Dr. Kathryn Kistler
Howard Hughes Medical Institute - Fred Hutchinson Cancer Research Center
Vaccine and Infectious Disease Department
1100 Eastlake Ave E
Seattle, WA 98109
UNITED STATES

An Atlas of Adaptive Evolution in Endemic Human Viruses
CELL-HOST-MICROBE-D-23-00539

Jun 26, 2023

Dear Dr. Kistler

I am enclosing the comments that the reviewers have made on your paper. The reviewers express interest in the topic and approach and raise a few points for consideration. We would be happy to consider a revised version of the manuscript provided that you satisfy the concerns of the reviewers. Additionally, in looking at the data, I should also note that at 5 figures/tables, the paper sits between a Short Article (4 tables/figures) and full research article (6-7 tables/figures). It seems that the main figures could be combined to present the findings more succinctly in the Short Article format. Please consider these changes to the data presentation when preparing the revised manuscript.

Thank you for the suggestion, but we would like to put forth this manuscript as a full research article and believe that ~5000 words and 5 figures fits well the guidelines laid on on the website (https://www.cell.com/cell-host-microbe/article-types). In order to address reviewer critiques, we have added text, a table and supplementary figures – we believe reducing the article to 4 figures and 4000 words would interfere with our ability to describe the research and to address the reviewers' points.

We would like to have the revised manuscript returned within 8 weeks; however, it would be useful for scheduling purposes if you could let us know as soon as possible when you anticipate resubmitting the paper. Please note that we take into account the published literature available on the day we make our final decision. If you need additional time to address the concerns that came up in the review process, please let us know so we can discuss a plan for moving your paper forward.

When you submit the revised version, do so through EM at: https://www.editorialmanager.com/cell-host-microbe/. From there, please follow the instructions for submitting a revised manuscript.

Before resubmitting, **please read our author's information concerning stylistic and**

formatting guidelines, **specifically our word and figure limits**. The revised version should conform to the general length restrictions for Cell Host & Microbe which for a Research Article is a file of 7,000 words (including figure legends, but excluding the reference list, STAR Methods, and any supplemental video or excel table legends), accompanied by no more than 7 figures/tables; for a Short Article the length restriction is 4,000 words with no more than 4 figures/tables; for a Brief Report the length restriction is 2,500 words. Please also include a point-by-point account of how you have responded to each of the reviewer's concerns. The abstract should not be longer than 150 words and should aim to make the general significance of the work clear to a broad scientific readership. Please also include a point-by-point account of how you have responded to each of the reviewer's concerns.

Please note that our article length guidelines have been recently adjusted to reflect that references are no longer counted towards the overall word count of the manuscript.

All of the panels of each figure should be printed together on a single page and should be organized as they will appear in the journal. Figures must be prepared in accordance with the Cell Press Data Processing Policy. For example, if you have digitally eliminated irrelevant or superfluous lanes from a gel or blot image, you must indicate the position of the deletion with a line or a space, and explain the manipulation in the figure legend. Details can be found one our Information for Authors page.

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The manuscript must also conform to our Supplemental Information Guidelines and our Cell Press methods reporting format, STAR Methods. If you have any questions, please don't hesitate to contact me.

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- **Mendeley Data**: Publish your original, unprocessed data through Mendeley Data. We will link your published paper and the dataset to each other. For more information, please click here.
- **STAR PROTOCOLS:** Complement your primary research article by publishing a step-by-step procedure with STAR Protocols, an open-access peer-reviewed journal from Cell Press. STAR Protocols aims to make the daily work of the scientific researcher easier by providing complete, authoritative, and consistent instructions on how to conduct experiments. The primary criteria for publication in STAR Protocols is usability

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I look forward to hearing from you and to reading the revised manuscript.

Best wishes,

Ella Hinson, Ph.D.
Scientific Editor, Cell Host & Microbe

Reviewers' Comments:

Reviewer #1: This latest manuscript, "An Atlas of Adaptive Evolution in Endemic Human Viruses" by Drs. Kistler & Bedford, presents a comprehensive survey of adaptive evolutionary signals based on available genomic data from endemic human viruses. Building upon their previous related work these past few years, especially their "Rapid and parallel adaptive mutations in spike S1 drive clade success in SARS-CoV-2" (2022) and "Evidence for adaptive evolution in the receptor-binding domain of seasonal coronaviruses OC43 and 229e" (2021), this manuscript under consideration presents a slightly updated test statistic compared to their previous work and a more expansive catalog of viruses to explore the important question of how commonplace antigenically-driven adaptive evolution is among human viruses.

The question the authors are exploring is an important and practical one, as it gets to the evolutionary reasons why intervention strategies (particularly vaccines) should differ between different viruses, and which viral species need to be handled similarly, regardless of their relatedness. This is the kind of material you would want members of ACIP to become intimately familiar with.

At a high level, my instinctual reaction is that I wish an "atlas" was much larger than this and included far more human-endemic viruses of importance, especially those that we routinely vaccinate against—but this reflects limitations in the state of genomic surveillance at large, not of the work of the authors, and is a stark reminder that there is much more genomic work to be done for a broad range of species. That said, this is impressive as it is, as it's already several fold more than the previous CoV papers mentioned above.

Thank you for your note of enthusiasm! As you point out, our panel contains 28 viruses, which is far from a comprehensive panel of endemic viruses. The reason our analysis is limited to this number is exactly as the reviewer states: limitations of genomic surveillance. For a proper

analysis of the rate of adaptation, we need sequences collected over a long span of time and not all endemic viruses have an adequate number of historical sequences available. We have added the following text to the “Genome-wide appraisal of rapidly-evolving viral proteins” section of the Results to convey this information to the reader:

> *While this "atlas" aims to examine continuous adaptive evolution across a range of viral diversity, the panel of endemic viruses is far from comprehensive and is largely limited by the availability of historical sequences which, for many viruses, is not adequate to make accurate rate estimates.*

In addition, this is mentioned in the Discussion:

> *We selected viruses to include in the panel based on the following criteria: 1) virus has been endemic for at least 12 years, 2) the genome is under 50 kilobases, and 3) there are at least 50 high-quality genomes available spanning at least 12 years. For many endemic viruses, the limiting factor is a dearth of historical sequences predating the mid-2000's. However, the COVID-19 pandemic has spurred an increased interest in monitoring and sequencing human pathogens and, if this trend continues, it is likely that there will be enough longitudinal data to add many more viruses to this panel in the years to come.*

I like the updated method (modified MK-test with updated outgroups for each sliding temporal window). It is sensible for viruses undergoing antigenically-driven adaptive evolution. The availability of code and data is excellent, as is the companion interactive visualizations to help explore the concepts and findings.

Questions:

1. Discussion paragraph 1 appears to be the first time we see the selection criteria for the list of viral species studied—can this be mentioned much earlier? (perhaps in the Intro paragraph "Here, we aim to survey"). The reason is that, for much of the reading of this paper, I'm bugged with the question: why didn't they include ? They did D68, but what about other entero/rhino/polios? HepC? HIV? If the simple answer is that the viruses presented here are all of the ones that meet the selection criteria (is that true?), that gets the question out of the way much faster.

Thank you for pointing this out! Yes, it is true that the reason other viruses, such as rhinoviruses and other enteroviruses, were not included is because there is not enough sequence data spanning enough time to make reasonable estimates of rates of adaptation at the genotype or subtype level for these viruses. As the reviewer suggests, we have added text that clarifies this to the first results section where we mention applying this method to a panel of viruses. We have also added Supplementary Figure 2, which shows the counts of sequences per year for each virus in the panel. The paragraph in Results about this now reads:

We next sought to survey a wide diversity of endemic human viruses for evidence of ongoing adaptive evolution. We focus on viruses that have been endemic in humans for at least 12 years because we are interested in continued adaptive evolution that persists during the endemic phase (rather than initial host adaptation that occurs early in a pandemic) and because a short temporal spread of sampled sequences decreases the accuracy of the estimated rate of adaptation (Kistler and Bedford, 2021). We analyze viruses at the subtype or genotype level, and require that each viral subtype in the panel has at least 50 genomes spread over a minimum of 12 years (Supplementary Figure 2 shows the temporal distribution of sequences for each virus). While this "atlas" aims to examine continuous adaptive evolution across a range of viral diversity, the panel of endemic viruses is far from comprehensive and is largely limited by the availability of historical sequences which, for many viruses, is not adequate to make accurate rate estimates. In total, we downloaded and curated sequence data for 28 human pathogenic viruses, which belong to 10 different viral families. This panel comprises both RNA and DNA viruses with a variety of modes of transmission including respiratory, fecal-oral, vector-borne, and via blood or bodily fluids.

2. Previous reviewers of your hCoV paper (eLife publishes open reviews) inquired about population structure in the data affecting the MK-like test. I would imagine the geographic and sampling biases vary widely from species to species. Would it be sensible to address this with a similarly modified FST-like test, that operates on the same sliding time windows, as an assurance of how panmictic the samples are within each time window? (do most of these viruses even have much geographic metadata available to be able to perform such a thing?)

As the reviewer points out, the potential issue here is that population structure can influence the estimation of a rate of adaptation. In particular, if a virus is actually composed of multiple geographically – or ecologically – persistent separate lineages, sweeping adaptive mutations occurring on one or both lineages will be obscured and will appear to this analysis as stable intermediate level polymorphisms, which are taken to be neutral. It is for this reason that we analyze viruses at the finest lineage/genotype level in the literature. Nevertheless, it is possible that some population structure exists within these finer-level lineage groupings. In the case that population structure is hiding ongoing adaptive evolution, we would expect to see high levels of amino acid substitution as substitutions accumulate in both lineages but a low rate of adaptive evolution as measured by our modified MK test. However, as shown in Supplementary Figure S3, we observe that all viruses with low rates of adaptive evolution also have lower rates of amino acid substitution. We have added an explanation of this to the Discussion, which now reads:

Relatedly, it is important to note that this method looks for fixations and near fixations, with the idea that positively-selected mutations will sweep through the population. This means that mutations that fix within a clade, but not the entire population, will not be considered potentially-adaptive and, thus, that this method is sensitive to how lineages are designated. For instance, if all influenza B viruses were analyzed together, rather than as separate B/Vic and B/Yam lineages, there would be no signal of adaptation in

the HA surface protein. In some cases it can be difficult to define what constitutes two distinct lineages versus two clades of the same lineage. In our analyses, we have divided each viral species into the lineage or genotype classifications used by the field of literature for that virus. As noted above, if a virus is comprised of multiple geographically – or ecologically –- distinct lineages, sweeping adaptive mutations occurring on one or both lineages will be obscured in our analysis as they will appear to be persistent polymorphisms. However, in such a case, we would still expect to see a high absolute rate of amino acid substitution as separate lineages would each accumulate substitutions. Here, all of the viruses with low rates of adaptation also have low rates of amino acid substitution (Figure S3), indicating that we are not missing adaptively-evolving viruses due to population structure.

In response to this query, we have also calculated F_{ST} at a geographic-region level (Africa, Asia, Europe, North America, Oceania, South America) for each virus (Review Table 1), where possible. To do this, we compute pairwise nucleotide distances between all contemporaneous tips from within the same region (π_w), and from between different regions (π_b). We then calculate F_{ST} as $(\pi_b - \pi_w)/\pi_b$, as done in [1]. We computed F_{ST} using two different definitions of contemporaneous sequences: those sampled within 5 years of each other, or within 1-year of each other (the shortest resolution the data allows). We were unable to compute F_{ST} for mumps, where the data is only from North America, and the influenza C, where the data contains only a couple years with sequences from multiple regions and so most time points give a divide-by-zero error.

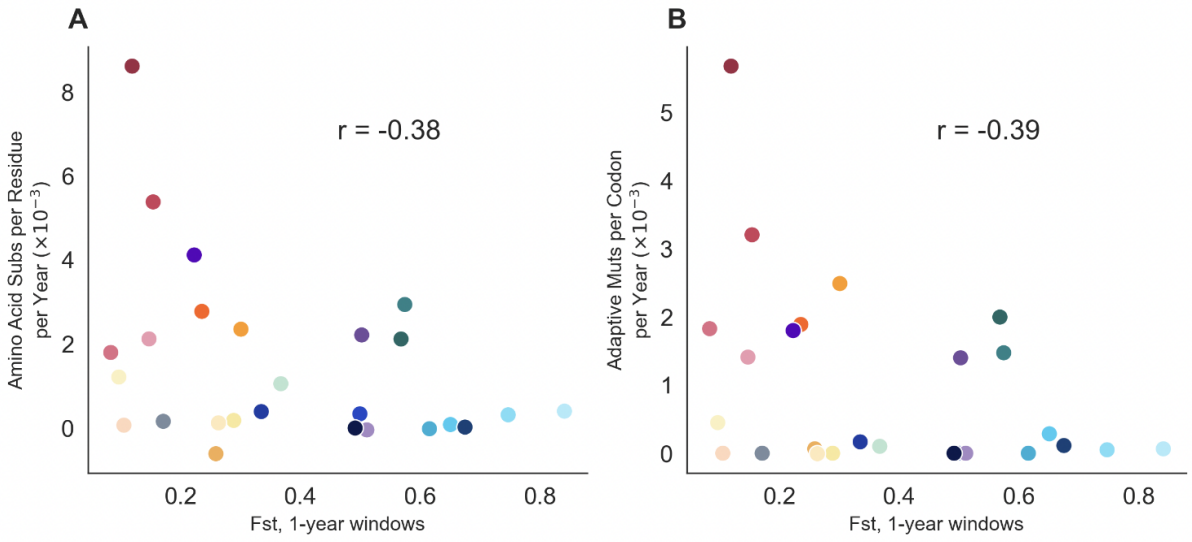
1. Bedford, Trevor, Sarah Cobey, and Mercedes Pascual. 2010. “Global migration dynamics Underlie Evolution and Persistence of Human Influenza A (H3N2).” *Plos Pathogens* 5 (6): e1000918.

We observe a fair spread of F_{ST} values across the 28 viruses, with some that resemble seasonal influenza in showing little geographic structure (parainfluenza-1, hepatitis B-A2, parvovirus B19-1A) and others that show more geographic structure (seasonal CoVs OC43-A and 229E, dengue). If we compare population structure via F_{ST} to estimated rate of adaptive evolution, we observe a negative correlation with Pearson coefficient of –0.39 (Review Figure 1B). However, we might generally expect that in viruses with greater rates of adaptive evolution, new variants will be appearing and spreading, both in frequency and in geographic extent. Thus it seems possible to explain this observed negative correlation either as: (1) artifactual due to population structure obscuring adaptive evolution as measured by our modified MK test, or (2) a real effect due to adaptive evolution erasing population structure.

As above, we can distinguish between hypotheses (1) and (2) by comparing the rate of adaptive evolution as measured by modified MK test to the overall rate of amino acid evolution (Review Figure 1A). Here we see the same strength of negative correlation between adaptive mutations and F_{ST} (Review Figure 1B) as we see for overall amino acid mutations and F_{ST} (Review Figure 1A). This suggests that causality goes in the direction of adaptive evolution diminishing population structure and that our adaptive evolution estimates are robust to the presence of geographic population structure.

Virus	Fst (5-year window)	Fst (1-year window)
Influenza A/H3N2	0.026	0.119
Influenza A/H1N1pdm	0.061	0.154
Influenza B/Vic	0.029	0.083
Influenza B/Yam	0.0364	0.147
Influenza C/Yamagata		
RSV-B	0.179	0.301
RSV-A	0.248	0.235
Parainfluenza-1	0.144	0.097
Measles	0.218	0.259
Mumps		
Parainfluenza-3	0.205	0.289
Dengue 2-AA	0.512	0.65
Dengue 4-II	0.78	0.841
Dengue 3-III	0.638	0.747
Dengue 1-V	0.494	0.615
OC43-A	0.304	0.568
229E	0.422	0.574
NL63	0.232	0.367
Rotavirus A/P[8]	0.207	0.335
Rotavirus A/P[4]	0.335	0.435
Norovirus GII.4	0.129	0.223
Enterovirus D68	0.495	0.502
Hepatitis A-IA	0.401	0.511
Hepatitis B-A2	0.093	0.105
Hepatitis B-D3	0.147	0.263
Parvovirus B19-1A	0.267	0.171
Adenovirus B-7	0.248	0.675
Adenovirus B-3	0.147	0.491

Review Table 1: F_{ST} values for each virus, computed used contemporaneous sequences sampled within 5-years of each other, or 1-year of each other.



Review Figure 1: Scatterplot of FST vs rate of amino acid substitution (A), or adaptive evolution (B). Each point is one virus, colored according to the colors used in the manuscript Supplementary Figure 3. Correlation coefficient for each plot is labeled.

3. In perusing the interactive visualization, which is quite helpful (https://blab.github.io/atlas-of-viral-adaptation/), it is interesting that the highest signal of adaptive evolution is not always from receptor binding domain or receptor binding domain containing genes for a given species (take all the Dengue clades/serotypes as an example). Do you have hypotheses for why other loci might have higher adaptive signal than RBDs? The web tool currently displays the test statistic on the y axis for the RBD or RBD-containing gene, with additional info in a mouseover hover box. Would it be possible to also show the rank-order of the RBD amongst all genes of that species in that hover box? Or some similar way to convey how often the behavior of this statistic is aligning with the core hypothesis/premise of this paper—antigenic evolution is the only explanation the authors offer for elevated test statistics.

We have added the genome-wide rank of the rate of adaptation in the receptor-binding protein or subunit to the hover box that appears on the main page of the website. As the reviewer points out, there are many viruses for which the receptor-binding gene or subunit does not have the fastest rate genome-wide. This is true of 13/18 of the viruses we predict to not be undergoing antigenic evolution. However, in none of these viruses do *any* of the genes exceed the threshold of continuous adaptive evolution that we estimated. In other words, we are not confident that the non-zero rates observed in these genes actually signify ongoing phenotypic change, and rather, we support the hypothesis that there is some noise to these rate estimates (especially for smaller datasets) and that no genes in these viruses are continually evolving adaptively.

4. I have been told that there is some lack of consensus for which gene in RSV A/B encodes the RBD (PMC8913501 suggests it is the F protein, but the test statistic the authors employ are consistent with G).

This is a good point. We have added a Methods section entitled “Genes analyzed for each virus” and accompanying Table 1 to clarify, for each virus, exactly which gene we are analyzing as the receptor-binding gene and the polymerase gene, along with a list of all other genes analyzed in the genome-wide analysis presented in Figure 3. We have also made a note in this section of the Methods that F is used for viral attachment to some receptors.

> *Table 1 shows all genes used in the genome-wide analysis presented in Figure 3 and indicates their classification as receptor-binding, polymerase, surface-located (but not receptor-binding), and non-surface-located (but not polymerase). For some viruses, multiple proteins have been reported to have receptor-binding capacity in different strains or circumstances. For instance, influenza NA [57, 22] and RSV F [14] proteins have been shown to bind receptors in some contexts. In these cases, we analyzed the canonical or primary in vivo receptor-binding protein. Viruses are listed in the order they appear in Figure 4.*

5. SARS-CoV-2 is treated specially here, and it makes sense that the time span of available data is nowhere near the 12 year minimum desired by the authors. That said, if a more customized analysis is being performed for it anyway, perhaps a discretized, non-sliding time window approach might work and the modified-MK test could be applied as before? For example, you could define four non-overlapping time windows corresponding to the time of dominance of any particular variant (e.g. Omicron-era, Delta-era, Alpha-era, pre-Alpha/Wuhan-era) and its outgroup could simply be the genotype of the VoC that preceded it (for Omicron, it would be Delta). This would allow for the basic MK idea of measuring "variation within vs variation between" on a SARS-CoV-2 lineage level.

We agree with the reviewer that a detailed study of evolutionary dynamics of SARS-CoV-2, including the pace and tempo of the conversion of polymorphism into divergence, would be very interesting. However, applying a McDonald-Kreitman-based analysis to distinct, competing lineages of SARS-CoV-2 violates some of the assumptions of neutrality made by the test. When parsing the data this way, mutations that appear as mid-frequency polymorphisms will often not be neutral, and thus the null hypothesis of the test will not be valid. Given this, analyzing the evolutionary dynamics during periods of dominance by different VOCs would require development of different methodology, putting it outside the scope of this paper, which intends to focus on the longer-term evolution of endemic viruses.

6. The discussion ends on the note of SARS-CoV-2 and the general question of the difference between a rapid, post-zoonosis period of host-adaptation-driven evolution (as the first few years of SARS-CoV-2 demonstrated) vs steady-state antigenic evolution of a long endemic virus. It begs the question of how to quantitatively discern between the two (is there a statistic that can tell us when viral evolution has transitioned from primarily one to the other?). While clearly

outside the scope of work that should be done within this manuscript, it would be worth some imagination by the authors in the discussion section as a possible direction for future work.

One could imagine discerning between the period of initial post-zoonosis adaptation and the ensuing period of ongoing antigenic evolution using the McDonald-Kreitman method we use throughout this paper by looking for an inflection point in the accumulation of estimated adaptive mutations. This can obviously only be done for viruses where we have sequenced samples spanning back to that initial period after zoonosis, such as influenza viruses H3N2 and H1N1pdm. If we examine the plot of adaptive mutations over time for these viruses (as shown in Figure 1C for H3N2, and Review Figure 2 below for both viruses), there is no obvious inflection point that exceeds the noise of the data. Further, if we calculate the rate of adaptation within the first 3 years (Review Figure 2 below, yellow line) versus all time points after the first 3 years (Review Figure 2 below, green line), we see that the H3N2 rate does not vary substantially over time and that the H1N1pdm rate is initially lower, but likely due to noise in the data. This analysis suggests that the initial rate of post-zoonosis adaptation is similar, or slower, than the subsequent period of antigenic drift, at least for these two influenza viruses. It is hard to say how this finding would extend to all viruses as the exact epidemiological circumstances of the virus spillover, how rapidly and extensively it spreads initially, and whether or not there is any prior immunity will be highly important.

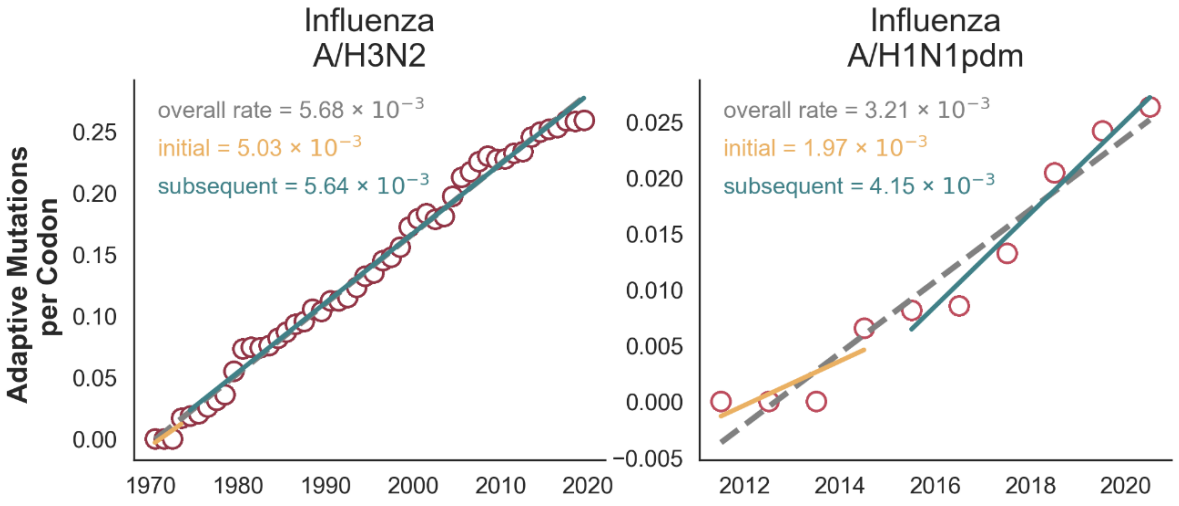
As an alternate approach to addressing this question, we extended the analysis of SARS-CoV-2 from Figure 5 of the manuscript to look at the accumulation of mutations at ACE2 contacts (potential sites of post-zoonosis adaptation) versus at known antibody escape sites. Our idea is that initial optimization to human ACE2 should appear as an early spike in mutations at sites in S1 that contact ACE2 and should subsequently level off (or at least slow down); on the other hand, adaptive mutations at antibody escape sites should not exist initially, but should ramp up as population immunity increases. However, our simple analysis does not bear this out (Review Figure 3 below). While the rate of mutation accumulation at escape sites (given by the slope) does increase over time as expected, we do not see an interpretable trend among ACE2-contacting residues.

This analysis is complicated and weakened by many factors such as: 1) many sites of immune escape occur at ACE2 contacts, 2) escape mutations are dependent on the genetic background they occur on and the circulating immunity at the time they are circulating, and we take neither factor into account in this analysis, 3) fitness effects of different mutations are not equal and it seems highly likely that initial human-adaptive mutations gave especially high fitness boosts while later mutations conferred only marginal gains– a simple tally of mutation accumulation does not account for this, 4) many initial post-zoonosis adaptations were outside of spike.

A more thorough analysis of the dynamics of SARS-CoV-2 evolution over time would likely need to be highly informed by experimental data on the phenotype of individual mutations. Because of the inescapable overlap between receptor-binding functionality and sites of antibody binding, we cannot tell from the sequence data alone what the biological functions of these mutations are.

We've added the following sentences to Discussion to highlight this avenue of research

Additionally, this suggests that overall rate of adaptation is not a simple proxy for initial post-spillover host adaptation vs longer-term continued antigenic drift. Future work on SARS-CoV-2 examining phenotypic effects of spike mutations on ACE2 binding vs immune escape and their adaptive impacts could distinguish crossover from initial host adaptation to later continued antigenic drift.



Review Figure 2: The estimated number of adaptive mutations per codon of HA1 are plotted over time for influenza A/H3N2 (left) and influenza A/H1N1pdm (right) in red circles. The overall rate of adaptation (gray) is the slope of the linear regression for all timepoints. The initial rate of adaptation (yellow) is found from the first 3 years, and the subsequent rate of adaptation (green) is determined from all timepoints after the initial 3 years. Note that in the H3N2 plot, the initial and subsequent rate lines overlay, and thus, obscure the overall rate line.

Review Figure 3: Top row shows the accumulation of amino acid substitutions over through the entire Receptor-Binding Domain (RBD, left, red), among sites that contact ACE2 (middle, yellow), or at sites that have been identified to be involved in antibody escape (right, green). Bottom row shows the slope, or rate of mutation accumulation within 4 month windows. ACE2 contacts were determined by Lan et al, 2020 [1]. Escape sites were determined from Deep-Mutational Scanning data by Greaney et al [2].

1. Lan, Jun, Jiwan Ge, Jinfang Yu, Sisi Shan, Huan Zhou, Shilong Fan, Qi Zhang, et al. 2020. “Structure of the SARS-CoV-2 Spike Receptor-Binding Domain Bound to the ACE2 Receptor.” *Nature* 581 (7807): 215–20.
2. Greaney, Allison J., Tyler N. Starr, and Jesse D. Bloom. 2022. “An Antibody-Escape Estimator for Mutations to the SARS-CoV-2 Receptor-Binding Domain.” *Virus Evolution* 8 (1): veac021.

Reviewer #2: Kistler and Bedford address a highly relevant topic in their investigation of the continuous antigenic evolution of 28 human viruses across 10 viral families. They present a comprehensive survey of the adaptation rates across the viruses and their genes, identifying 10 viruses that undergo antigenic evolution. Additionally, they compare the overall rates of amino acid fixation and demonstrate that the antigenic evolution of SARS-CoV-2 is considerably faster than that of other human viruses.

To achieve this, they build upon the well-established McDonald-Kreitman test by introducing modifications that allow for the estimation of adaptation rates in viral genomes. The paper is well-written and clear. The paper enhances our understanding of the continual antigenic evolution of endemic human viruses. The presentation of these results as an interactive plot is also fantastic.

I have two relatively minor concerns:

It is crucial to clearly distinguish continual antigenic evolution from other forms of adaptation early on in the paper. Currently, this is only discussed in the later part of the Discussion on page 14, stating, "In this study, we have focused on continuous antigenic evolution within viral lineages over the past ~50 years. It is important to note that this is a very particular type of

evolution in which antigenic variation is selected for repeatedly, leading to selective sweeps within a single lineage." I also suggest modifying the title to include the term 'continuous' to reflect this focus.

As the reviewer suggests, we have modified the title of this paper to be “An Atlas of Continuous Adaptive Evolution in Endemic Human Viruses”. We have also edited a few sections of the text to clarify the type of evolution we are analyzing, as the reviewer requested. The edited sentences are:

1. In the introduction: “Here, we aim to survey the potential for antigenic evolution or other continuous adaptive evolution across a broad diversity of endemic human viruses.”
2. In the first section of the Results (“An extension of the McDonald-Kreitman method for estimating rates of adaptation in viral genomes”), where we explain the method used for calculating rates of adaptation:

> *To identify endemic human viruses that are evolving antigenically, we calculated rates of adaptation across the genomes of a wide diversity of viruses using an extension of the McDonald-Kreitman test (McDonald and Kreitman 1991, Williamson 2003). This method divides an alignment of viral sequences into temporal windows, and compares the isolates in each window to a fixed outgroup, which represents the historical genome sequence of that virus (Bhatt et al 2010, Bhatt et al 2011). The number of adaptive mutations in each time window are calculated as an excess of fixed (or nearly fixed) nonsynonymous mutations above the neutral expectation. The rate of adaptation is then computed as the slope of the linear regression fitting adaptive mutations versus time. This temporal aspect means that recurrent fixations, or selective sweeps, over time will yield a high rate of adaptation while a single adaptive fixation will not. Thus, this method is well-suited toward our goal of detecting continuous adaptation, such as antigenic evolution.*

Using the two influenza B virus lineages as an example, the authors note in the discussion that the combined analysis of diversifying lineages can affect their estimates because positively selected mutations may not always sweep through the population. Are the authors confident whether the smaller datasets presented here have adequate global coverage to exclude any diversification or extended regional circulation that might affect the presented estimates?

If a virus is composed of multiple geographically – or ecologically – separate lineages, sweeping adaptive mutations occurring on one or both lineages will be obscured and will appear to this analysis as intermediate level polymorphisms, which are taken to be neutral. In this case where population structure is hiding ongoing adaptive evolution, we would expect to see high levels of amino acid substitution but a low rate of adaptive evolution. However, as shown in Supplementary Figure S3 of the manuscript, we observe that all viruses with low rates of adaptive evolution also have lower rates of amino acid substitution. We have further explanation to the paragraph of the Discussion that the reviewer is mentioning – it now reads:

Relatedly, it is important to note that this method looks for fixations and near fixations, with the idea that positively-selected mutations will sweep through the population. This means that mutations that fix within a clade, but not the entire population, will not be considered potentially-adaptive and, thus, that this method is sensitive to how lineages are designated. For instance, if all influenza B viruses were analyzed together, rather than as separate B/Vic and B/Yam lineages, there would be no signal of adaptation. In some cases it can be difficult to define what constitutes two distinct lineages versus two clades of the same lineage. In our analyses, we have divided each viral species into the lineage or genotype classifications used by the field of literature for that virus. As noted above, if a virus is comprised of multiple geographically- or ecologically-distinct lineages, sweeping adaptive mutations occurring on one or both lineages will be obscured in our analysis as they will appear to be intermediate level polymorphisms. However, in such a case, we would still expect to see a high absolute rate of amino acid substitution. Here, all of the viruses with low rates of adaptation also have low rates of amino acid substitution (Figure S3), indicating that we are not missing adaptively-evolving viruses due to population structure.