Coronavirus Emergence and Evolution: 6+*In vitro* Analysis of Spike Protein Cleavage of SARS-CoV-2, AcCoV-JC34, and Murine Hepatitis Virus

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ABSTRACT

Members of the *Coronaviridae* family of viruses represents a highly diverse group that emerges from a wide range of animal reservoirs. Animal reservoirs may vary, from bats and birds to mammals and rodents. Interestingly, some members of *Coronaviridae* contain a distinct furin cleavage site (FCS) (RXR/KR) at the S1/S2 domain of the spike glycoprotein. The FCS is recognized by the prototypical proprotein convertase, furin, and previous studies show that SARS-CoV-2 contains an FCS, which happens to be atypical in terms of the positioning of the basic (R) residues (RRAR). The FCS can often be found in human, avian and rodent coronaviruses, but are not typically present in SARS-like viruses, including in bat reservoirs. AcCoV-JC34 is a little understood coronavirus (sub-genus *Luchacovirus)* isolated from *Apodemus chevrieri* (Chevrier's field mouse) in Yunnan, China, and is predicted to contain a putative FCS. In this study, first we determined whether the presence of the FCS on AcCoV-JC34 allows for furin cleavage, as it does for SARS-CoV-2. Next, we analyzed the spike protein cleavage of murine hepatitis virus (MHV) strains using furin, and compared our data to prior studies with the goal of understanding disease pathology in mice. To examine these cleavage events, we generated predicted furin cleavage scores using ProP computer software for the S1/S2 domain of selected coronaviruses. We then utilized a fluorogenic peptide cleavage assay in which linear peptide mimics of the S1/S2 domain of selected coronaviruses were mixed with furin protease and buffwe. From the data, we obtained the comparative activity of the protease. We found that although AcCoV-JC34 appears to have an appropriate series of amino acid residues for furin cleavage, our data suggests that it is not cleaved by furin. Further, for MHV, furin cleavage is strain-dependent and can vary across pH values. Overall, this work informs a study of coronavirus evolution, emergence, and pathogenesis with respect to the S protein.

MATERIALS AND METHODS:

Prediction of furin cleavage via ProP Software

First, to predict whether furin cleavage occurs at the S1/S2 domain of the S protein, we use ProP computer software as discussed in Duckert et al. (2004). According to Duckert et al. (2004) a score greater than 0.5 suggests moderate cleavage by furin protease. Using data from NCBI of the S1/S2 domain of selected coronaviruses, within the ProP computer software we recorded the series of amino acid residues of the S protein for coronaviruses under investigation, and then received a furin cleavage score.

Fluorogenic Peptide Cleavage Assay

The fluorogenic peptide cleavage assay, along with furin and trypsin buffer dilutions, were conducted essentially as in Jaimes et al. (2017). First, we designed and ordered linear peptides that mimic the S1/S2 domain of the S glycoprotein for selected coronaviruses (Biomatik, Kitchener, Ontario). Information about the series of amino acid residues within the S1/S2 domain were obtained from NCBI, by searching for the accession numbers/complete genomes of AcCoV-JC34, SARS-CoV-2, MHV-1, MHV-2, MHV-3/MHV-JHM, MHV-S, and MHV-A59, followed by a multiple sequence alignment**.** Each of the peptide mimics used contained a fluorescence resonance energy transfer (FRET) pair, 7-methoxycoumarin-4-yl-acetly (MCA) and 2,4-dinitrophenol (DNP), on the N-terminus and C-terminus ends of the peptide. MCA is a light sensitive molecule whose light is quenched in the presence of DNP. However, if a protease recognizes a site on the peptide, it is able to cleave it, and upon cleaving DNP no longer quenches MCA's light and that signal is detected by a fluorescent plate reader. From this signal, we acquire information about the activity of the protease, and can do a comparison between peptides within the same biological replicate. For this experiment, the comparison of

SARS-CoV-2 and AcCoV-JC34 was completed with three biological replicates, and three technical replicates within one biological replicate. For the MHV peptides three biological replicates were completed for each of the furin buffer pH values (four total) under investigation. The pH of the buffer solution was determined using a pH meter.

In a 96-well plate, each well contained one of our selected S1/S2 peptides mixed in a solution of diluted protease with its buffer. The proteases used in this experiment includes the prototypical proprotein convertase, furin, and the prototype serine endopeptidase trypsin (Both ordered from New England BioLabs). In order to ensure that the assay is working optimally, we used trypsin protease as a positive control because it is not as selective in terms of substrate recognition within the S protein of coronaviruses (Millet & Whittaker, 2014). Next, the 96-well plate is placed within a fluorescence plate reader, set at 37 degrees Celsius to measure cleavage, and then monitored by an increase in fluorescence based on the separation of the FRET pair for one hour.

Similar to Jaimes et al. (2017), from the fluorescent plate reader, data was saved as a .txt file, and analyzed within Excel (Microsoft). We averaged three technical replicates together, to obtain a graph where relative fluorescent units were plotted on the y-axis and time on the x-axis. From the slope of the graph, we obtained the Vmax of furin and trypsin protease, and compared the activity of furin and trypsin cleavage for each peptide mimic within one biological replicate. Further, three biological replicates were averaged together for the Vmax of the protease as well.

Chapter 1: *A comparison of AcCoV-JC34 and SARS-CoV-2 Furin Cleavage Site* [1]

As evident by the current COVID-19 pandemic, which has resulted in the death of over 6,000,000 people globally, members of the *Coronaviridae* family of viruses represents an emerging public health threat (John Hopkins University, 2022). While there is debate whether severe acute respiratory syndrome-2 (SARS-CoV-2) (a betacoronavirus) emerged via a zoonotic transfer from a *Rhinolophus affinis* bat to humans, the actual source of the pandemic remains elusive. Even the World Health Organization suggests that another intermediate host may be responsible (World Health Organization, 2020). The COVID-19 pandemic is not the first time a coronavirus has "jumped" from animals to humans and caused widespread illness and disease. For example, the epidemic from 2001 caused by SARS-CoV-1 could have emerged from *Rhinolophus* bats or civets; and Middle Eastern Respiratory Syndrome (MERS-CoV) of 2012 is believed to have arisen from camels (Wang & Eaton, 2007; Han et al. 2016).

A way that coronaviruses establish themselves within the human population is through zoonotic transmission events from animal reservoirs. There are two conditions that may give rise to a zoonotic transmission event: 1) Humans being within close proximity or consuming animals, and 2) The accumulation of mutations within the virus that allows it to jump from host to host. Particularly for coronaviruses, mutations in the spike (S) protein are concerning because even the slightest change in an amino acid residue can lead to an expansion in host range and/or increased pathogenicity (Millet & Whittaker, 2014). Further, as infected individuals spread the virus to others hosts or potential reservoirs, there is a higher probability in the emergence of variants of

[1] Data and Figure 1 of Chapter 1 as seen in bioRxiv in 2021 by Choi, A., Singleton, D. T., Stout, A. E., Millet, J. K., & Whittaker, G. R. (2021). In vitro and computational analysis of the putative furin cleavage site (RRARS) in the divergent spike protein of the rodent coronavirus AcCoV-JC34 (sub-genus luchacovirus). *BioRxiv*, 2021.12.16.473025. https://doi.org/10.1101/2021.12.16.473025

concern. This leads researchers to ask, how does a mutation in the coronavirus S protein affect cell or tissue tropism? And as mutations continue to accumulate in the virus and viruses continue to spread, which animals may act as newly emerged reservoirs?

Furin is a member of the proprotein convertase family of proteases, and is essential for viral entry of coronaviruses. Furin cleaves the S1/S2 domain of the spike (S) protein which will lead to fusion of a virus particle with the host cellular membrane. For SARS-CoV-2, previous literature shows that furin recognizes a specific series of amino acid residues at a furin cleavage site (FCS). However, as mentioned by Stout et al. (2021) the RRAR sequence is atypical for a human coronavirus, and within the *Coronaviridae* family this specific sequence appears is shared with rodent *Luchacovirus* AcCoV-JC34; which was identified in Yunnan Province, China (Ge et al., 2017). The appearance of the FCS in both human coronavirus SARS-CoV-2 and rodent coronavirus AcCoV-JC34 may provide support for a mechanistically-shared spillover potential where, due to the accumulation of mutations in the S protein from a similar animal reservoir, both S proteins are cleaved by the same protease to mediate viral entry.

The aim of Section 1 of this thesis was to examine and compare proteolytic cleavage by furin using peptides mimicking the cleavage site of AcCoV-JC34 and SARS-CoV-2. Additionally, we tested cleavage by trypsin as a positive control. Due to previous studies suggesting the presence of a putative FCS within AcCoV-JC34's S1/S2 domain, we believed that AcCoV-JC34 is cleaved by furin in a similar fashion to how SARS-CoV-2 is cleaved by furin.

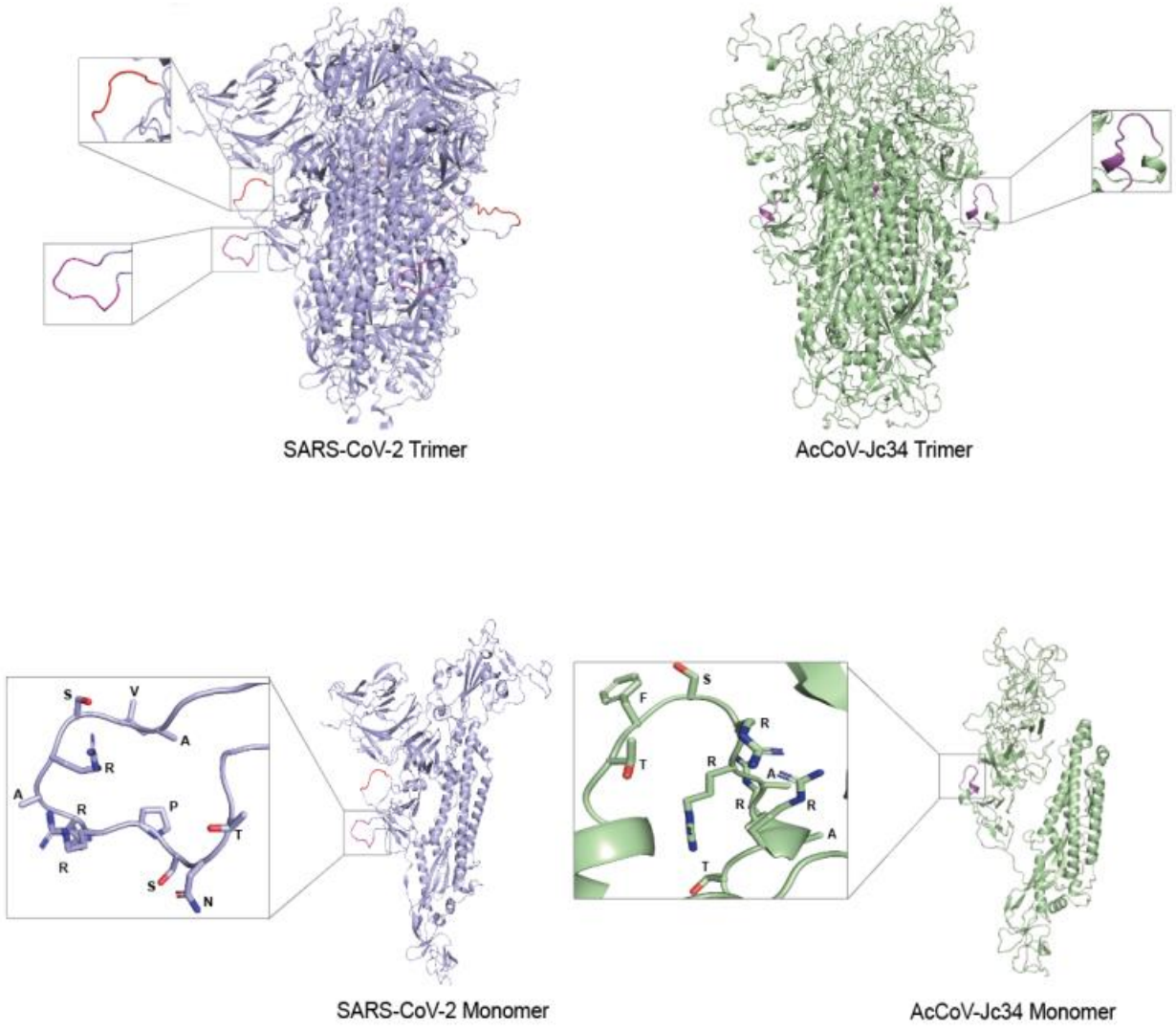

Figure 1. Predicted Structure of SARS-CoV-2 and AcCoV-JC34 S Protein. As shown in Choi et al. (2021), the above figure depicts the structural models of the Spike (S) protein for SARS-CoV-2 and AcCoV-JC34. As observed in the zoomed-in image, both S proteins contain the RRAR sequence that is recognized by furin protease.

VIRUS	S1/S2 SEQUENCE	PROP SCORE
SARS-COV-1	AGICASYHTVSLLR|STSQKS	0.12
SARS-COV-2	ASYQTQTNSP**RRAR**|SVASQS	**0.62**
UKRN3	CDSTDVTTFMTKAR|ATTFVD	0.12
ACCOV-JC34	VVTFS**RRAR**|ARTLT	0.28

Figure 2. CoV Sequences of S1/S2 Domain and Furin Cleavage Scores. Sequence analysis of select Coronavirus Spike proteins were generated using ProP 1 software. (|) denotes the position of the predicted S1/S2 cleavage site. The bolded series of amino acid residues containing Arginine (R) and Alanine (A) represents the FCS that is recognized by furin to mediate cleavage.

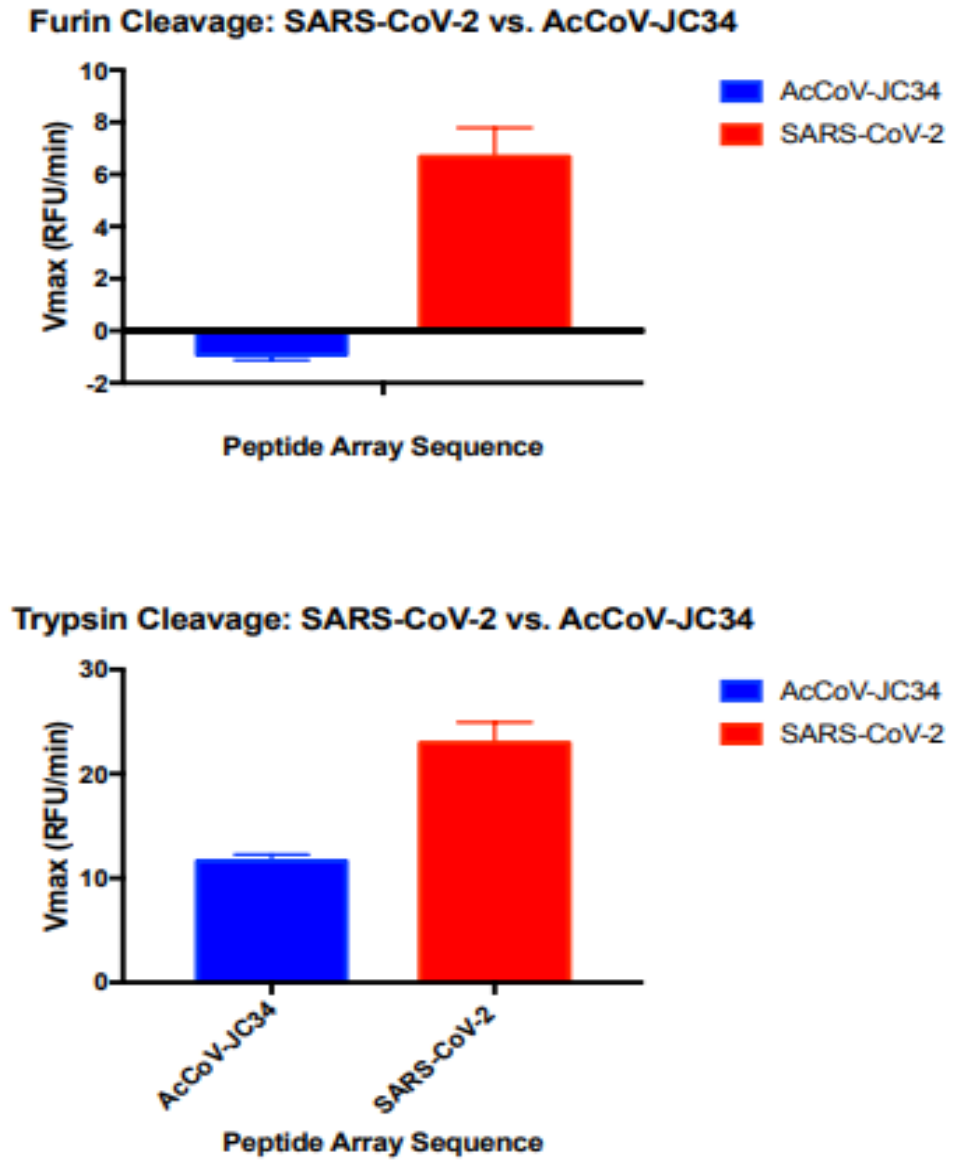

Figure 3. Activity of Furin Cleavage on CoV Peptide Mimics. The figure above depicts the activity of furin and trypsin protease when mixed in buffer with SARS-CoV-2 WT and AcCoV-JC34 linear peptide mimics of the S1/S2 site. Furin protease cleaved only SARS-CoV-2, while trypsin protease cleaved both peptides.

Results and Conclusions I: Furin Cleavage of SARS-CoV-2 and AcCoV-JC34

As mentioned previously, in the Materials and Methods section, Duckert et al. (2004) indicate that a ProP score above 0.5 is the threshold value of furin cleavage. In figure 1, the predicted ProP score of 0.62 for SARS-CoV-2 suggests that it is moderately cleaved by furin. AcCoV-JC34 is weakly predicted as cleaved by furin with a ProP score of 0.28. As a comparison, SARS-CoV-1 and UkRN3 (closely related to SARS-CoV-2 and AcCoV-JC34 respectively) have a shared low furin cleavage score of 0.12.

Further, figure 3 shows the average activity of furin and trypsin protease with AcCoV-JC34 and SARS-CoV-2. In terms of furin cleavage, SARS-CoV-2 was cleaved by furin while AcCoV-JC34 was not. For SARS-CoV-2, the Vmax value was 6.68 RFU/min and for AcCoV-JC34 it was -0.91 RFU/min. Keeping in mind that both SARS-CoV-2 and AcCoV-JC34 contain the same series of amino acid residues within the S1/ S2 site that furin recognizes, RRAR, this result is surprising. Especially, since ProP predicts that AcCoV-JC34 should be weakly cleaved by furin. With trypsin, both AcCoV-JC34 and SARS-CoV-2 showed cleavage. We tabulated Vmax values as 11.63 RFU/min for AcCoV-JC34 and 22.96 RFU/min for SARS-CoV-2. When considering that trypsin has non-specific recognition of the S1/S2 domain for cleavage, and prefers at least a single arginine residue within the S1/S2 domain to cleave, this result is expected (Millet & Whittaker, 2014).

Overall, we wanted to determine whether cleavage in the S1/S2 domain of SARS-CoV-2 is similar to that of AcCoV-JC34. As mentioned by Stout et al. (2021), for coronaviruses while the receptor binding domain can inform host tropism and be revealing of a spillover event, the recognition of host cell proteases to mediate viral entry is increasingly becoming apparent as evidence for these factors as well. AcCoV-JC34 is noted as having a distinct spike protein compared to other alphacoronaviruses, and we aimed to investigate whether this uniqueness is due to the presence of a FCS (Stout et al. 2021). For SARS-like human coronaviruses, a FCS is atypical. Interestingly, SARS-CoV-2 contains a FCS within its S protein, and is hypothesized to have emerged from bats due to genome sequence similarities (Stout et al., 2021). However, we believed that if the predicted FCS on AcCoV-JC34 is cleaved by furin similar to how furin cleaves SARS-CoV-2, then those results may provide support of a spillover event from rodents.

However, the data suggests that AcCoV-JC34 is not cleaved by furin despite having what appears to be an appropriate series of basic amino acid residues.

Chapter 2: *Cleavability of MHV Strains by furin and The Histidine Switch*

Murine Hepatitis Virus (MHV) is a betacoronavirus often used as a model to study the entry-level mechanisms in coronavirus biology (Millet & Whittaker, 2014). MHV exists as different strains that range in their virulence and carry different organotropisms (Körner et al. 2020). For example, MHV-1 and MHV-S are described as less virulent compared to strains MHV- 2, MHV-3, and MHV-A59; while MHV-JHM is neurotropic (Körner et al. 2020). Similar to SARS-CoV-2, within the S protein, MHV strains contain an RXXR or XRAR sequence (X denoting any amino acid), suggesting that it too may contain a FCS and is cleaved by furin. If similar to SARS-CoV-2, MHV strains are cleaved by furin, then that may provide further insight into how rodents may act as a reservoir for newly emerging human coronaviruses like SARS-CoV-2 and its variants (Stout et al., 2021). Additionally, due to carrying differing disease pathologies and a range in their virulence we tested five different strains of MHV.

In section 2 of this study, we examined cleavage by furin of five different strains of MHV. Similar to section 1, this was carried out using ProP computer software to generate predicted furin cleavage scores, and a fluorogenic peptide cleavage assay to assess cleavability by proteases. We believed that based off of ProP scores that moderate furin cleavage should occur for strains MHV-1, MHV-JHM/MHV3*, and MHV-A59. Additionally, furin cleavage should be minimal or absent for MHV-2 and MHV-S. Further, we assessed furin cleavage at different pH values to investigate whether pH would have an effect on the activity of furin cleavage and whether neighboring amino acid residues cause a ‘Histidine Switch’. Previous studies show that the presence of Histidine within the glycoproteins of viruses can affect the

conformational changes the glycoprotein undergoes during viral entry in a pH-dependent manner (Kalani et al., 2013). Further, other studies with SARS-CoV-2 variants have looked at the role of lowering pH to observe the effects of Histidine's ionizable side chain which has a pKa close to neutral (Lubinski et al. , 2021; Nelson and Cox, 2000). Thus, when considering that our selected MHV strains contain furin cleavage sites with a neighboring upstream Histidine residue, this led us to test pH values within a range of 5.5, 6.3, 6.8, and 7.8.

*Strains MHV-JHM and MHV-3 have the same amino acid sequence in the S1/S2 domain.

VIRUS	S1/S2 SEQUENCE	PROP SCORE
MHV-1	STSH**RARR**|STS	**0.84**
MHV-2	STS**HRAR**|SSVS	0.21
MHV-JHM/MHV-3*	SKS**RRAR**R|SVS	**0.87**
MHV-S	STA**HRAR**|TSVS	0.10
MHV-A59	SKS**RRAH**R|SVS	**0.77**

Figure 4. MHV Sequences of S1/S2 Domain and Furin Cleavage Scores. * Strains MHV-JHM and MHV-3 have the same amino acid sequence in the S1/S2 domain.

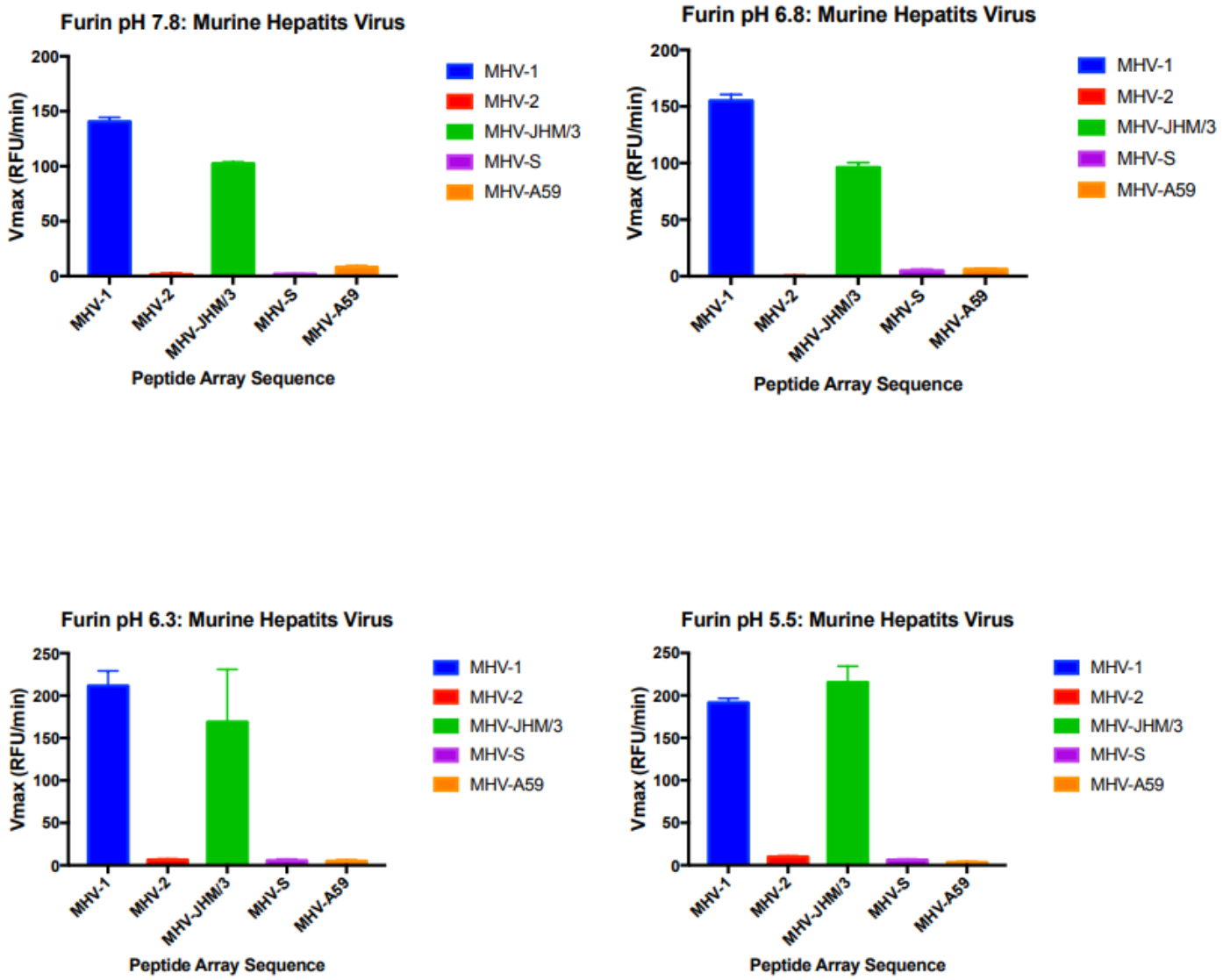

Figure 5. Activity of Furin Cleavage on CoV Peptide Mimics. The figure above depicts the activity of furin protease when mixed in its buffer with linear peptide mimics of murine hepatitis virus strains. The data shows that across pH, MHV-1 and MHV-JHM/MHV-3 are cleaved by furin, while MHV-2, MHV-S, and MHV-A59 are not.

Results & Conclusions II: Furin Cleavage of Murine Hepatitis Virus Strains

As mentioned previously, Duckert et al. (2004) postulate that a ProP score above 0.5 is predictive of moderate cleavage by furin. Figure 4 exemplifies a predicted ProP score of 0.84 for MHV-1, 0.21 for MHV-2, 0.87 for MHV-JHM/MHV-3, 0.10 for MHV-S, and 0.77 for MHV-A59. These results suggest that MHV-1, MHV-JHM/MHV-3, and MHV-A59 are predicted as cleaved by furin. On the other hand, MHV-2 and MHV-S are predicted as not cleaved by furin.

Figure 5 depicts the differing pH values of furin buffer tested when mixed with MHV peptides mimics, and the Vmax for furin cleavage of the MHV strains. As outlined in Jaimes et al. (2019), due to the set-up of the fluorogenic peptide cleavage assay, it is not appropriate to compare cleavability of the protease across strain and pH values, but we can compare cleavability between strains within the same pH value. However, we will note general trends observable for specific MHV strains across pH values. As predicted, MHV-1 and MHV-JHM/MHV-3 showed consistent cleavage by furin irrespective of pH, while MHV-2 and MHV-S showed minimal cleavage. These results were expected, especially because MHV-1 and MHV-JHM/MHV-3 contain the specific series of amino acid residues recognized as the FCS. Surprisingly, MHV-A59 showed minimal cleavage by furin even though its ProP score was greater than the 0.5 threshold. When considering that the amino acid sequence of MHV-A59 within the S1/S2 is RRAH, one may suggest that furin cleavability was affected by the neighboring Histidine. Previous studies have shown that Histidine can cause pH-dependent structural changes for viral glycoproteins, such as with Hemagglutinin of influenza virus, where it can act as a ‘pH sensor’ and alter the helical structure of the glycoprotein (Kalani et al., 2013). When keeping this in mind, the S1/S2 site of MHV-A59 may be more or less exposed due to the presence of the Histidine, and affect cleavability in a pH-dependent manner. This is further supported with the trend of MHV-A59 furin activity increasing with an increase in pH, but once

again one cannot compare across pH. Overall, the data suggests that furin cleavage of MHV is strain-dependent and can vary across pH values. Additionally, MHV-A59 although predicted as cleaved by furin protease is minimally cleaved.

DISCUSSION

Overall, we had two aims: 1) To examine and compare proteolytic cleavage by furin using peptides mimicking the S1/S2 domain of AcCoV-JC34 and SARS-CoV-2 and 2) To analyze the cleavability by furin protease of peptides mimicking the S1/S2 domain for five MHV strains. With respect to Aim 1, AcCoV-JC34, despite having an appropriate series of amino acid residues, is not cleaved by furin similar to SARS-CoV-2. It is unclear as to why AcCoV-JC34 is not cleaved by furin. But one may propose that the neighboring amino acid residues around the FCS may be hindering furin's ability to recognize it. As mentioned in Choi et al. (2021), upstream of the FCS in SARS-CoV-2 is a Proline that may create a structural turn allowing better access for furin to access the site compared to AcCoV-JC34. Additionally, in AcCoV-JC34, there exists an arginine residue downstream that may prevent furin from recognizing the FCS due to its tight binding pocket (Choi et al. 2021).

With respect to Aim 2, cleavability by furin was not dependent on the relative virulence of the MHV strains. Although MHV-A59 is considered more virulent, furin minimally cleaved the peptide mimic sub-threshold compared to MHV-1 that is considered less virulent which furin cleaved moderately. These results may suggest that other aspects of the viral genome are responsible for the increased virulence of MHV-A59 compared to MHV-1. Further, as mentioned above MHV-A59 is minimally cleaved by furin, which was an unexpected result given that it had a moderately predicted FCS. Within the S protein of MHV-A59, the FCS contains a RRAH sequence rather than the RRAR sequence seen in MHV-1 and MHV-

JHM/MHV-3. We predict that the Histidine is inhibiting furin's ability to recognize the FCS for MHV-A59 due to the "Histidine switch". However, our study does not definitively conclude this, and this lack in cleavability by furin may be the result of other neighboring amino acid residues upstream or downstream.

Limitations of this study Lastly, as outlined in Jaimes et al. (2017) the linear peptide mimics used in this study do not reflect the true tertiary structure of the spike glycoprotein of coronaviruses in vivo. Thus, for the unexpected results (such as AcCoV-JC34 and MHV-A59) further analysis may require a cell-fusion assay along with western blot analysis to determine if furin truly cleaves the S protein in its tertiary structure.

However, these experiments are a significant first step in analyzing furin cleavage for select rodent coronaviruses. After all, as mentioned in Tsoleridis et al. (2019), alphacoronaviruses, like AcCoV-JC34 and MHV have used rodents as a host for quite some time. And when considering SARS-CoV-2's atypical FCS, while its still up for debate, rodents carry the potential to have acted as an animal reservoir and may be revealing of a spillover event. Further, it is possible that the S protein for AcCoV-JC34 and the MHV strains are cleaved more optimally by an alternative protease of at a secondary site within the S1/S2 domain. When considering that furin is part of a family of 7 proprotein convertases, in the future we plan to study other family members related to furin, to compare and contrast spike protein cleavage within the *Coronaviridae* family.

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APPENDIX

Virus	**Velocity of Protease (RFU/min)**	
	Furin	*Trypsin*
AcCoV-JC34	-0.91	11.63
SARS-CoV-2	6.68	22.96

Table 1. Velocity of Furin Cleavage on CoV Peptide Mimics. The table above depicts the velocity of furin and trypsin protease when mixed in buffer with select CoV linear peptide mimics.

MHV Strain	**Velocity of Furin Protease (RFU/min)**			
	pH 5.5	*pH 6.3*	*pH 6.8*	*pH 7.8*
MHV-1	191.5	211.49	155.16	140.53
MHV-2	9.86	6.63	0.07	1.58
MHV-S	6.41	5.66	4.86	1.92
MHV-JHM/MHV-3	215.45	169.02	96.09	102.4
MHV-A59	3.39	5.12	6.29	8.30

Table 2. Velocity of Furin Cleavage on CoV Peptide Mimics. The table above depicts the velocity of furin and trypsin protease when mixed in buffer with select CoV linear peptide mimics.

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