study integrating active surveillance, genomics and phylodynamics to investigate SARS-CoV-2 transmission in non-human hosts. The dataset is substantial, the analyses are generally rigorous, and Figures 4 and 5 are particularly effective at communicating the evolutionary narrative. The documentation of a delta (AY.39) lineage re-emerging in mink after apparent disappearance in humans is especially compelling. I recommend only minor revisions and hope that my comments below improve the manuscript.**

- In the Methods (p.20), the study states that procedures were performed under an IACUC protocol. However, earlier in the manuscript the voluntary surveillance framework is noted. It would be helpful to explicitly explain whether WTD samples were collected under separate wildlife ethics approvals and the lack of animal ethics approvals generally.

The text has been revised to expand on this (Lines 551-553):

"Deer sequences were obtained from SARS-CoV-2-positive nasal swabs collected post-mortem from hunter-harvested deer; therefore, collection of these samples was exempt from animal use oversight."

- The study reports 760 rRT-PCR positives across three farms, yet only 293 genomes were

sequenced. Later, 526 PCR-positive samples were sent for for sequencing, but only 293 passed QC. Were the 526 samples selected based on Ct threshold? If so, what cutoff? What proportion failed sequencing due to low viral load versus QC filtering? Were sequenced samples evenly distributed across PCR positive samples?

> The text was revised to discuss the criteria for the selection of samples, which was based on CT threshold and downstream QC. The text now reads (Lines 483-494):
>
> *"Original sample material for 526**/760 (69.2%)** PCR positive nasal swab samples were sent to the National Veterinary Services Laboratories (NVSL) for whole genome sequencing. **Samples were selected for sequencing based on a cycle threshold (Ct) value of less than 34.0 on either two or three of the targeted genes.** The Nextera XT DNA Sample Preparation Kit was used in accordance with manufacturer instructions to prepare cDNA libraries from viral RNA that was amplified via PCR. The 500 cycle MiSeq Reagent Kit v2 was used to perform sequencing. Sequences were assembled using IRMA v0.6.7 and DNAStar SeqMan NGen v14.0.1. **A total of 462/526 (87.8%) assemblies were obtained from NVSL, of which 401 were selected based on a ≥80% genome coverage threshold.** Pangolin v3.1.1134 was used to assign variant lineages for all sequences. Variant annotation, validation, and quality assessment of the consensus sequences were performed using VADR35. **A total of 293** genomes that passed quality control were used in all downstream analyses and were deposited in GenBank. Accession numbers are available on Table S3."*

- Given the finding of elevated evolutionary rates in mink and deer, it would be highly informative to examine host-associated mutational signatures using mutational spectrum analysis (e.g., as in Nat Commun 2023; s41467-023-42916-w). Specifically, are there shifts in transition/transversion ratios in mink versus humans? Do mink-adapted mutations cluster in particular sequence contexts?

> Reviewer 1 had a similar comment. We agree with both reviewers that this is a good suggestion and performed a mutational spectrum analysis in the revised manuscript. (See above.)

- The global phylogeny (Figure 3) includes sequences up to April 24, 2023. It might be worthwhile to incorporate publicly available sequences from ~12 months after the study period to assess whether any additional AY.39 genomes cluster near the Farm P mink clade. And this would determine whether mink-adapted mutations appear subsequently in humans or other animals.

> This is a good point. To address this, we searched for additional AY.39 sequences on the GISAID database collected after April 24, 2023. We did not identify any newly available

AY.39 genomes submitted after the study period, which confirms that the AY.39 dataset used in the original analysis is complete.

- The manuscript argues that AY.39 was circulating undetected during the 4-8 month gap. It would be worth further discussion of the undersampling of low-prevalence human transmission chains and regional sampling bias of sequence data in GISAID.

This is a good suggestion, and we revised the Discussion (Lines 351-355) as follows. Unfortunately, we cannot say anything more specific about regional biases in genomic surveillance data in North America without revealing clues about the location of our sampling, which we are not disclosing to protect our mink farming partners in this study.

*"Rather, our results are consistent with AY.39 circulating and replicating for an extended period (4–8 months) in a natural host. AY.39 could have been transmitted at low levels in humans prior to its emergence in mink,* ***which may have gone undetected due to undersampling of low-prevalence transmission chains and regional biases in genomic surveillance data."***

- Figure 2: Currently, farm panels (N, O, P) are separated. It would be helpful if all farm epidemic curves were superimposed in a single comparative panel. Or, alternatively, include a normalised prevalence panel (percent positive per farm per week) to allow direct visual comparison. This would improve clarity when comparing outbreak dynamics between farms.

This is a good suggestion. We revised Figure 2 to include a normalized prevalence panel with percent positive per farm per week (Figure 2a).

- Line 49: "some the country's largest mink-producing states" → should read "some of the country's largest mink-producing states."

The text was revised as suggested.

- Line 59: "led to led to widespread breakthrough infections" → duplicated phrase.

The text was revised to eliminate the duplicated phrase.

- Line 194: "sampled in in humans" → duplicated word.

The text was revised to eliminate the duplicated word.