One Health 13 (2021) 100282

Contents lists available at ScienceDirect

One Health

journal homepage: www.elsevier.com/locate/onehlt

Furin cleavage sites in the spike proteins of bat and rodent coronaviruses: Implications for virus evolution and zoonotic transfer from rodent species

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ARTICLE INFO

Keywords:
Coronavirus
Furin
Spike protein
Bats
Rodents

ABSTRACT

Bats and rodents comprise two of the world's largest orders of mammals and the order Chiroptera (bats) has been implicated as a major reservoir of coronaviruses in nature and a source of zoonotic transfer to humans. However, the order Rodentia (rodents) also harbors coronaviruses, with two human coronaviruses (HCoV-OC43 and HCoV-HKU1) considered to have rodent origins. The coronavirus spike protein mediates viral entry and is a major determinant of viral tropism; importantly, the spike protein is activated by host cell proteases at two distinct sites, designated as S1/S2 and S2'. SARS-CoV-2, which is considered to be of bat origin, contains a cleavage site for the protease furin at S1/S2, absent from the rest of the currently known betacoronavirus lineage 2b coronaviruses (*Sarbecoviruses*). This cleavage site is thought to be critical to its replication and pathogenesis, with a notable link to virus transmission. Here, we examine the spike protein across coronaviruses identified in both bat and rodent species and address the role of furin as an activating protease. Utilizing two publicly available furin prediction algorithms (ProP and PiTou) and based on spike sequences reported in GenBank, we show that the S1/S2 furin cleavage site is typically not present in bat virus spike proteins but is common in rodent-associated sequences, and suggest this may have implications for zoonotic transfer. We provide a phylogenetic history of the *Embecoviruses* (betacoronavirus lineage 2a), including context for the use of furin as an activating protease for the viral spike protein. From a One Health perspective, continued rodent surveillance should be an important consideration in uncovering novel circulating coronaviruses.

1. Introduction

The *Coronaviridae* are single-stranded, positive sense, enveloped RNA viruses [1]. They are classified, by genera, as *Alpha-*, *Beta-*, *Gamma-*, and *Deltacoronaviruses* [2]. The *Alpha-* and *Betacoronaviruses* are most often associated with mammalian species, while the *Gamma-* and *Deltacoronaviruses* remain largely avian-associated [3]. Across bat and rodent species, both *Alpha-* and *Betacoronaviruses* have been identified. Members of the *Coronaviridae* are further classified into subgenera. Specifically, in bats, this has included the subgenera, *Setracovirus, Myotacovirus, Rhinacovirus, Colacovirus, Pedacovirus, Decacovirus, Minunacovirus,* and *Nyctocovirus*, from the *Alphacoronavirus* genus, in addition to *Nobecovirus, Sarbecovirus, Merbecovirus* and *Hibecovirus* from the *Betacoronavirus* genus. Coronaviruses from rodents, fall within two subgenera: the *Luchacoviruses* (within the *Alphacoronavirus* genus) and *Embecoviruses* (*Betacoronavirus* genus) [5] (Table 1).

Current classification schemes for the *Coronaviridae* utilize the replicase domain of the ORF1ab gene; however, the spike protein, is a critical mediator of viral tropism and an important contributor to the viruses natural history and potential for spillover [6]. The rodent *Alphacoronaviruses* have previously been shown to form a monophyletic group [7]. The *Embecovirus* subgenera, previously classified as lineage 2a, additionally includes human coronavirus HKU-1, several mammalian coronaviruses such as equine coronavirus and dromedary camel coronavirus HKU23 [8], but lacks any representation of Chiroptera-associated coronaviruses [4]. With discovery of the China *Rattus* coronavirus (ChRCoV) HKU24, Lau and colleagues have provided evidence that rodent coronaviruses are the ancestors to the *Embecovirus* subgenus [4,9]. While bats are frequently implicated as the source of new and emerging coronaviruses, rodents need also to be considered as a

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https://doi.org/10.1016/j.onehlt.2021.100282
Received 15 June 2021; Accepted 16 June 2021
Available online 22 June 2021

Table 1
Coronaviridae subgenera associated with bats and rodents

	Order Chiroptera	Order Rodentia
α CoV	*Colacovirus*	*Luchacovirus*
	Decacovirus	
	Duvinacovirus	
	Minunacovirus	
	Myotacovirus	
	Nyctocovirus	
	Pedacovirus	
	Rhinacovirus	
	Settracovirus	
β CoV	*Hibecovirus*	*Embecovirus*
	Merbecovirus	
	Nobecovirus	
	Sarbecovirus	

potential source of zoonotic spillovers, given previous observations. The natural ability of coronaviruses to recombine adds to the emergence and transmission of new viruses and there are many examples of recombination reported: the prototypic coronavirus mouse hepatitis virus (MHV), an *Embecovirus,* has been noted for its ability to recombine [10,11] and the dromedary camel coronavirus HKU23 appears to have had a recombinant history, based on similarity between portions of HKU23 and RodentCoV-IM2014 [12].

Globally, over 2000 rodent species have been described and encompass a wide range of habitats [13]. Rodents have previously been implicated in transmitting numerous pathogens to humans, including hantavirus, Lassa virus, *Francisella tularensis* and *Yersinia pestis*. In a study involving rodents destined for human consumption, coronaviruses were frequently identified [14]. Among some of the rodents sampled, bat coronavirus 512/2005 and infectious bronchitis virus were identified; although this may not be an indication of active infection, as the authors note, it does provide evidence of ongoing transmission opportunities across animal species [14]. Two coronaviruses of human consequence have been hypothesized to have rodent origins: HCoV-OC43 and HCoV-HKU1, with HCoV-OC43 emerging via a bovine intermediate [15]. Surveillance efforts have recently identified OC-43, and sequences that cluster with OC-43, in a small number of free-living rodent species [16,17]. In addition to zoonotic rodent transmission concerns, Yang and colleagues have hypothesized a potential role of rodents in the emergence of swine acute diarrhea syndrome coronavirus (SADS-CoV) [18]. Other hypotheses regarding SADS-CoV suggest it may have origins with the bat coronavirus HKU2, which, based on the replicase gene, has previously been classified as a *Rhinacovirus*, genus Alphacoronavirus, despite the spike protein resembling that of *Betacoronaviridae* [19]. A virus identified in *Apodemus chevrieri*, AcCoV-JC34 has been considered to have a unique spike protein compared with other alphacoronaviruses, being only 1126 residues and phylogenetically clustering with the bat coronavirus HKU-2 [20]. Coronaviruses in rodents have been identified across several countries and from a number of rodent host species, including *Bandicota savilei, Rattus exulans, Rattus tanezumi, Rattus norvegicus, Rattus argentiventer, Leopoldamys neilli, Rattus andamanensis, Rattus losea, Maxomys surifer, Mus cervicolor, Berylmys berdmorei, Mus caroli, Mus cookie, Niviventer fulvescens, Berylmys bowersi, Dremomys refigenis,* and *Menetes berdmorei* [16,21] (Table 2 and Supplementary Table 1).

The spillover of coronaviruses from animal species to humans has the potential for major global health implications. Following the 2002–2003 severe acute respiratory syndrome coronavirus (SARS-CoV) outbreak, surveillance studies began to recognize the role that bat species play as reservoirs for novel coronaviruses [22,23]. Ten years later, Middle East respiratory coronavirus (MERS-CoV) emerged in humans and clusters phylogenetically with two bat viruses, *Tylonycteris* bat coronavirus HKU4 (BtCoV-HKU4) and *Pipistrellus* bat coronavirus HKU5 (BtCoV-HKU5) [24] in the *Merbecovirus* clade (group 2c coronaviruses). Further investigation identified NeoCoV from South African *Neoromicia capensis*

Table 2
Surveillance studies investigating coronavirus shedding across rodent species.

Species	Location	Time period	# Positive/ Sample size	Ref
Apodemus sp.	France	2014–2016	16/206	[78]
Apodemus agrarius	China	2011–2013	10/444	[5]
	China	2014–2015	1/49	[17]
Apodemus chevrieri	China	2011	21/98	[20]
	China	2014–2015	24/193	[17]
Apodemus ilex	China	2011	1/17	[20]
Apodemus latronum	China	2014–2015	3/6	[17]
Apodemus sylvaticus	United Kingdom	Not given	0/9	[79]
Arvicola terrestris	France	2014–2016	0/35	[78]
Bandicota indica	China	2014–2015	2/5	[17]
Deomys ferrugineus	Democratic Republic of Congo, Republic of Congo	2006–2018	1/1	[80]
Eothenomys cachinus	China	2014–2015	1/1	[17]
Eothenmys fidelis	China	2011	1/62	[20]
Eothenomys miletus	China	2014–2015	3/131	[17]
Field rat (*Rattus* sp. and *Bandicota* sp.)	Viet Nam	2013–2014	239/702**	[14]
Hystrix sp.	Viet Nam	2013–2014	20/331***	[14]
Malacomys longipes	Democratic Republic of Congo, Republic of Congo	2006–2018	1/38	[80]
Micromys minutus	China	2011–2013	0/2	[5]
Microtus sp.	France	2014–2016	0/9	[78]
Microtus agrestis	United Kingdom	Not given	3/11	[79]
Microtus fortis	China	2011–2013	0/305	[5]
	China	2014–2015	0/10	[17]
Mus musculus	China	2011–2013	0/7	[5]
	United Kingdom	Not given	0/394 (Liver) 0/58 (Gut)	[79]
	China	2014–2015	0/1	[17]
Myodes glareolus	United Kingdom	Not given	1/1	[79]
	Poland	Not given	1/300	[79]
	France	2014–2016	5/80	[78]
Niviventer confucianus	China	2011–2013	1/85	[5]
Niviventer eha	China	2014–2015	0/2	[17]
Niviventer fulvescens	China	2010–2012	0/97****	[9]
Niviventer niviventer	China	2014–2015	0/2	[17]
Rattus sp.	Viet Nam	2013–2014	1/1***	[14]
Rattus andamanensis	China	2010–2012	0/170****	[9]
	China	2014–2015	1/1	[17]
Rattus edwardsi	China	2011–2013	0/2	[5]
Rattus lossea	China	2011–2013	14/301	[5]
Rattus losea	China	2014–2015	0/15	[17]
Rattus fulvescens	China	2011–2013	0/4	[5]
Rattus nitidus	China	2014–2015	0/2	[17]
Rattus norvegicus	United Kingdom	Not given	4/95* (Liver)	[79]
	China	2010–2012	3/359****	[9]
	China	2011–2013	4/262	[5]
	China	2014–2015	2/101	[17]
Rattus tanezumi	China	2010–2012	0/9****	[9]
	China	2011–2013	1/53	[5]
	China	2014–2015	2/159	[17]
Rattus rattus	China	2010–2012	0/24****	[9]
Rattus rattus sladeni	China	2014–2015	0/18	[17]
Rhizomys sp.	Viet Nam	2013–2014	6/96***	[14]
Sciuridae sp.	Viet Nam	2013–2014	0/1	[14]

* Authors noted that some animals had both liver and gut samples positive (2).
** 26 of the animals were co-infected; viruses identified were murine coronavirus and Longquan Aa coronavirus.
*** Virus identified were bat coronavirus 512/2005 or infectious bronchitis virus.
**** Specifically tested for HKU24 via RT-PCR.

bats and suggested it as the sister group to the MERS-CoV-camel clade [25]. MERS-CoV involved the dromedary camel as an intermediate host in the spillover to humans [26–28]. The most recent SARS-CoV-2 outbreak has been traced to bat origins, with a high similarity to a previously identified bat coronavirus RaTG13 (96% genome-wide sequence identity) [29], as well as several other bat coronaviruses, RpYN06 (94.5% sequence identity) [30] and bat-SL-CoVZC45 and bat-SL-CoVZXC21 [31]. The role of an intermediate host in the spread of SARS-CoV-2 to humans remains an open area of investigation.

A major factor for understanding coronavirus tropism lies with the spike protein, consisting of S1 and S2 domains that carry out the receptor binding and membrane fusion functions of the virus, respectively [1]. SARS-CoV-2 and SARS-CoV both utilize ACE-2 as their receptor, while MERS-CoV utilizes DPP4 [32–34]. Equally important as a usable receptor is the presence of a functional protease for S activation [35]. Numerous proteases have been investigated across the *Coronaviridae*, including TMPRSS2, which acts to activate SARS-CoV-2, as well as furin, trypsin, and cathepsins [36]. While receptor binding is well recognized as a factor in host tropism, it is becoming more recognized that host cell proteases can also influence coronavirus species susceptibility and "spillover" events [37,38].

A notable difference between SARS-CoV-2 and RaTG13 (and all other *Sarbecoviruses*) is the insertion of a predicted cleavage site for a furin-like protease (P-R-R-A-R) between the S1/S2 regions of the spike protein of SARS-CoV-2 [39–41] with loss of this site associated with virus attenuation and decreased tramnsmission [42,43]. However, the P-R-R-A-R motif is sub-optimal for furin cleavage, indicating it may not be fully adapted for humans. RmYN02, identified in *R. malayanus* has 93.3% genomic nucleotide identity with SARS-CoV-2 and while the identity is only 71.9% (nucleotide sequence) in the spike protein, RmYN02 has a closely homologous sequence (P-A-A-R) at the S1/S2 cleavage site; however, this is lacking basic amino acids needed for cleavage by furin-like proteases [44]. Comparatively, MERS-CoV is highly unusual in that it contains both an S1/S2 furin cleavage site and an S2' furin cleavage site [35]. Among two closely related bat coronaviruses, BatCoV-HKU5 and BatCoV-HKU4, HKU5 is also cleaved by furin, though HKU4 may be more closely related to MERS-CoV and can utilize the same receptor, DPP4 [45,46].

Furin is a ubiquitously expressed serine protease that functions across numerous physiological processes [47]. In addition to several other proprotein convertases, furin has the ability to cleave and activate viral proteins [48]. Differences in furin-like activity across animal hosts may impact viral processing or alter viral tropism. Furin cleavage sites are not unique to the *Coronaviridae*. The highly pathogenic influenza strain, H5N1, for instance, also contains a polybasic furin cleavage site in the hemagglutinin H5 protein, with major implications for disease outcome [49]. Other viral proteins that contain furin cleavage sites include human immunodeficiency virus (HIV) envelope glycoprotein gp160, herpesvirus glycoprotein B, tick-borne encephalitis virus envelope protein prM, and Ebola virus GP [47,50,51]. Additionally, within the *Nidovirales* (the order within which the *Coronaviridae* are placed), a furin cleavage site has been predicted in an insect nidovirus isolated from *Culex* mosquitoes [52].

In the case of coronaviruses, it is known that furin cleavage sites often occur naturally and that this is highly dependent on the specific virus family or lineage [57]. While uncommon for coronaviruses of bats, furin cleavage sites are commonly found in coronaviruses of rodents and it is perhaps fitting to note that proteolytic processing of the coronavirus spike protein was first recognized in the model rodent coronavirus, murine hepatitis virus, MHV-A59 [53], with later analyses demonstrating the importance of furin for the proteolytic cleavage and function of its spike protein [54]. Here, we provide a comprehensive analysis of furin cleavage sites found in rodent coronaviruses, with a focus on the *Embecovirus* subgenus within the *Betacoronavirus* family, and discuss implications for virus evolution and zoonotic transfer from rodent species from a One Health perspective.

1.1. Furin cleavage across bat and rodent species

Based on the known ability of bats and rodents to harbor coronaviruses, comparing the spike protein and furin cleavage sites of viruses across these two hosts may help elucidate origins of novel coronaviruses and guide future surveillance efforts. Through the use of two publicly available programs used to accurately predict furin cleavage sites, PiTou and ProP [55,56], we analyzed furin cleavage across spike sequences in rodent and chiropteran species. PiTou utilizes a hidden Markov model, specifically targeting 20 amino acid residues surrounding furin cleavage sites and important for binding and solvent accessibility [56]. The final score in PiTou is based on log-odds probability. ProP utilizes an artificial neural network to predict furin cleavage [55]. In ProP, sites are given a score of between 0 and 1, with a score above 0.5 being a predicted furin cleavage site.

The protease furin cleaves at a distinct multi-basic motif containing paired arginine residues; furin requires a minimal motif of R-X-X-R, with a preference for an addition basic residue; i.e., R-X-B-R [48]. While most studies have focused on human furin, previous work has indicated furin-like proteases in the megabat *Pteropus* [57]. The presence of suitable protease activators to enable viral infections further adds to the mystery that enables bats to act as viral reservoirs without seemingly showing clinical signs. Many, but not all, coronaviruses contain a cleavage site at S1/S2, which primes the spike protein for fusion and may increase its affinity for the viral receptor but may also make it structurally unstable. As such S1/S2 is considered dispensable for virus infection. The presence of S1/S2 furin cleavage sites in viruses uniquely identified in bats is shown in Fig. 1A (see also Supplementary Table 2). Across the *Coronaviridae*, the S2' cleavage site is also present, with cleavage here considered a required event for viral infection [58]. Bat sequences which have predicted furin cleavage sites at S2' are additionally shown in Fig. 1B.

The S1/S2 cleavage site was first noticed in coronaviruses infecting laboratory rodents; for example, the mouse hepatitis virus (MHV-1) strain JHM contains a strong S1/S2 cleavage site in addition to a second predicted furin cleavage site just distal. The presence of S1/S2 furin cleavage sites in viruses uniquely identified in rodents is shown in Fig. 2A. Most often, a second, overlapping, S1/S2 furin cleavage site was predicted by PiTou and not by ProP, though an exception was rodent coronavirus RtBi-CoV/FJ2015, in which both PiTou and ProP predicted adjacent furin cleavage sites. This redundancy may indicate a needed feature for rodent-associated *Coronaviridae*. Unsurprisingly, the majority of the rodent coronaviruses with S1/S2 furin cleavage sites were classified as *Embecoviruses*, a subgenera known for this feature [59]. A weakly predicted cleavage site was also detected in the *Alphacoronavirus,* AcCoV-JC34. A total of 21 unique potential S1/S2 furin cleavage sites are shown in Fig. 2A. It is important to note, however, that isolate specific differences exist; for example, two isolates of HKU24 possess disparate predicted furin cleavage sites. Lastly, the virus with the strongest predicted furin cleavage site, based on PiTou, was RtR-CoV-T12006A. ProP also gave a strong furin cleavage prediction score (0.88), the highest score observed from ProP, but shared by several other viruses. S2' furin cleavage sites additionally occur naturally in rodent coronaviruses and are shown in Fig. 2B. It is worthwhile to note, the rodent coronaviruses with S2' furin cleavage sites also possess S1/S2 furin cleavage sites. Compared to S1/S2 furin cleavage sites in rodent coronaviruses, S2' furin cleavage sites are less common. Given the well-conserved nature of the S2' cleavage site, it is unsurprising that the predicted scores in the rodent coronaviruses are relatively similar to the predicted scores from bat associated coronaviruses.

1.2. Phylogenetic analysis

The majority of rodent coronaviruses investigated here are from the *Embecovirus* clade. This clade additionally includes two human coronaviruses, HCoV-OC43 and HCoV-HKU1 [15]. As previously reported and supported by our analysis, HCoV-HKU1 likely emerged through a rodent

A.

Isolate	Subgenus	S1/S2 Sequence	Furin Score	
			PiTou	ProP
Bat Hp-betacoronavirus/Zhejiang2013	HibeCoV	714 - CVNYTADTRLRTAR | AADRAL - 733	8.19	0.61
(Putative) Zaria bat coronavirus	HibeCoV	696 - DTCLNITRGRVGSR | SAGHLK - 715	2.27	0.20
Bat coronavirus HKU5-1	MerbeCoV	732 - LCAIPPTTSSRVRR | ATSGAS - 751	10.26	0.82
BtPa-BetaCoV/GD2013	MerbeCoV	733 - LCAIPPTTSSRLRR | ATSGVP - 752	10.21	0.81
Pipistrellus abramus bat CoV HKU5-related	MerbeCoV	738 - LCAIPPTTSTRVRR | ATSGVS - 757	8.40	0.72
Bat coronavirus HKU5-2	MerbeCoV	737 - LCAIPPTTSTRFRR | ATSIPD - 756	7.06	0.59
Pipistrellus bat coronavirus HKU5	MerbeCoV	737 - LCAIPPTTSTRFRR | ATSGVS - 756	7.01	0.73
Coronavirus Neoromicia/PML-PHE1/RSA/2011	MerbeCoV	732 - LCAIPPNTNLRSGR | STFGLG - 751	2.17	0.56
Bat coronavirus (BtCoV/A434/2005)	Unclassif.	725 - LCAIPPTISTRLRR | ATSGVS - 744	8.11	0.61
Bat coronavirus PREDICT/PDF-2180	Unclassif.	733 - LCAIPPNTNLRVGR | STFGLG - 752	1.28	0.54

B.

Isolate	Subgenus	S2' Sequence	Furin Score	
			PiTou	ProP
Pipistrellus abramus bat CoV HKU5-related	MerbeCoV	877 - LQIPQVTTGERKYR | SAIEDL - 896	0.35	0.571
Bat coronavirus HKU5-1	MerbeCoV	871 - LQIPQVTTGERKYR | STIEDL - 890	-0.56	0.507
Coronavirus BtRt-BetaCoV/GX2018	NobeCoV	796 - MGCLGSSCNSRNYR | SALSDL - 815	-5.18	0.527
Rousettus bat coronavirus HKU 9	NobeCoV	789 - MGCLGSNCGGKSHR | SALSEL - 808	0.09	0.16
Bat coronavirus 1B	MinunaCoV	876 - FDLTLALPRQRQSR | SAIEDL - 895	5.3	0.592
229E-related bat coronavirus	DuvinaCoV	865 - IPSLPTSGGSRVAGR | SAIEDI - 884	0.8	0.212
BtMf-AlphaCoV/GD2012-b	Unclassif.	876 - FDLTLALPRERQSR | SAIEDL - 895	4.98	0.525
Bat coronavirus MfulBtCoV/3709	Unclassif.	879 - FDLTLALPRQRQSR | SAIEDL - 898	5.3	0.592

Fig. 1. Examples of predicted furin cleavage sites identified in coronaviruses associated with chiropteran species. Over 150 spike sequences from bat associated CoVs were screened for furin cleavage sites, including those in both the *Alpha-* and *Betacoronavirus* genera, using the programs PiTou and ProP. A. Unique S1/S2 furin cleavage sites predicted in bat associated coronaviruses. B. Unique S2' furin cleavage sites predicted across bat associated coronaviruses. Associated NCBI accession numbers are as follows: Bat Hp-betacoronavirus/Zhejiang2013 (YP_009072440), (Putative) Zaria bat coronavirus (ADY17911), Bat coronavirus HKU5–1 (ABN10875), BtPa-BetaCoV/GD2013 (AIA62343), Pipistrellus abramus bat coronavirus HKU5-related (QHA24687), Bat coronavirus HKU5-2 (ABN10884), Pipistrellus bat coronavirus HKU5 (AGP04938), Coronavirus Neoromicia/PML-PHE1/RSA/2011 (AGY29650), BtCoV/A434/2005 (ABG11962), Bat coronavirus PREDICT/PDF-2180 (YP_009361857), BtRt-BetaCoV/GX2018 (QJX58383), *Rousettus* bat coronavirus HKU 9 (AVP25406), Bat coronavirus 1B (ACA52157), 229E-related bat coronavirus (ALK28781), BtMf AlphaCoV/GD2012-b (AIA62241), Bat coronavirus MfulBtCoV/3709 (AMB43191), Bat coronavirus MfulBtCoV/3736-1 (AMB43194), Bat coronavirus MfulBtCoV/3759-1 (AMB43195), Bat coronavirus MsBtCoV/4001-1 (AMB43196), Bat coronavirus MsBtCoV/4068 (AMB43198), *Rhinolophus* bat coronavirus HKU32 (QCX35178), *Rousettus* bat coronavirus GCCDC1 (QKF94914), *Hipposideros* bat coronavirus HKU10 (AFU92131), Bat coronavirus HKU9-3 (ABN10927), Bat coronavirus HKU9-1 (ABN10911), Alphacoronavirus Bat-CoV/P.kuhlii/Italy/3398-19/2015 (YP_009755890), NL63-related bat coronavirus (YP_009824967), *Chaerephon* bat coronavirus/Kenya/KY41/2006 (ADX59458), Miniopterus bat coronavirus/Kenya/KY33/2006 (ADX59488), SARS-like coronavirus BatCoV/BB9904/BGR/2008 (ALJ94036), and *Rousettus* bat coronavirus/Kenya/KY06/2006 (ADX59474).

A.

Isolate or strain	Classification	S1/S2 Sequence	Furin Score	
			PiTou	ProP
Murine hepatitis virus strain JHM	EmbeCoV	756 - GLCVDYSKSRRARR | SVSTGY - 775	13.70	0.88
		755 - AGLCVDYSKSRRAR | RSVSTG - 774	4.48	0.37
Longquan RI rat coronavirus Longquan-189	EmbeCoV	740 - GFCVDYSTARRKRR | DLSTGY - 759	12.69	0.84
		739 - SGFCVDYSTARRKR | RDLSTG - 758	4.02	0.45
Betacoronavirus HKU24, Longquan-723	EmbeCoV	751 - GYCVDYSATRRSKR | DLNTGY - 770	12.63	0.79
Longquan Rl rat coronavirus Ruili-66	EmbeCoV	740 - GFCVDYSTARRKRR | EISTGY - 759	11.38	0.80
Betacoronavirus HKU24, Lijiang-41	EmbeCoV	750 - GYCVDYSATRRAKR | DLNTGY - 769	10.34	0.75
Rat coronavirus Parker	EmbeCoV	743 - GLCVNYSTAHRARR | SVSTGY - 762	10.00	0.88
Rat sialodacryoadenitis coronavirus SDAV-681	EmbeCoV	743 - GLCVNYSIAHRARR | SVSTGY - 762	9.77	0.86
Longquan Rl rat coronavirus Longquan-708	EmbeCoV	747 - GLCVNYSTSHRARR | SISTGY - 766	9.25	0.86
Betacoronavirus HKU24, HKU24-R05005I	EmbeCoV	750 - GYCVDYSSTWRAKR | DLNTGY - 769	-5.47	0.74
MHV Strain A59	EmbeCoV	701 - MGAGLCVDYSKSRR | ADRSVS - 720	0.58	0.17
		704 - GLCVDYSKSRRADR | SVSTGY - 723	-5.00	0.79
Coronavirus AcCoV-JC34	LuchaCoV	495 - CNSSDVVTFSRRAR | ARTLTD - 514	0.15	0.28
RtRe-CoV/Tl2006A	Unclassif.*	737 - GLCVDYSKARRSRR | SVSTGY - 756	15.45	0.88
		736 - SGLCVDYSKARRSR | RSVSTG - 755	2.16	0.22
RtRt-CoV/Tb2018	Unclassif.*	737 - GLCVDYSKARRARR | SVSTGY - 756	13.17	0.87
		736 - SGLCVDYSKARRAR | RSVSTG - 755	3.48	0.31
Rodent coronavirus RtMm-CoV/GD2015	Unclassif.*	740 - GFCVDYSTARRKRR | ALSTGY - 759	13.07	0.80
		739 - SGFCVDYSTARRKR | RALSTG - 758	6.91	0.59
Rodent coronavirus RtAp-CoV/Tibet2014	Unclassif.*	750 - GYCVDYSSARRSKR | ALSTGY - 769	12.96	0.82
Rodent coronavirus RtMm-CoV-1/IM2014	Unclassif.*	735 - GYCVDYSTSRRNKR | SLSTGY - 754	12.81	0.88
Rodent coronavirus RtBi-CoV/FJ2015	Unclassif.*	740 - GFCVDYSSARRKRR | DLSTGY - 759	12.71	0.84
		739 - SGFCVDYSSARRKR | RDLSTG - 758	4.43	0.51
Rodent coronavirus RtAs-CoV/IM2014	Unclassif.*	756 - CVDYQSQSTRRARR | AVDAPT - 775	12.20	0.78
		755 - YCVDYQSQSTRRAR | RAVDAP - 774	3.80	0.38
Rodent coronavirus RtMruf-CoV-2/JL2014	Unclassif.*	752 - GYCVDYSKTRRAKR | ATSTGY - 771	11.14	0.77
		749 - MGSGYCVDYSKTRR | AKRATS - 768	1.34	0.16
RtRe-CoV/Tl2009	Unclassif.*	749 - GLCVNYSTAHRARR | SISTGY - 768	8.71	0.85
Rodent coronavirus RtNn-CoV/SAX2015	Unclassif.*	743 - GFCVDYSTAHRERR | EISTGY - 762	5.90	0.76

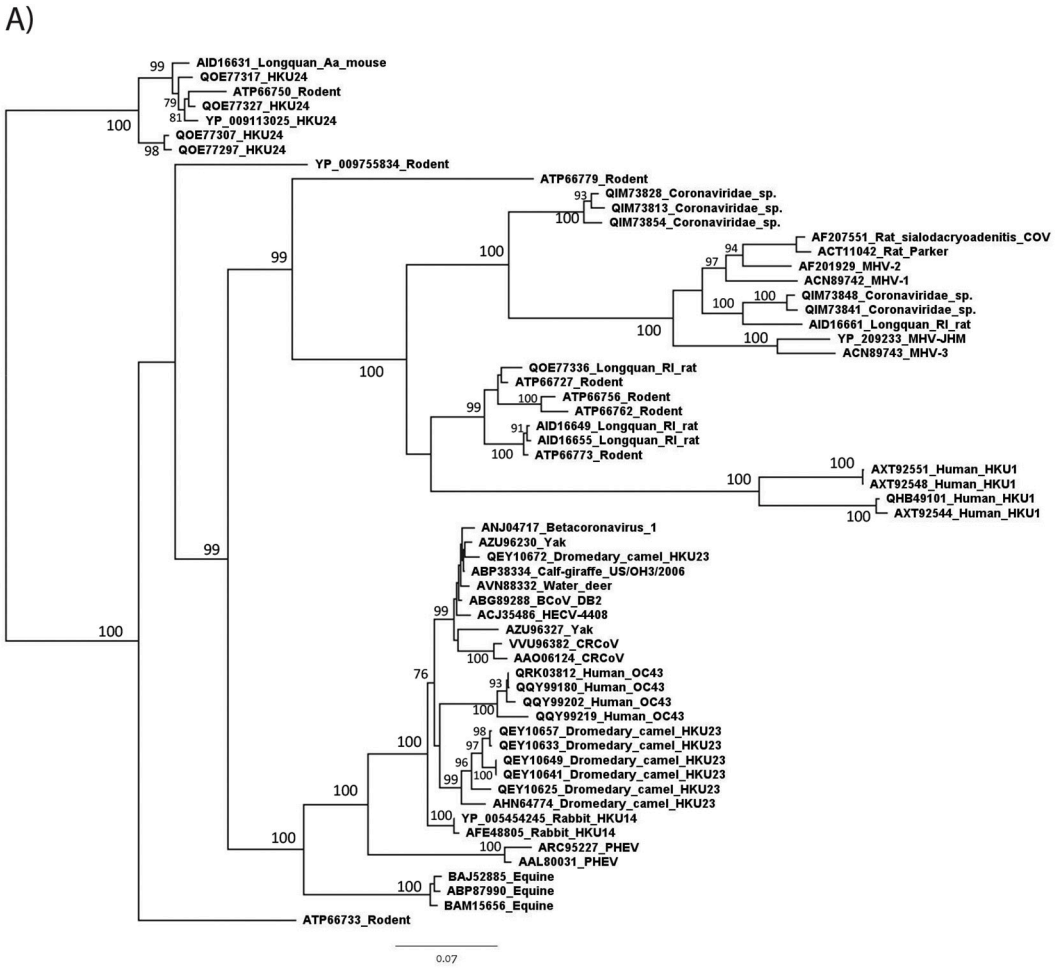
B.

Isolate	S2' Sequence	Furin Score	
		PiTou	ProP
RtRt-CoV/Tb2018	887 - SSCSEGTTVSRTGR | SAIEDL - 906	5.53	0.613
RtRe-CoV/Tl2006A	887 - SSCAEGTTVSRTGR | SAIEDL - 906	5.44	0.613

Fig. 2. S1/S2 furin cleavage sites are commonly found in rodent associated coronaviruses. A. Examples of predicted S1/S2 furin cleavage sites in rodent associated coronaviruses. Longquan RI rat coronavirus Longquan-189 (AID16649) shared the same S1/S2 furin cleavage site as Longquan Rl rat coronavirus Longquan-370 (AID16655). Murine hepatitis virus strain JHM (YP_209233) shares the same S1/S2 furin cleavage sites as several other MHV strains (ACN89763, ACN89705, ACN89696, ACN89722), as well as Murine coronavirus MHV-3 (ACN89743) in which sites occur at residues 770 and 769, versus 769 and 768. Longquan Rl rat coronavirus Longquan-708 (AID16661) shares the same S1/S2 cleavage site as Murine coronavirus MHV-1 (ACN89742), in which the predicted furin cleavage site occurs at residue 758. Betacoronavirus HKU24 Lijiang-41 (QOE77297) shares the same S1/S2 furin cleavage site as Betacoronavirus HKU24 Lijiang-53 (QOE77307) and Betacoronavirus HKU24 Ruili-874 (QOE77327), in addition to several other accession numbers (AYR18625, AYR18679, AYR18634, AYR18607, AYR18652, AYR18670, and AYR18643). Coronavirus AcCoV-JC34 (YP_009380521) shares the same S1/S2 furin cleavage site as Lucheng Rn rat coronavirus Lijiang-170 (QOE77268), which were only predicted by PiTou. RtRt-CoV/Tb2018 (QIM73854) shared the same S1/S2 predicted cleavage sites as RtRt-CoV/Tk2011 (QIM73813). Rodent coronavirus RtMm-CoV/GD2015 (ATP66756) shared the same S1/S2 predicted cleavage site as Rodent coronavirus RtRn-CoV/YN2013 (ATP66727). RtRe-CoV/Tl2009 (QIM73841) shared the same S1/S2 furin cleavage site as RtRt-CoV/Tn2018 (QIM73848). B. S2' furin cleavage sites predicted in rodent association coronaviruses, both of which were noted as unclassified, but our analysis supported as belonging to the *Embecovirus* subgenera. *Our analysis supports classification as an *Embecovirus*.

host [15]. Specifically, the phylogenetic history of the spike protein of *Embecovirus*es is shown in Fig. 3A. The tree is rooted at the HKU24 group based on earlier reports [9] as well as our own findings, which involved rooting the *Embecovirus* clade with three different *Merbecovirus* CoV – including those from a hedgehog and two bats – using a 2000 amino acid alignment after Gblocks [60] removal of ambiguously aligned positions of Orf1ab, and in all cases HKU24 fell at the base of the *Embecovirus* group. The phylogeny depicts a basal split in the *Embecovirus*es into a predominately rodent clade and a predominately ungulate clade, with spike sequences from horses at the base of that ungulate group. Historically, rodents have been considered major sources of pathogens and our evidence support their role in the emergence of two human coronaviruses (HCoV-OC43 and HCoV-HKU1).

Furin scores using both ProP and PiTou are summarized in Fig. 3B, based on PiTou scoring for all the *Embecoviruses* spike genes analyzed in Fig. 3. Furin scores are represented in a heat map coloring, with lower



Accession #	ProP	PiTou
AID16631		
QOE77317	0.786	12.6274
ATP66750	0.819	12.9607
QOE77327	0.753	10.3431
YP_009113025	0.742	
QOE77307	0.753	10.3431
QOE77297	0.753	10.3431
YP_009755834	0.768	11.1362
ATP66779	0.876	12.8092
QIM73828	0.879	15.4498
QIM73813	0.869	13.1656
QIM73854	0.869	13.1656
AF207551	0.864	9.76696
ACT11042	0.879	10.001
AF201929		
ACN89742	0.861	9.24972
QIM73848	0.848	8.71322
QIM73841	0.848	8.71322
AID16661	0.861	9.24972
YP_209233	0.881	13.7021
ACN89743	0.881	13.7021
QOE77336	0.8	11.3783
ATP66727	0.795	13.07
ATP66756	0.795	13.07
ATP66762	0.758	5.89886
AID16649	0.839	12.6919
AID16655	0.839	12.6919
ATP66773	0.835	12.7078
AXT92551	0.744	11.7701
AXT92548	0.744	11.7701
QHB49101	0.878	14.4111
AXT92544	0.882	14.6348
ANJ04717	0.827	13.0402
AZU96230	0.849	13.2666
QEY10672	0.774	11.8709
ABP38334	0.849	13.2666
AVN88332	0.849	13.2666
ABG89288	0.849	13.2666
ACJ35486	0.774	11.8709
AZU96327	0.809	12.0557
VVU96382	0.849	13.2666
AAO06124	0.709	2.56004
QRK03812	0.751	11.0189
QQY99180	0.751	11.0189
QQY99202	0.72	12.5802
QQY99219	0.753	10.0989
QEY10657	0.815	10.2952
QEY10633	0.812	10.5098
QEY10649	0.737	
QEY10641	0.751	9.1237
QEY10625	0.727	9.12074
AHN64774	0.718	8.89978
YP_005454245	0.629	7.19044
AFE48805	0.629	7.19044
ARC95227	0.758	11.3482
AAL80031	0.715	6.83027
BAJ52885	0.789	11.9171
ABP87990	0.815	12.1435
BAM15656	0.621	2.04567
ATP66733	0.776	12.1951

Fig. 3. Phylogenetic tree of spike protein sequences from the *Embecovirus* clade. A) The tree was constructed using RaxML [81], 1000 bootstrap replicates, from a spike protein alignment, that removed any ambiguously aligned positions using Gblocks [60]. Bootstrap support percentages for nodes receiving less than 75% are not shown. B) Sequences are colored based on the predicted furin cleavage score from PiTou.

score in red and higher score in blue. Unscored residues are depicted with a gray box.

Almost all *Embecoviruses* contain furin cleavage sites at the S1/S2 interface, with two notable exceptions (shown with black boxes in Fig. 3B). MHV-2 (AF201929) is the cause of a laboratory-acquired infection, first identified in 1952 as acute hepatitis associated with mouse leukemia [61]. It is a distinct virus in cell culture, with a spike protein known to be cleaved by cathepsins [62] and differs significantly from the more widely studied MHV-I and may represent a tissue-specific variant (S1/S2 site H**R**A**R**SS for MHV-2 vs. H**R**A**RR**S for MHV-1; basic residues in bold, predicted mutation underlined). As such, MHV-2 may show parallels with a tropism shift associated with a loss of furin cleavage seen with feline coronaviruses [63]. The other notable exception is Longquan Aa mouse coronavirus (LAMV) (AID16631). Comparatively HKU24 (ATP66750) contains an S1/S2 furin cleavage and aligns with specific residues LL-GAP**R**E for LAMV. In this case, the S1/S2 site seems to represent an indel at that region, and would be of high interest for further follow up with regard to its evolution.

In some cases, there is discrepancy between the ProP and PiTou scores, notably for sequences YP_009113025 and QEY10649. YP_009113025 is a variant of HKU24 with an S1/S2 site of W**R**A**RR**D, with a bulky hydrophobic residue (W) at P5 and QEY10649 is an HKU23 (dromedary camel) virus with an S1/S2 site D**RR**x**RR**A is a with a negatively-charged/acidic residue (D) at P6. In both cases, we consider that these flanking residues are the cause of the scoring discrepancy, with PiTou downgrading the score because of this. The impact of such flanking residues with altered biochemical properties remains to be resolved, but equivalent changes have also been seen in modified furin cleavage observed with feline coronaviruses, as measured with enzymatic assays with purified proteases and peptide mimics of the S1/S2 cleavage site [63]. Overall, while a furin cleavage site is prevalent in *Embecovirus* spike protein, the composition of the site does vary and a more detailed study of these variations may inform our understanding of infection and zoonotic transfer of these viruses.

2. Discussion

Continued surveillance in bat and rodent species, concomitant with laboratory experimentation and computational analysis, is essential for understanding the overall disease ecology of coronaviruses in these two host groups. Understanding species cross-over potential for coronaviruses often focuses on known features, such as mutation within the receptor-binding domains or in genomic recombination events, but other factors, such as alterations in protease cleavage sites within spike, may also be important.

The Chiroptera and Rodentia encompass vast lineages and inhabit unique ecological niches from bats roosting in caves to mice living in apartment buildings. The spillover of coronaviruses between these species and into humans, livestock, and other species remains a threat to One Health and global health. Across species, it is also important to consider host adaptations that may facilitate their ability to act as viral reservoirs. In bats, the ability to harbor and shed coronaviruses, with

limited or inapparent clinical signs has remained an ongoing question [38,64]. By comparison, less has been investigated in wild rodent species harboring coronaviruses, though in laboratory settings it is apparent that rodents can show disease associated with coronavirus infection, including Golden Syrian Hamsters (*Mesocricetus auratus*) infected with SARS-CoV-2, which have proven a robust and effective model for SARS-CoV-2 infection [65,66].

SARS-CoV-2 has remained a global challenge and like most diseases, rodent models are helpful in guiding our understanding. In laboratory mice, transgenic humanized ACE2 mice better recapitulate SARS-CoV-2 infection and pathology [67,68]. However, new SARS-CoV-2 variants of concern (VOCs) have expanded the host range of SARS-CoV-2, allowing infection of laboratory mice [69]. Challenges of deer mice (*Peromyscys maniculatus*) with SARS-CoV-2 have demonstrated infection and transmission [70,71]. Additionally, experimentally challenged bank voles (*Myodes glareolus)* appear susceptible to SARS-CoV-2, despite not showing clinical signs [72]. Understanding coronavirus adaptation and transmission among rodent species is an important area of investigation both from the One Health perspective as well as understanding spillover between rodents. Additional important areas of study include how/why some rodents harbor unique viruses, as well as what are the underlying host differences that previously allowed one rodent species (laboratory mice) to be largely unaffected by SARS-CoV-2 while another rodent species (hamsters) readily develop disease. In mice infected with MHV, disease has been associated with encephalomyelitis, wasting, and mortality in naïve animals [73,74]. Further, a case report has described a wasting syndrome in guinea pigs with a presumed coronavirus infection [75].

The activation of the spike protein is a complex process and furin is a commonly used protease to activate fusion machinery. Among the *Alphacoronaviruses*, the presence of an S1/S2 furin cleavage site is infrequently observed, though a notable example is in feline coronavirus, which normally possesses a furin cleavage site at the S1/S2 boundary, but loss of basic residues is often associated with the systemic disease feline infectious peritonitis [63]. A second *Alphacoronavirus*, canine coronavirus 23/03, also possesses a furin cleavage site, but the role in disease is less well defined [76]. In bat spike sequences from *Alphacoronaviruses*, no obvious S1/S2 furin cleavage site was identified based on ProP and PiTou predictions. Two proposed alphacoronavirus from rodents, AcCoV-JC34 and Lucheng Rn rat coronavirus Lijiang-170, had a shared, weakly predicted furin cleavage site (S-R-R-A-R), based on the PiTou program. A caveat to using bioinformatics programs lies in the correlation between a software prediction and biological plausibility. A predicted site, for example, may not be accessible to furin and thus, non-functional and the action of other proteases, needs also to be considered. It is also presently unclear how the presence or absence of a cleavage site and its relative cleavability score translates into a given biological process, such a transmissibility, cross-species tropism or pathogenicity. Nevertheless, our study provides a predictor that can be integrated with experimental validation and further surveillance.

Based on our studies reported here, it is possible that viruses with S1/S2 furin cleavage sites are more commonly found in *Coronaviridae* circulating in rodents. Over three times as many spike sequences were publicly available and screened from bats (179) compared to rodents (55). While MHV was included in our screening, 41 rodent viruses were from previous surveillance work and can be considered naturally occurring. Of these 41 rodent sequences, 32 (78%) had potential S1/S2 furin cleavage sites. In the spike sequences from bats, only 11 of 179 (6%) sequences had predicted S1/S2 furin cleavage sites. For some CoVs, such as HKU5, numerous accessions are publicly available through sources such as NCBI, so it is challenging to strictly compare the frequency of furin cleavage sites across the species that sequences come from, but it does seem apparent that rodents regularly harbor viruses with strong S1/S2 furin cleavage sites. With regard to the S2' furin cleavage site, the proportion of rodent versus bat sequences possessing this site is relatively similar, being found in 3 of 41 (7.3%) naturally occurring rodent spike sequences and in 13 of 179 (7.3%) bat sequences. Host differences may additionally contribute to these observed differences. Both rodent and bat associated coronaviruses have been associated with human disease and both must continue to be investigated, including in regular surveillance studies. Recently, a web-based, risk assessment tool for evaluating viruses of wildlife origin designated Lassa virus, a rodent-associated virus, as the virus most likely to spillover into human populations (ranked #1); notably, several rodent-related coronaviruses were ranked in the top 50, specifically Murine CoV (#18), Longquan Aa CoV (#23), Rodent CoV (#27), and HKU-1 (37) [77].

The *Coronaviridae* lend themselves well to zoonotic spillover. The unique spike protein determines host tropism and is largely a balance between receptor binding ability and the presence of an acceptable host cell protease for spike protein activation. Bats and rodents have both been implicated as reservoirs for ancestral coronavirus species. The common presence of predicted furin cleavage sites across rodent coronaviruses, suggests rodents warrant further consideration as a putative source of new viral emergences.

Acknowledgments

We thank all members of the Whittaker Lab for helpful discussions during the preparation of this manuscript.

Funding

AES is supported by NIH Comparative Medicine Training Program T32OD011000.

Declaration of interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.onehlt.2021.100282.

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