



Article Conserved Molecular Signatures in the Spike, Nucleocapsid, and Polymerase Proteins Specific for the Genus *Betacoronavirus* and Its Different Subgenera

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Abstract: The genus Betacoronavirus, consisting of four main subgenera (Embecovirus, Merbecovirus, Nobecovirus, and Sarbecovirus), encompasses all clinically significant coronaviruses (CoVs), including SARS, MERS, and the SARS-CoV-2 virus responsible for current COVID-19 pandemic. Very few molecular characteristics are known that are specific for the genus Betacoronavirus or its different subgenera. In this study, our analyses of the sequences of four essential proteins of CoVs, viz., spike, nucleocapsid, envelope, and RNA-dependent RNA polymerase (RdRp), identified ten novel molecular signatures consisting of conserved signature indels (CSIs) in these proteins which are specific for the genus Betacoronavirus or its subgenera. Of these CSIs, two 14-aa-conserved deletions found within the heptad repeat motifs 1 and 2 of the spike protein are specific for all betacoronaviruses, except for their shared presence in the highly infectious avian coronavirus. Six additional CSIs present in the nucleocapsid protein and one CSI in the RdRp protein are distinctive characteristics of either the Merbecovirus, Nobecovirus, or Sarbecovirus subgenera. In addition, a 4-aa insert is present in the spike protein, which is uniquely shared by all viruses from the subgenera Merbecovirus, Nobecovirus, and Sarbecovirus, but absent in Embecovirus and all other genera of CoVs. This molecular signature provides evidence that viruses from the three subgenera sharing this CSI are more closely related to each other, and they evolved after the divergence of embecoviruses and other CoVs. As all CSIs specific for different groups of CoVs are flanked by conserved regions, their sequences provide novel means for identifying the above groups of CoVs and for developing novel diagnostic tests. Furthermore, our analyses of the structures of the spike and nucleocapsid proteins show that all identified CSIs are localized in the surface-exposed loops of these protein. It is postulated that these surface loops, through their interactions with other cellular proteins/ligands, play important roles in the biology/pathology of these viruses.

Keywords: *Betacoronavirus* and its subgenera *Sarbecovirus; Merbecovirus* and *Nobecovirus;* molecular markers; conserved signature indels (CSIs); spike; nucleocapsid and RNA-dependent RNA polymerase (RdRp) proteins; evolution of betacoronaviruses; heptad repeat motifs 1 and 2

1. Introduction

Coronaviruses (CoVs) are a part of the subfamily *Orthocoronavirinae* [1,2]. The members of this family have been divided into four genera, viz., *Alphacoronavirus, Betacoronavirus, Gammacoronavirus,* and *Deltacoronavirus,* based on their branching in phylogenetic trees and genomic structures [1–4]. Of these four genera, only members of the *Alphacoronavirus* and *Betacoronavirus* genera infect mammals, whereas Gamma- and Delta-CoVs mainly infect birds [1,2]. Of the CoVs infecting humans, Alpha-CoVs causes only mild respiratory diseases, whereas all CoVs causing severe respiratory illnesses in humans, and responsible for different coronaviruses epidemics/pandemics, viz., SARS, MERS, and COVID-19, belong to the genus *Betacoronavirus* [1,2]. The genus *Betacoronavirus* is made up of four main lineages,



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Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https:// creativecommons.org/licenses/by/ 4.0/). which are now recognized as distinct subgenera with the names *Embecovirus*, *Merbecovirus*, *Nobecovirus*, and *Sarbecovirus* [1–5]. Of these four subgenera, SARS coronavirus (SARS-CoV) and the COVID-19 virus (named as SARS-CoV-2) are both members of the subgenus *Sarbecovirus*, whereas the MERS-CoV is a part of the *Merbecovirus* subgenus [1,3,5,6]. Thus, in terms of the propensity of CoVs to cause severe diseases in humans, betacoronaviruses and their different subgenera are of central importance. Therefore, it is of much interest to know how the genus *Betacoronavirus* and its different subgenera differ from each other and other CoVs. In this context, it is of much interest to identify molecular markers that are specific for different genera/subgenera of CoVs [1,2,7].

In our recent work, we have reported analyses of the spike (S) and nucleocapsid (N) protein sequences from different sarbecoviruses to identify molecular signatures consisting of conserved signature indels (CSIs) in the S and N proteins, which were specific for different lineages of sarbecoviruses [8]. These studies identified multiple CSIs specific for different lineages of sarbecoviruses including some specific for a cluster consisting of SARS-CoV-2-related CoVs [7,8]. In addition, several signatures were identified, which were specifically shared by other lineages of sarbecoviruses [8]. The molecular markers identified in these studies provided novel means for identifying several distinct clades of sarbecoviruses in molecular terms [7,8]. In addition, the distribution patterns of these molecular signatures in different sarbecoviruses also provided evidence indicating that the SARS-CoV-2 and a pangolin virus (Pangolin CoV-MP_789), whose receptor binding domain is most similar to the SARS-CoV-2 [9–12], originated by recombination events involving specific sarbecoviruses [8].

Our current understanding of the evolutionary relationships amongst different genera/subgenera of CoVs is primarily based on phylogenetic trees of sequences of the spike and RNA-dependent RNA polymerase (RdRp) proteins [5,11–14]. In phylogenetic trees, although different genera and subgenera of CoVs form distinct clades, based on these analyses, it remains unclear how different genera and subgenera of CoVs are related to each other [5,11-14]. Hence, other molecular-sequence-based approaches that can more reliably elucidate the evolutionary relationships among different genera and subgenera of CoVs should be useful in advancing our understanding of these viruses. With this objective, in the present study, we have extended our earlier work on CoVs to identify molecular signatures that are specific for either the entire *Betacoronavirus* genus or those specifically shared by members of different subgenera of *Betacoronavirus*. The conserved signature indels (CSIs) (insertions/deletions) in genes/proteins that are specific for either a given group of organisms/viruses or commonly shared by more than one group/lineage [15,16] provide an important class of molecular markers, that has proven very useful for evolutionary, diagnostic, and taxonomic studies [8,15,17–20]. The CSIs that are useful for evolutionary studies are generally of specific lengths and are flanked on both sides by conserved regions to ensure their reliability as genetic markers [15,16]. In the present study, we have analyzed the sequences of the spike (S), nucleocapsid (N), envelope (E), and RdRp proteins from different CoVs to look for the presence of CSIs that are specifically shared by either some or all subgenera of *Betacoronavirus*. Results of these studies, which are reported here, have identified ten novel CSIs in the S, N, and RdRp proteins that are either specific for the genus Betacoronavirus or one or more of its subgenera, viz., Merbecovirus, Nobecovirus, and Sarbecovirus. The identified molecular markers in addition to providing novel and reliable means for distinguishing the above groups of viruses from each other, as well as all other CoVs, also serve to clarify the evolutionary relationships among different subgenera of Betacoronavirus.

2. Materials and Methods

2.1. Identification of Conserved Signature Indels in Protein Sequences

The identification of CSIs in protein sequences was carried out as described in our recent work [7,8,16]. Briefly, to identify CSIs, sequences for the S, N, E, and RdRp proteins for representative CoVs from different genera and subgenera of CoVs were retrieved from

the NCBI database (https://www.ncbi.nlm.nih.gov/genome/, accessed on 30 November 2021) [21] and the GISAID (Global Initiative on Sharing Avian Flu Data) database of SARS CoV-19 sequences [22]. Multiple sequence alignments for these proteins were created using the ClustalW algorithm from the MEGA X (Molecular Analysis Genetic Analysis) software package [23]. These sequence alignments were inspected for any insertion or deletion (indel) in a conserved region specifically present in either all betacoronaviruses or members of its different subgenera. The indels of interest were required to be flanked by at least 4–5 conserved amino acids on each side within the neighbouring 40–50 residues [16,24]. The indels not flanked by conserved regions were not further considered, as they generally do not provide reliable molecular characteristics [16,25]. As the focus of this work was on betacoronaviruses, indels that were specific for other genera of CoVs were not investigated in this study. For the indels that were of interest, query sequences encompassing the conserved indels and their flanking 40-50 amino acids were subjected to a second BLASTp (Basic Local Alignment Search Tool, p refers to protein) search against the NCBI nr (non-redundant) database. All significant hits obtained from these searches were examined to determine the lineage specificities of the identified CSIs. The SIG_CREATE and SIG_STYLE programs described in our earlier work [16] (available on the GLEANS (Gupta Lab Evolutionary Analysis Software), www.gleans.net (accessed on 30 November 2021)) were utilized to create the formatted signature files for different CSIs that are presented here [16]. Sequence information in different figures is shown for only a limited number of viruses (strains) from different genera/subgenera. However, unless otherwise specified, the described CSIs are specific for the indicated lineages, and they are not present in other genera/subgenera of CoVs. Phylogenetic trees based on sequences of the RdRp and spike proteins from representative strains from different lineages of CoVs were constructed using MEGA X as described in our recent work [8].

2.2. Analysis of the Available Protein Structures to Map the Structural Locations of CSIs

The structural locations of the identified CSIs were mapped in the spike and nucleocapsid (N) protein structures using the experimentally solved three-dimensional (3D) structures obtained from the Protein Data Bank (PDB) [26]. The superimpositions of the 3D structures were carried out using PyMOL (Version 1.7.4; Schrödinger, LLC, (New York, NY, USA) to examine the structure, features, and location of identified CSIs in the spike and N-protein structure. In the absence of experimentally solved structures, computational techniques such as comparative protein structure modelling or homology modelling can be utilized to generate the 3D structure of target proteins [27]. Use of homology models to analyze the structural locations of CSIs has been described in several of our previously published works [28–31].

3. Results

3.1. Phylogenetic Relationships among Coronaviruses

Figure 1 shows a phylogenetic tree based on the sequences for RdRp protein from representative CoVs from the subfamily *Orthocoronavirinae*. In accordance with earlier studies [1–3], members of the four *Orthocoronavirinae* genera, viz., *Alphacoronavirus, Betacoronavirus, Gammacoronavirus,* and *Deltacoronavirus,* form distinct clades in this tree. Additionally, within the genus *Betacoronavirus,* four main clusters corresponding to its four subgenera i.e., *Embecovirus, Sarbecovirus, Merbecovirus,* and *Nobecovirus* are also observed. These clusters are marked in the tree along with their commonly known clade designations (i.e., clades A, B, C, and D). Similar branching of the *Orthocoronavirinae* viruses is seen in a phylogenetic tree based on the spike protein (Supplementary Figure S1) and in earlier phylogenetic studies based on spike and RdRp proteins [1–3]. The trees shown in Figure 1 and Figure S1 provide a phylogenetic framework to understand the significance of various identified molecular signatures.



Figure 1. A maximum-likelihood distance tree based on sequence alignment of the RNAdependent RNA polymerase (RdRp) protein from representative viruses/strains from different genera/subgenera of CoVs. The tree was bootstrapped 100 times and the % bootstraps for different branches are indicated on the nodes. The clades corresponding to different genera and subgenera within the genus *Betacoronavirus* are labeled.

3.2. Molecular Markers (CSIs) Specific for the Genus Betacoronavirus and Its Different Subgenera

The main objective of this work was to identify molecular signatures (CSIs) in the S, N, E, and RdRp proteins that are specific for the genus *Betacoronavirus* or its different subgenera. These studies have identified several novel CSIs in the S, N, and RdRp proteins. However, no useful CSI was detected in the envelope protein. We discuss below the group specificity and characteristics of the identified CSIs.

Our analyses have identified two large CSIs in the spike protein that are commonly shared by all betacoronaviruses. Figure 2 shows partial sequence alignments of two different conserved regions from the S2 subunit of the spike protein where these CSIs are found. The CSIs in these sequence alignments are colour-highlighted and, in both cases, they consist of 14-aa deletions within conserved regions of the spike protein. The dashes (-) in these alignments indicate identity with the amino acid on the top line. Sequence information in Figure 2 is shown for only a limited number of viral strains from different genera and subgenera of CoVs, however all other members of the indicated genera/subgenera also contained or lacked the indicated CSIs. As seen from Figure 2, both these CSIs (marked **0** and (**2**) are present in the spike protein homologs from different subgenera of betacoronaviruses but barring one exception they are not found in any other genera of CoVs. The Omicron variant of SARS-CoV-2 contains large numbers of changes in the spike protein including several insertions and deletions [32]. However, in the sequence region where these two CSIs are found, no changes are observed in the omicron variant (results not shown). Besides the betacoronaviruses, the only other virus which contains these two CSIs is avian coronavirus belonging to the genus *Gammacoronavirus* (see Figures 1 and S1). However, other viruses from this genus do not contain these CSIs. The most likely explanation to account for the distribution of these two CSIs in different CoVs is that the genetic changes giving rise to these CSIs occurred in a common ancestor of the betacoronaviruses, and these changes were then retained by all members of this genus. The presence of these two CSIs in the avian coronavirus can result from either a genetic recombination with a betacoronavirus [33] or by means of independent occurrence of these genetic changes in this virus.

			1172		1205
	CSARS-CoV-2	YP_009724390	INASVVNIQKEID		RLNEVAKNLNESLIDLQELGKY
Lineage B	BatcoronavirusRaTG13	QHR63300			
Beta CoV	Pangolincoronavirus	QIA48641			PP
Bela-Cov -	Bat SARS-like coronavirus	AT098181			
(Sarbecovirus)	SARS coronavirus ExoN1	AFM43867			I
	BtRs-BetaCoV/ GX2013	AIA62320			
1072 P.O.	<pre>Pipistrellus_bat_coronavirus_HKU5</pre>	YP_001039962	TTLL-LNT-LM		V-SV-QYKN-
Lineage C	BtPa-BetaCoV/ GD2013	AIA62343	TTLL-LNT-LM		V-SV-QYKN-
Beta-CoV -	MERS_coronavirus	ATQ39390	TTLLDLSG-ML		A-QV-AYKN-
(Merbecovirus	Betacoronavirus_England_1	YP_007188579	TTLLDLTY-ML		S-QQ-V-AYKN-
Υ.	Human_CoV_Jordan-N3/ 2012	AHY21469	TTLLDLTY-ML		S-QQ-V-AYKN-
	└Bat_coronavirus_HKU9-5-1	ADM33558	F-F-M-DVN-QLA		T - TD - VQQ K - Y KQ - NV -
Lineage D	Rousettus_bat_coronavirus_HKU9	YP_001039971	F-F-T-DVTAQ-K		S-HD-VNQQ-FKK-NV-
Beta-CoV	Coronavirus_BtRt-BetaCoV/GX2018	QDF43840	F-FTGSEQLA		T - TD - VNH K - F KQMNV -
(Nultración)	Bat_coronavirus_HKU9-4	ABN10935	F-F-T-EL-SA		T VQQ K - F KQMNV -
(Nobecovirus)	Rousettus_bat_coronavirus	AVP25406	F-F-L-DVNEQLA		I-TD-VNQQ-YKQT-
	Murine_coronavirus	AF011507	L-VTFLDLTY-MN		- IQDAI - K Y - N - K - V - T -
Lineage A	Rabbit_coronavirus_HKU14	AFE48827	VTFLDD-MN		Q-AL-VQ-Y-N-KDI-T-
Data CaV	Human_coronavirus_0C43	AXX83351	VTFLDL-V-MN		Q-AI-VQ-Y-N-KDI-T-
Bela-Cov -	Camel_coronavirus_HKU23	ATI09449	VTFLDL-D-MN		Q-AI-VH-Y-N-KDI-T-
(Embecovirus)	Human_coronavirus_HKU1	ABD75513	TFLDLYY-MN		VIQ-SI-SS-F-N-K-I-T-
	_ Rat_coronavirus_Parker	YP_003029848	L-VTFLDLSY-MN		- IQDAI Y - N - K - I - T -
	Human_coronavirus_229E	BAL45640	Y-QTIL-LTSS	TRENKSAELNYTVQ	K-QSLID-I-ST-VKW-NRV
Alpha CoV	Camel_alphacoronavirus_Camel229E	A0128259	Y-QTIL-LTSS	TLENKSAELNYTVQ	QTLID-I-ST-VKW-NRV
Alpha-Cov -	Alpaca_respiratory_coronavirus	AFI49431	Y-QTIL-LTSS	TLENKSAELNYTVQ	QTLID-I-ST-VKW-NRV
	L Human_coronavirus_NL63	BBL54116	F-LTYL-LSS-LK	QLEAKTASLFKTTV	E-QGLIDQI-STYVKL-NRF
	Duck_coronavirus	AKF17724	YTIPIL-LEDD-C	RLNSSINALWNYSS	IID-INAADTY-N-EQ-N-V
Gamma CoV -	Turkey_coronavirus	CBA13338	YSVKILDL-NC	MLNSSIQQLWNVSS	T-DDINSADTF-N-EQ-N-I
Gamma-COv -	Avian_coronavirus	ANY59000	YTIP-LSN		RIQG-IQGDET-SIL
	European_turkey_coronavirus	ALQ43515	YSVKILDL-NC	MLNSSIQQLWNVSS	T-DDINSADTF-N-EQ-N-I
	Falcon_coronavirus_UAE-HKU27	BBC54822	Y-NTIL-LTN	DLNGRAENLTQIVA	N-QDYIDKI-ATIVEW-NRV
Delta-CoV	Pigeon_coronavirus_UAE-HKU29	BBC54842	Y-NTIL-LTTN	DLNGRAENLTQIVE	N-Q-YIDKI-ATIEW-NRV
Dena-COV -	Bulbul_coronavirus_HKU11-934	YP_002308479	Y-YTIL-LSQK	DLQLKSQNLTDIAE	IMETYIQKTIVW-N-V
	White-eye_coronavirus_HKU16	YP_005352838	F-HTIL-LSQE	DLQIRSQNLSATAE	L-QQYIDNT-VEW-NRV

Figure 2. Cont.

			915	2	950
	SARS-CoV-2	YP 009724390	VLYENQKLIANQFN		SAIGKIQDSLSSTASALGKLQD
Lincore D	BatcoronavirusRaTG13	QHR63300			
Lineage B	Pangolincoronavirus	QIA48641			
Beta-CoV -	Bat SARS-like coronavirus	AT098181	Q		KSQETT-ST
(Sarbecovirus)	SARS coronavirus ExoN1	AFM43867	Q		KSQETT-ST
	BtRs-BetaCoV/ GX2013	AIA62320	Q		KSQETT-ST
	┌Murine coronavirus	AF011507	SMSA		N-L-AEGFDA-NI-S
	Rabbit coronavirus HKU14	AFE48827	SQA		N-LDAEGFDA-NA-I-A
Lineage A	Human coronavirus OC43	AXX83351	SQA		N-LDAEGFDA-NV-I-A
Beta-CoV	Camel coronavirus HKU23	ATI09449	SQA		N-LTANGFDA-NV-I-S
(Embecovirus)	Human coronavirus HKU1	ABD75513	NK A		K-LLSNGFTA-NA-I-S
(Lineccontinus)	_Rat_coronavirus_Parker	YP_003029848	SMSS		NAEGFDA-NA-I-S
	Pipistrellus_bat_coronavirus_HKU5	YP_001039962	SIK		Q-L-AM-TGFTT-NL-FN-V
Lineage C	BtPa-BetaCoV/ GD2013	AIA62343	SK		Q-L-AM-TGFTT-NL-FN-V
Beta-CoV -	MERS_coronavirus	ATQ39390	SK		Q-L-AM-TGFTT-NE-FQ-V
(Merhecovirus)	Betacoronavirus_England_1	YP_007188579	SK		Q-L-AM-TGFTT-NE-FH-V
(meroccovirus)	Human_CoV_Jordan-N3/ 2012	AHY21469	SK		Q-L-AM-TGFTT-NE-FQ-V
	Bat_coronavirus_HKU9-5-1	ADM33558	VS		N-LTNKGFDA-NES-M-L
Lineage D	Rousettus_bat_coronavirus_HKU9	YP_001039971	VSS		N-LVNKGFTE-SIS-M
Beta-CoV	Coronavirus_BtRt-BetaCoV/GX2018	QDF43840	VQQS		K-LTSKGFDA-NLSQM-A
(Nahaamimua)	Bat_coronavirus_HKU9-4	ABN10935	VDSS		N-LNQKGFDA-NS-I-A
(Nobecovirus)	_Rousettus_bat_coronavirus	AVP25406	MA		N-LTGKGFDA-NMA-M-S
	Human_coronavirus_229E	BAL45640	QIL-AS	KAMTNIVDAFTGVN	DTQTSQA-QTV-TN-I
Alpha CoV -	Camel_alphacoronavirus_Camel229E	A0128259	QIL-AS	KAMTNIVDAFTGVN	DTQTSQAIQTV-TN-I
Alpha-Cov	Alpaca_respiratory_coronavirus	AFI49431	QIL-AS	KAMTNIVDAFTGVN	DTQTSQAIQTV-TN-I
	Human_coronavirus_NL63	BBL54116	QIL-AS	KAINNIVASFSSVN	DTQTAEAIHTVTIN-I
1	- Duck_coronavirus	AKF17724	LDNS	NALEKIQSALDVVS	AGFQEVAKGFETVSV S - V
Gamma-CoV	Turkey_coronavirus	CBA13338	MDNS	KALKNIQSALDTVS	QGFLEVSKGFE-VSMT-I
Gamma-COV -	Avian_coronavirus	ANY59000	L-MKEKAS		RHM-EGFKSLQQI
I	— European_turkey_coronavirus	ALQ43515	MDNS	NALKNIQSALDTVS	QGFVEVAKGFE-VSMT-I
	Falcon_coronavirus_UAE-HKU27	BBC54822	QQIL-DS	SAMGNISLAFKEVS	EGLSQVSG-ITTV-NI-I-T
	Pigeon_coronavirus_UAE-HKU29	BBC54842	QQIL-DS	SAMGNISLAFKEVS	EGLSQVSGAITTV-NT-I-T
Delta-CoV -	Bulbul_coronavirus_HKU11-934	YP_002308479	QIL-ES	SAVGNISLALSAVN	DKQTSELTV-T-IN-I-T
	Sparrow deltacoronavirus	AWV67125	0TI-FS	OAVGNTSLALSSVN	DOOTSEA-NTV-N-TN-T-T

Figure 2. Partial sequence alignments of two conserved regions from the spike protein showing two different CSIs that are specific for the genus *Betacoronavirus*. The CSIs present in these sequence alignments are highlighted in blue and they are labeled **0** and **0**. Both CSIs are commonly shared by all members of the genus *Betacoronavirus*, but barring one exception, avian coronavirus, they are not found in any other CoV. Dashes (–) in these and all other sequence alignments denote identity with the amino acid shown in the top sequence. The numbers on the top indicate the locations of these sequence regions within the indicated proteins. The accession numbers of different proteins are given in the second column.

Molecular Markers (CSIs) Specific for Different Groups (Subgenera) of Betacoronavirus

The subgenus *Merbecovirus* of *Betacoronavirus* includes the virus responsible for the Middle East respiratory syndrome (MERS) [1,2]. Although the viruses from this subgenus form a distinct clade in phylogenetic trees (see Figure 1), there is no known molecular signature that is specific for this group of CoVs. Our analyses have identified two CSIs, in the RdRp and N proteins, that are uniquely shared by different members of this subgenus. Partial sequence alignments of the RdRp and N proteins showing the CSIs that are specific for the subgenus *Merbecovirus* are presented in Figure 3.

In the sequence alignment of RdRp protein shown in Figure 3A, a 2-aa insertion (marked **③**) is present in a conserved region (highlighted in cyan) that is commonly shared by all merbecoviruses, but it is absent in all other betacoronaviruses as well other genera of CoVs. Likewise, in the partial sequence alignment of the N-protein presented in Figure 3B, a 1-aa deletion (marked **④**) is present, which is again a unique characteristic of the members of the subgenus *Merbecovirus*. The genetic changes responsible for these CSIs are postulated to have occurred in a common ancestor of the subgenus *Merbecovirus*, and they provide novel and reliable means for distinguishing members of this subgenus from all other CoVs.

(A)				17		5	57
(A)	Tylonycteris_bat_coronavirus_HKU	4 AWH65	886	ARLEPCSSGL	TTDVVYRAFDICNF	KA	RVAGIGKYYKTNTCRFV
	Bat_coronavirus_HKU4-4	ABN10	865				
Lineage C	BtPa-BetaCov/ GD2013	A1A62	342	IA	Y		K
Beta-CoV	Pipistrellus_bat_coronavirus_HKU	AWH05	919	IA	E V		K
(Manh accuring)	Human betacoronavirus 2c Jordan-	N3/ 2012 AHV21	468		S		к
(Merbecovirus)	Betacoronavirus England 1	YP 00	7188577	I	SY		K
	MERS CoV-1	AWH65	952	IA	SY	1	K
	Rabbit_coronavirus_HKU14	AFE48	811	VAT	SQLA	- :	SQ
	Human_coronavirus_0C43	AGT51	429	VA	SQLY-A	1	N LHL - V - C Q
Lineage A	Bovine_coronavirus	BBM61	254	VA	SQLA	:	SQ
Beta-CoV	CamelcoronavirusHKU23	ALA50	077	VA	SQLA	-	3Q
(Emhecovirus)	HumancoronavirusHKU1	ABD75	567	VA	SQLI		NRLV-CQ
(Entoccorti us)	Murine_coronavirus_MHV-3	ACN89	748	VA	DQLA		NRLV-CQ
	Bat-coronavirus-BM48-31/ BGB/ 2008	YP_00	3858583		SY.F		KFAFLCQ
	BtBf-BetaCoV/ HeB2013	ATA62	289	TGT-T	SY-E		KFA-FLCQ
Lineage B	SARScoronavirusExoN1	ACZ72	179	TGT-T	SY-E	1	KFA-FLCQ
Data Call	Bat-SARS-coronavirus-HKU3-9	ADE34	778	T GT - T	SY-E	1	KFA-FLCQ
Beta-Cov	Pangolin coronavirus	QIA48	613	TGT-T	SY-E	1	KFA-FLCQ
(Sarbecovirus)	Bat coronavirus RaTG13	QHR63	299	TGT-T	SY-D	1	KFA-FLCQ
· · · ·	-Covid-19-Wuhan-Hu-1	YP_00	9725307	TGT-T	SY-D	- 0	KFA-FLCQ
Lineage D	Coronavirus_BtRt-BetaCoV/GX2018	QDF43	839	V-QG	QPT	1	KF-LHL-N-CYQ
Beta-CoV	Bat_coronavirus_HKU9-10-2	ADM33	581	V-LGV	QPT		KF-LHL-N-CYQ
Beta-COV	Rousettus_bat_coronavirus_HKU9	YP_00	1039970	V-LGV	QP-I-LT		KF-LHL-N-CYQ
(Nobecovirus)	Rousettus_bat_CoV/ Kenya/ KY06/ 200	6 ADX59	481	V-LGV	QP-I-LT		KF-LHL-N-CYQ
	Phanada_goose_coronavirus	YP_00	9755920	I-LID-V			JAMFUNL-H-GAQ
Gamma CoV -	Avian coronavinus	QUA76	203	I-LAN-C			ES MEONI P CA O
	Turkey coronavirus	ACV87	243		DPKVK		ESMETNE-B-CAQ
	-Bulbul coronavirus HKU11-796	AC.112	043	00P-V	-P-A-KHVH-N		ITSFISTCSR
	Gentoo penguin virus	01587	929	-QQQP-N	QP-A-KHVH-N	-	TTSFLSTCAK
Delta CoV	Falcon coronavirus UAE-HKU27	BBC54	821	-QRQP-N	QP-A-KHVH-N	1	TTSFLSTCAK
222-001-000-0-02-000-035-02-00-0	Pigeon coronavirus UAE-HKU29	BBC54	841	-QQQP-N	QP-A-KHVH-N	- 2	TTSFLSTCAK
	Human_coronavirus_229E	A0G74	782	NDID	YCVRAFDVYNKD		ASF NL -S-CVK
	Camel_alphacoronavirus	YP_00	9194637	NDID	YCVRAFDVYNKD		ASFNL-S-CVK
Alpha CoV	Chaerephon_bat_CoV/ Kenya/ KY22/ 20	06 ADX59	501	NDTD	RVVRAFDIYNKD		CFL-V-CVE
	LNL63-related_bat_coronavirus	APD51	481	NDVD	VCVRAFDIYNKG		- SFL CL - V - CA K
(B)			119	6	`		158
(B)	-Middle East respiratory syndro	VP 009047211	119 DG1/W0/HED/				158 2. GTKL PK
(B)	-Middle East respiratory syndro	YP_009047211	119 DGIVWVHED	GATDAPSTF	GTRNPNNDSAIVTG	F A	158 P GTKLPK
(B)	-Middle East respiratory syndro Human betacoronavirus 2c EMC/2	YP_009047211 AG006008	119 DGIVWVHED	GATDAPSTF	GTRNPNNDSAIVTC	F AI	158 Р GTKLPK
(B)	-Middle East respiratory syndro Human betacoronavirus 2c EMC/2 Betacoronavirus England 1	YP_009047211 AG006008 YP_007188586	119 DGIVWVHED	GATDAPSTF	GTRNPNNDSAIVTC	0F AI	158 P GTKLPK
(B)	-Middle East respiratory syndro Human betacoronavirus 2c EMC/2 Betacoronavirus England 1 Coronavirus Neoromicia/PML-PHE	YP_009047211 AG006008 YP_007188586 AIG13103	119 DGIVWVHED0 E	GATDAPSTF	GTRNPNNDSAIVTG	0F AI	158 P GTKLPK
(B)	-Middle East respiratory syndro Human betacoronavirus 2c EMC/2 Betacoronavirus England 1 Coronavirus Neoromicia/PML-PHE Hypsugo bat coronavirus HKU25	YP_009047211 AG006008 YP_007188586 AIG13103 ASL68960	119 DGIVWVHED EIE	GATDAPSTF	GTRNPNNDSAIVTC	PF AI	158 P GTKLPK
(B) Lineage C Beta-CoV	-Middle East respiratory syndro Human betacoronavirus 2c EMC/2 Betacoronavirus England 1 Coronavirus Neoromicia/PML-PHE Hypsugo bat coronavirus HKU25 BtVs-BetaCoV/SC2013	YP_009047211 AG006008 YP_007188586 AIG13103 ASL68960 AHY61344	119 DGI VWVHEDO E 	GATDAPSTF	GTRNPNNDSAIVTC	PF AI	158 P GTKLPK Q R
(B) Lineage C Beta-CoV (Merbecovirus)	-Middle East respiratory syndro Human betacoronavirus 2c EWC/2 Betacoronavirus England 1 Coronavirus Neoronicia/PML-PHE Hypsugo bat coronavirus HKU25 BtVs-BetaCoV/SC2013 Pipistrellus bat coronavirus	YP_009047211 AG006008 YP_007188586 AIG13103 ASL68960 AHY61344 AWH65917	119 DGI VWVHEDO EIE N E N	GATDAPSTF	GTRNPNNDSAIVTC	2F AI	158 P GTKLPK Q R T
(B) Lineage C Beta-CoV (<i>Merbecovirus</i>)	-Middle East respiratory syndro Human betacoronavirus 2c EMC/2 Betacoronavirus England 1 Coronavirus Neoromicia/PML-PHE Hypsugo bat coronavirus HKU25 BtVs-BetaCoV/SC2013 Pipistrellus bat coronavirus Tylonycteris bat coronavirus	YP_009047211 AG006008 YP_007188586 AIG13103 ASL68960 AHY61344 AWH65917 AGP04896	119 DGIVWVHEDO EIE N E IN Y-E	GATDAPSTF	GTRNPNNDSAIVTC	2F AI	158 P GTKLPK Q R T LI
(B) Lineage C Beta-CoV (Merbecovirus)	-Middle East respiratory syndro Human betacoronavirus 2c EMC/2 Betacoronavirus England 1 Coronavirus Neoromicia/ PML-PHE Hypsugo bat coronavirus HKU25 Btvs-BetacoV/SC2013 Pipistrellus bat coronavirus Tylonycteris pachypus bat coro	YP_009047211 AG006008 YP_007188586 AIG13103 ASL68960 AHY61344 AWH65917 AGP04896 QHA24685	119 DGIVWVHEDO EIE 	GATDAPSTF	GTRNPNNDSAIVTC	PF AI	158 P GTKLPK R R LI
(B) Lineage C Beta-CoV (<i>Merbecovirus</i>)	-Middle East respiratory syndro Human betacoronavirus 2c EUK/2 Betacoronavirus England 1 Coronavirus Neoromicia/PML-PHE Hypsugo bat coronavirus HKU25 BtVs-BetaCoV/SC2013 Pipistrellus bat coronavirus Tylonycteris pachypus bat coro Ledegehog coronavirus 1	YP_009047211 AG006008 YP_007188586 AIG13103 ASL68960 AHY61344 AWH65917 AGP04896 QHA24685 QCC20721	119 DGIVWVHEDO EIE N E Y-E Y-E Y-E	3ATDAPSTF 	GTRNPNNDSAIVTC	2 F AI	158 P GTKLPK Q R T LI Q
(B) Lineage C Beta-CoV (<i>Merbecovirus</i>)	-Middle East respiratory syndro Human betacoronavirus 2c EMC/2 Betacoronavirus England 1 Coronavirus Neoromicia/PML-PHE Hypsugo bat coronavirus HKU25 BtVs-BetaCoV/SC2013 Pipistrellus bat coronavirus Tylonycteris bat coronavirus Tylonycteris pachypus bat coro _Hedgehog coronavirus 1 Severe acute respiratory syndr	YP_009047211 AG006008 YP_007188586 AIG13103 ASL68960 AHY61344 AWH65917 AGP04896 QHA24685 QCC20721 AP040586	119 DGI WWHEDU 	3ATDAPSTF	GTRNPNNDSAIVTC	PF AI	158 P GTKLPK
(B) Lineage C Beta-CoV (Merbecovirus)	-Middle East respiratory syndro Human betacoronavirus 2c EHC/2 Betacoronavirus England 1 Coronavirus Neoromicia/PML-PHE Hypsugo bat coronavirus HKU25 Btvs-Betacov/Sc2013 Pipistrellus bat coronavirus Tylonycteris pachypus bat coro Hedgehog coronavirus 1 -Severe acute respiratory syndr Bat coronavirus RTG13	YP_009047211 AG006008 YP_00718586 AIG13103 ASL68960 AHV61344 AWH65917 AGP04896 QHA24685 QC220721 AP040586 QHR63308	119 DGI VWVHEDU 	2 3ATDAPSTF 	GTRNPNNDSA I VTC	2F AI	158 P GTKLPK Q R T LI LI Q 2S 2T
(B) Lineage C Beta-CoV (<i>Merbecovirus</i>)	-Middle East respiratory syndro Human betacoronavirus 2c EUK/2 Betacoronavirus England 1 Coronavirus Neoromicia/PML-PHE Hypsugo bat coronavirus HKU25 BtVs-BetaCoV/SC2013 Pipistrellus bat coronavirus Tylonycteris pachypus bat coro Ledegheg coronavirus 1 - Severe acute respiratory syndr Bat coronavirus RaTG13 Pangolin coronavirus	YP_009047211 AG006008 YP_007188586 AIG13103 ASL68960 AHY61344 AWH65917 AGP04896 QHA24685 QCC20721 AP040586 QHR63308 QHR63308 QIA48648	119 DGI VWVHEDU 	2 3ATDAPSTF 	GTRNPNNDSAIVTC	RF AI	158 P GTKLPK R T LI LI 2S 2T 2A
(B) Lineage C Beta-CoV (Merbecovirus)	Middle East respiratory syndro Human betacoronavirus 2c ENC/2 Betacoronavirus England 1 Coronavirus Neoromicia/PML-PHE Hypsugo bat coronavirus HKU25 Btvs-BetaCoV/SC2013 Pipistrellus bat coronavirus Tylonycteris pachypus bat coro Hedgehog coronavirus 1 - Severe acute respiratory syndr Bat coronavirus RaTG13 Pangolin coronavirus	YP_009047211 AG006008 YP_007188586 AIG13103 ASL68960 AHY61344 AWH65917 AGP04896 QHA24685 QC20721 AP040586 QHR63308 QIA48648 AHX37566	119 DGI VWVHEDO 	2 3ATDAPSTF 	GTRNPNNDSAIVTC	2F AI L PI L PI	158 P GTKLPK
(B) Lineage C Beta-CoV (Merbecovirus)	-Middle East respiratory syndro Human betacoronavirus 2c EHC/2 Betacoronavirus England 1 Coronavirus Neoromicia/ PML-PHE Hypsugo bat coronavirus HKU25 Btvs-Betacov/SC2013 Pipistrellus bat coronavirus Tylonycteris pachypus bat coro Hedgehog coronavirus 1 - Severe acute respiratory syndr Bat coronavirus RaTG13 Pangolin coronavirus Rhinolophus affinis coronaviru Coronavirus BtR1-Betacov/SC201	YP_009047211 AG008008 YP_007188586 AIG13103 ASL68960 AHY61344 AWH65917 AGP04896 QHA24885 QCC20721 AP040586 QHA24853 QIA48648 AIX37566 QDF43818	119 DGI VWVHEDO 	2 3ATDAPSTF 	GTRNPNNDSAIVTC	RF AI 	158 P GTKLPK Q T LI LI LI Q QS 2T 2A 2T 2T
(B) Lineage C Beta-CoV (<i>Merbecovirus</i>)	"Middle East respiratory syndro Human betacoronavirus 2c EUK/2 Betacoronavirus England 1 Coronavirus Neoromicia/PML-PHE Hypsugo bat coronavirus HKU25 BtVs-BetaCoV/SC2013 Pipistrellus bat coronavirus Tylonycteris pachypus bat coro Hedgehog coronavirus 1 - Severe acute respiratory syndr Bat coronavirus RaTG13 Pangolin coronavirus Rhinolophus affinis coronaviru Coronavirus BtR1-BetaCoV/SC201 BtHF-BetaCoV/JL2012	YP_009047211 AG006008 YP_007188586 AIG13103 ASL68960 AHY61344 AWH65917 AGP04996 QHA24685 QCC20721 AP040586 QHR63308 QHA8648 AHX37566 QDF43818 AIA62285	119 DGI VWVHEDO 	GATDAPSTF	GTRNPNNDSAIVTC	RF AI L PI L PI L PI L PI	158 P GTKLPK R LI LI LI 0S 2T 2A 2T 2T 2T 2T
(B) Lineage C Beta-CoV (Merbecovirus)	Middle East respiratory syndro Human betacoronavirus 2c EMC/2 Betacoronavirus England 1 Coronavirus Neoromicia/ PML-PHE Hypsugo bat coronavirus HKU25 Btvs-BetacoV/SC2013 Pipistrellus bat coronavirus Tylonycteris pachypus bat coro Hedgehog coronavirus 1 Severe acute respiratory syndr Bat coronavirus RaTG13 Pangolin coronavirus Rhinolophus affinis coronaviru Coronavirus BHAI-BetaCoV/SC201 BtHF-BetaCoV/HE2013	YP_009047211 AG006008 YP_007188586 AIG13103 ASL68960 AHY61344 AWH65917 AGP04896 QC220721 AP040586 QC420721 AP040586 QIA48648 AHX37566 QDF43818 AIA62285 AIA62218	119 DGI VWVHEDO 	2 3ATDAPSTF 	GTRNPNNDSAIVTC	2F AI 	158 P GTKLPK Q R LI LI LI 0S QS 2T 2T 2T 2T 2T
(B) Lineage C Beta-CoV (Merbecovirus)	-Middle East respiratory syndro Human betacoronavirus 2c EUK/2 Betacoronavirus England 1 Coronavirus Neoromicia/PML-PHE Hypsugo bat coronavirus HKU25 BtVs-Betacov/SC2013 Pipistrellus bat coronavirus Tylonycteris bat coronavirus Tylonycteris pachypus bat coro LHedgehog coronavirus 1 - Severe acute respiratory syndr Bat coronavirus RTG13 Pangolin coronavirus Rhinolophus affinis coronaviru Coronavirus BtR1-BetaCoV/SC201 BtH7-BetaCoV/JL2012 BtB3-BetaCoV/HB2013 Bat CoV 2012	YP_009047211 AG006008 YP_007188586 AIG13103 ASL68960 AHY61344 AWH65917 AGP04896 GHA2485 GC20721 AP040586 GHA8485 GLA8648 AHX37566 GDF43818 AIA62285 AIA62285 AIA62285 AIA62218 ON0468	119 DGI WWHED EIE N 	3ATDAPSTF	GTRNPNNDSAIVTC	2F AI 	158 P GTKLPK Q R T LI LI LI Q QS QT QT QT QT QT QT
(B) Lineage C Beta-CoV (Merbecovirus)	Middle East respiratory syndro Human betacoronavirus 2c ENC/2 Betacoronavirus England 1 Coronavirus Neoromicia/PML-PHE Hypsugo bat coronavirus HKU25 Btvs-BetaCoV/SC2013 Pipistrellus bat coronavirus Tylonycteris pachypus bat coro Hedgehog coronavirus 1 Severe acute respiratory syndr Bat coronavirus RaTG13 Pangolin coronavirus Rhinolophus affinis coronaviru Coronavirus BtR1-BetaCoV/SC201 BtRf-BetaCoV/HuB2013 Bat CoV Z79/2005 Bat Sever Bc/0 Bf1/2004	YP_009047211 AG006008 YP_007188586 AIG13103 ASL68960 AHY61344 AWH65917 AGP04896 0HA24685 0C220721 AGP04896 0HA24685 0HA24685 0HA246845 AHX37566 0DF43818 AIA62285 AIA62218 000468 ABD75315	119 DGIVWVHEDG EIE R R 	3ATDAPSTF	GTRNPNNDSAIVTC	NF AI	158 P GTKLPK
(B) Lineage C Beta-CoV (Merbecovirus)	-Middle East respiratory syndro Human betacoronavirus 2c EHC/2 Betacoronavirus England 1 Coronavirus Neoromicia/PML-PHE Hypsugo bat coronavirus HKU25 Btvs-BetaCoV/SC2013 Pipistrellus bat coronavirus Tylonycteris pachypus bat coro Hedgehog coronavirus 1 Severe acute respiratory syndr Bat coronavirus RTG13 Pangolin coronavirus Rhinolophus affinis coronaviru Coronavirus BtR1-BetaCoV/SC201 BtHS-BetaCoV/HuB2013 Bat CoV 279/2005 Bat SARS CoV Rf1/2004 Bat SARS CoV Rf1/2004	YP_009047211 AG008008 YP_007188586 AIG13103 ASL68960 AHY61344 AWH65917 AGP04896 QC20721 AP040586 QC420721 AP040586 QIA48648 AHX37566 QIF43818 AIA62285 AIA62318 Q00468 ABD75315	119 DGIVWVHEDO EIE N N EN EY-W-E EATE EATE EATE EATE EATE EATE EATE EATE	3ATDAPSTF	GTRNPNNDSAIVTC	17 AI 	158 P GTKLPK Q R LI LI LI 0S 0T 0T 0T 2T 2T 2T 2T 2T 2T
(B) Lineage C Beta-CoV (Merbecovirus)	-Middle East respiratory syndro Human betacoronavirus 2c EUK/2 Betacoronavirus England 1 Coronavirus Neoronicia/PML-PHE Hypsugo bat coronavirus HKU25 BtVs-BetaCoV/SC2013 Pipistrellus bat coronavirus Tylonycteris bat coronavirus Tylonycteris bat coronavirus Jylonycteris bat coronavirus Adedgehog coronavirus 1 -Severe acute respiratory syndr Bat coronavirus RTG13 Pangolin coronavirus Rhinolophus affinis coronaviru Coronavirus BtR1-BetaCoV/SC201 BtRf-BetaCoV/JL2012 BtR5-BetaCoV/H2005 Bat SARS coronavirus HKU3-4 Det COP Coronavirus HKU3-4	YP_009047211 AG006008 YP_007188566 AIG13103 ASL68960 AHY61344 AWH65917 AGP04896 QHA24685 QCC20721 AP040586 QHR63308 QLA48648 AHX37566 QDF43818 AIA62285 AIA62318 Q00468 ABD75315 ADE34730 ADE34730	119 DGIVWVHEDG EIE 	3ATDAPSTF	GTRNPNNDSAIVTC	17 АГ 	158 P GTKLPK
(B) Lineage C Beta-CoV (Merbecovirus)	Middle East respiratory syndro Human betacoronavirus 2c ENC/2 Betacoronavirus England 1 Coronavirus Neoromicia/PML-PHE Hypsugo bat coronavirus HKU25 Btvs-BetaCoV/SC2013 Pipistrellus bat coronavirus Tylonycteris pachypus bat coro Hedgehog coronavirus 1 Severe acute respiratory syndr Bat coronavirus RATG13 Pangolin coronavirus Rhinolophus affinis coronaviru Coronavirus BtR1-BetaCoV/SC201 BtR5-BetaCoV/HB2013 Bat Cov 279/2005 Bat SARS coronavirus HKU3-4 Bat SARS coronavirus HKU3-9	YP_009047211 AG006008 YP_007188586 AIG13103 ASL68960 AHV61344 AWH65917 AGP04896 QC20721 AP040586 QIA424685 QC20721 AP040586 QIA48648 AHX37566 QDF43818 AIA62285 AIA62218 Q00468 ABD75315 ADE34730 ADE34730	119 DGIVWVHED0 EIE M N N N N N N N N E	3ATDAPSTF	GTRNPNNDSAIVTC	17 АГ 	158 P GTKLPK
(B) Lineage C Beta-CoV (Merbecovirus) Lineage B Beta-CoV (Sarbecovirus)	Middle East respiratory syndro Human betacoronavirus 2c EUK/2 Betacoronavirus 2c EUK/2 Betacoronavirus England 1 Coronavirus Neoromicia/ PML-PHE Hypsugo bat coronavirus HKU25 Btvs-Betacov/SC2013 Pipistrellus bat coronavirus Tylonycteris pachypus bat coro Hedgehog coronavirus 1 - Severe acute respiratory syndr Bat coronavirus RTG13 Pangolin coronavirus Rhinolophus affinis coronaviru Coronavirus BtR1-BetaCoV/SC201 BtRf-BetaCoV/JL2012 BtRs-BetaCoV/JL2012 Btat SARS Cov Rf1/2004 Bat SARS coronavirus HKU3-4 Bat SARS coronavirus HKU3-4 BatSARS coronavirus HKU3-4	YP_009047211 AG008008 YP_007188586 AIG13103 ASL68960 AHY61344 AWH65917 AGP04896 QHA24885 QCC20721 AP040586 QHA2485 QC20721 AP040586 QHA24853 QLA48648 AIX37566 QDF43818 AIA62285 AIA62285 AIA62285 AIA62218 QO0468 ABD75315 ADE34730 ADE34787 AIA62298	119 DGIVW/HED EIE N N	3ATDAPSTF	GTRNPNNDSAIVTC	алан ан а	158 P GTKLPK
(B) Lineage C Beta-CoV (Merbecovirus) Lineage B Beta-CoV (Sarbecovirus)	Middle East respiratory syndro Human betacoronavirus 2c ENC/2 Betacoronavirus England 1 Coronavirus Neoromicia/PML-PHE Hypsugo bat coronavirus HKU25 BtVs-BetaCoV/SC2013 Pipistrellus bat coronavirus Tylonycteris pachypus bat coro Hedgehog coronavirus 1 Severe acute respiratory syndr Bat coronavirus RaTG13 Pangolin coronavirus Rhinolophus affinis coronaviru Coronavirus BtR1-BetaCoV/SC201 BtRf-BetaCoV/HuE2013 Bat CoV Z79/2005 Bat SARS coronavirus HKU3-4 Bat SARS coronavirus HKU3-9 = btRf-BetaCoV/HE2013	YP_009047211 AG006008 YP_007188586 AIG13103 ASL68960 AHV61344 AWH65917 AGP04896 0HA24685 0C20721 AGP04986 0HR63308 0IA48648 AHX37566 0DF43818 AIA62285 AIA62285 AIA62285 AJA62318 000468 ABD75315 ADE34730 ADE34730 AJA62298 AVP25399	119 DGIVW/HEDG EIE N N N	3ATDAPSTF	GTRNPNNDSA I V TC	Ж А	158 P GTKLPK
(B) Lineage C Beta-CoV (Merbecovirus) Lineage B Beta-CoV (Sarbecovirus)	-Middle East respiratory syndro Human betacoronavirus 2c EHC/2 Betacoronavirus England 1 Coronavirus Neoromicia/PML-PHE Hypsugo bat coronavirus HKU25 Btvs-BetacoV/SC2013 Pipistrellus bat coronavirus Tylonycteris pachypus bat coro Hedgehog coronavirus 1 - Severe acute respiratory syndr Bat coronavirus RTG13 Pangolin coronavirus Rhinolophus affinis coronaviru Coronavirus BtR1-BetaCoV/SC201 BtHS-BetaCoV/HUB2013 Bat CoV 279/2005 Bat SARS cov Rf1/2004 Bat SARS coronavirus HKU3-4 Bat SARS coronavirus HKU3-9 Bat Satoronavirus HKU3-4 Bat Coronavirus HKU9-4	YP_009047211 AG008008 YP_007188586 AIG13103 ASL68960 AHV61344 AWH65917 AGP04896 QHA24685 QC20721 AP040586 QHA24685 QC20721 AP040586 QHA24685 AD04386 AB075315 AIA62285 AJIA62318 Q00468 ABD75315 ADE34787 AJA62298 ADE34787 AJA62299 ABN10939	119 DGIVWVHED0 EIE M N N	3ATDAPSTF	GTRNPNNDSAIVTC	E AI	158 P GTKLPK
(B) Lineage C Beta-CoV (Merbecovirus) Lineage B Beta-CoV (Sarbecovirus) Lineage D Beta-CoV	-Middle East respiratory syndro Human betacoronavirus 2c EUK/2 Betacoronavirus 2c EUK/2 Betacoronavirus England 1 Coronavirus Neoromicia/PML-PHE Hypsugo bat coronavirus HKU25 BtVs-Betacov/SC2013 Pipistrellus bat coronavirus Tylonycteris pachypus bat coro Hedgehog coronavirus 1 -Severe acute respiratory syndr Bat coronavirus RTG13 Pangolin coronavirus Rhinolophus affinis coronaviru Coronavirus BtR1-BetaCoV/SC201 BtRf-BetaCoV/JL2012 BtRs-BetaCoV/JL2012 BtRS-BetaCoV/HB2013 Bat CoV 2079/2005 Bat SARS coronavirus HKU3-4 Bat SARS coronavirus HKU3-4 Bat SARS coronavirus HKU3-4 Bat coronavirus HKU3-4 Bat coronavirus HKU3-4 Bat coronavirus HKU3-4 Bat coronavirus HKU3-4 Bat coronavirus HKU3-4	YP_009047211 AG008008 YP_007188586 AIG13103 ASL68960 AHV61344 AWH65917 AGP04896 QC20721 AP040586 QC20721 AP040586 QIA48648 AIX37566 QDF43818 AIA62285 AIA62285 AIA62285 AIA62218 ADE34787 AJA62298 AVP25399 ABN10923	119 DGIVW/HED EIE N N	3ATDAPSTF S-	GTRNPNNDSAIVTC	E AI 	158 P GTKLPK
(B) Lineage C Beta-CoV (Merbecovirus) Lineage B Beta-CoV (Sarbecovirus) Lineage D Beta-CoV	Middle East respiratory syndro Human betacoronavirus 2c ENC/2 Betacoronavirus England 1 Coronavirus Neoromicia/PML-PHE Hypsugo bat coronavirus HKU25 Btvs-BetaCoV/SC2013 Pipistrellus bat coronavirus Tylonycteris pachypus bat coro Hedgehog coronavirus 1 Severe acute respiratory syndr Bat coronavirus RaTG13 Pangolin coronavirus Rhinolophus affinis coronaviru Coronavirus BtR1-BetaCoV/SC201 BtR5-BetaCoV/HeB2013 Bat Cov 279/2005 Bat SARS coronavirus HKU3-4 Bat SARS coronavirus HKU3-4 Bat SARS coronavirus HKU3-9 -BtRf-BetaCoV/HeB2013 -BtRf-BetaCoV/HeB2013 Bat coronavirus HKU3-4 Bat coronavirus HKU3-4 Bat coronavirus HKU9-4 Bat coronavirus HKU9-2 Bat coronavirus HKU9-10-2	YP_009047211 AG006008 YP_007188586 AIG13103 ASL68960 AHY61344 AWH65917 AGP04896 0C20721 AP040586 0LHA24685 0LHA24685 0LHA24685 0LHA24685 ALH37566 0LF43818 AIA62285 ALA62218 000468 ABD75315 ADE34730 AD2	119 DGIVWVHED EIE M N E E E	SATDAPSTF	GTRNPNNDSA I VTC	аF АІ 	158 P GTKLPK R R LI LI LI 0S 0T 0T 0T 0T
(B) Lineage C Beta-CoV (Merbecovirus) Lineage B Beta-CoV (Sarbecovirus) Lineage D Beta-CoV (Nobecovirus)	-Middle East respiratory syndro Human betacoronavirus 2c EHC/2 Betacoronavirus England 1 Coronavirus Neoromicia/PML-PHE Hypsugo bat coronavirus HKU25 Btvs-BetaCoV/SC2013 Pipistrellus bat coronavirus Tylonycteris bat coronavirus Tylonycteris pachypus bat coro Hedgehog coronavirus 1 Severe acute respiratory syndr Bat coronavirus RTG13 Pangolin coronavirus Rhinolophus affinis coronaviru Coronavirus BtR1-BetaCoV/SC201 BtHF-BetaCoV/HuB2013 Bat CoV 2012 BtRs-BetaCoV/HuB2013 Bat SARS cov Rf1/2004 Bat SARS coronavirus HKU3-4 Bat SARS coronavirus HKU3-9 Batt FetaCoV/HuB2013 Rousetlus bat coronavirus HKU3-9 Bat coronavirus HKU9-4 Bat coronavirus HKU9-4 Bat coronavirus HKU9-10-2 Bat coronavirus HKU9-3	YP_009047211 AG008008 YP_007188586 AIG13103 ASL68960 AHV61344 AWH65917 AGP04896 GC20721 AP040586 GC20721 AP040586 GUH863308 GIA48648 AHX37566 GDF43818 AIA62285 AIA62285 AIA62285 AD5315 ADE34787 AJA62298 AVP25399 ABN10923 ADM33586 ABN10931	119 DGIVW/HED EIE M Y-E Y-E Y-V-W-E EIATE EI-ATE EI-ATE EI-ATE EATE E	SATDAPSTF	GTRNPNNDSAIVTC	2F AI 	158 P GTKLPK
(B) Lineage C Beta-CoV (Merbecovirus) Lineage B Beta-CoV (Sarbecovirus) Lineage D Beta-CoV (Nobecovirus)	-Middle East respiratory syndro Human betacoronavirus 2c EMC/2 Betacoronavirus England 1 Coronavirus Neoromicia/PML-PHE Hypsugo bat coronavirus HKU25 Btvs-BetacoV/SC2013 Pipistrellus bat coronavirus Tylonycteris bat coronavirus Tylonycteris bat coronavirus Tylonycteris bat coronavirus -Severe acute respiratory syndr Bat coronavirus RaTG13 Pangolin coronavirus Rhinolophus affinis coronaviru Coronavirus BtR1-BetaCoV/SC201 BtRf-BetaCoV/HuB2013 Bat CoV 279/2005 Bat SARS coronavirus HKU3-4 Bat SARS coronavirus HKU3-4 Bat SARS coronavirus HKU3-4 Bat coronavirus HKU9-2 Bat coronavirus HKU9-2 Bat coronavirus HKU9-2 Bat coronavirus HKU9-5-1	YP_009047211 AG006008 YP_007188586 AIG13103 ASL68960 AIG13103 ASL68960 AIV61344 AWH65917 AGP04986 QHA24685 QC20721 AGP04986 QHA24685 QH484848 AIX37566 QDF43818 AIX62285 AIA62318 Q00468 ABD75315 ADE34730 ADE34787 AIA62298 AVP25399 ABN10939 ABN10931 ADM33562	119 DGIVWVHEDG EIE N E Y-E Y-E EY-E EY-E EATE E	3ATDAPSTF	GTRNPNNDSA I V TC 	аға	158 P GTKLPK
(B) Lineage C Beta-CoV (Merbecovirus) Lineage B Beta-CoV (Sarbecovirus) Lineage D Beta-CoV (Nobecovirus)	Middle East respiratory syndro Human betacoronavirus 2c EMC/2 Betacoronavirus England 1 Coronavirus Neoromicia/PML-PHE Hypsugo bat coronavirus HKU25 Btvs-BetaCoV/SC2013 Pipistrellus bat coronavirus Tylonycteris pachypus bat coro Hedgehog coronavirus 1 Severe acute respiratory syndr Bat coronavirus RTG13 Pangolin coronavirus Rhinolophus affinis coronaviru Coronavirus BtR1.BetaCoV/SC201 BtR5-BetaCoV/HB2013 Bat SARS CoV Rf1/2004 Bat SARS coronavirus HKU3-4 Bat Soronavirus HKU3-4 Bat coronavirus HKU3-4 Bat coronavirus HKU3-4 Bat coronavirus HKU3-4 Bat coronavirus HKU9-2 Bat coronavirus HKU9-10-2 Bat coronavirus HKU9-10-2 Bat coronavirus HKU9-10-2 Hat coronavirus HKU9-10-2 Hat coronavirus HKU9-10-2 Hat coronavirus HKU9-10-2 Hat coronavirus HKU9-10-2 Hat coronavirus HKU9-10-2 Bat coronavirus HKU9-10-2 Human coronavirus HKU9-10-2	YP_009047211 AG006008 YP_007188586 AIG13103 ASL68960 AHV61344 AWH65917 AGP04896 0C620721 AP040586 0CH820721 AP040586 0LHA24685 ALX37566 0DF43818 AIA62285 AIA62218 Q00468 ABD75315 AIA62218 Q00468 ABD75315 AIA62298 ABE34730 ADE3456 ADM33586 ADM33562 AJ024067	119 DGIVWVHED0 EIE N E E	SATDAPSTF SATDAPSTF <td< td=""><td>GTRNPNNDSA I VTC </td><td></td><td>158 P GTKLPK </td></td<>	GTRNPNNDSA I VTC 		158 P GTKLPK
(B) Lineage C Beta-CoV (Merbecovirus) Lineage B Beta-CoV (Sarbecovirus) Lineage D Beta-CoV (Nobecovirus)	-Middle East respiratory syndro Human betacoronavirus 2c EUK/2 Betacoronavirus England 1 Coronavirus Neoromicia/PML-PHE Hypsugo bat coronavirus HKU25 Btvs-BetaCoV/SC2013 Pipistrellus bat coronavirus Tylonycteris bat coronavirus Tylonycteris pachypus bat coro Hedgehog coronavirus 1 - Severe acute respiratory syndr Bat coronavirus RTG13 Pangolin coronavirus Rhinolophus affinis coronaviru Coronavirus BtR1-BetaCoV/SC201 BtRf-BetaCoV/JL2012 BtRs-BetaCoV/JL2012 Bts.BetaCoV/JL2012 Bts.BetaCoV/HB2013 Bat Cov 2079/2005 Bat SARS Coronavirus HKU3-4 Bat SARS coronavirus HKU3-4 Bat coronavirus HKU3-4 Bat coronavirus HKU9-3 -BtRf-BetaCoV/HB2013 -Rousetus bat coronavirus HKU9-3 Bat coronavirus HKU9-3 Bat coronavirus HKU9-3 Bat coronavirus HKU9-3 Bat coronavirus HKU9-3	YP_009047211 AG008008 YP_007188586 AIG13103 ASL68960 AHV61344 AWH65917 AGP04896 QHA24885 QCC20721 AP040586 QHA2485 QCC20721 AP040586 QIA48648 AIX37566 QDF43818 AIA62285 AIA62285 AIA62285 AIA62285 AIA62298 ADE34730 ADE34787 AIA62298 AVP25399 ABN10923 ABN10923 ADM33586 ADB10931 ADM33582 AJA20407 ALA50084	119 DGIVW/HED EIE N N N	3ATDAPSTF	GTRNPNNDSAIVTC		158 P GTKLPK
(B) Lineage C Beta-CoV (Merbecovirus) Lineage B Beta-CoV (Sarbecovirus) Lineage D Beta-CoV (Nobecovirus)	-Middle East respiratory syndro Human betacoronavirus 2c ENC/2 Betacoronavirus England 1 Coronavirus Neoromicia/PML-PHE Hypsugo bat coronavirus HKU25 Btvs-BetaCoV/SC2013 Pipistrellus bat coronavirus Tylonyteris pachypus bat coro Hedgehog coronavirus 1 - Severe acute respiratory syndr Bat coronavirus RaTG13 Pangolin coronavirus Rhinolophus affinis coronaviru Coronavirus BtR1-BetaCoV/SC201 BtR5-BetaCoV/HeB2013 Bat SARS CoV Rf1/2004 Bat SARS coronavirus HKU3-4 Bat SARS coronavirus HKU3-4 Bat Scoronavirus HKU3-4 Bat coronavirus HKU3-4 Bat coronavirus HKU9-4 Bat coronavirus HKU9-2 Bat coronavirus HKU9-5-1 -Bat coronavirus HKU9-5-1	YP_009047211 AG006008 YP_007188586 AIG13103 ASL68960 AHV61344 AWH65917 AGP04896 0C220721 AP040586 0DF43818 AIA62285 AIA62285 AIA62285 AIA62285 AIA62285 AIA62285 AJA62285 AJA62298 AVP25399 ABN10939 ABN10931 ADM33586 ADM33586 ADM33586 ADM33586 ADM33586 ADM33562 AJ024067 ALA50084 BA553430	119 DGIVW/HED EIE N E E E	3ATDAPSTF	GTRNPNNDSA I V TC 	аға	158 P GTKLPK
(B) Lineage C Beta-CoV (Merbecovirus) Lineage B Beta-CoV (Sarbecovirus) Lineage D Beta-CoV (Nobecovirus) Lineage A Beta-CoV (Nobecovirus)	-Middle East respiratory syndro Human betacoronavirus 2c EHC/2 Betacoronavirus England 1 Coronavirus Neoromicia/PML-PHE Hypsugo bat coronavirus HKU25 Btvs-BetaCoV/SC2013 Pipistrellus bat coronavirus Tylonycteris pachypus bat coro Hedgehog coronavirus 1 - Severe acute respiratory syndr Bat coronavirus RTG13 Pangolin coronavirus Rhinolophus affinis coronaviru Goronavirus BtR1-BetaCoV/SC201 BtHF-BetaCoV/L2012 BtRs-BetaCoV/HUB2013 Bat SARS coV RH1/2004 Bat SARS coronavirus HKU3-4 Bat SARS coronavirus HKU3-4 Bat SARS coronavirus HKU3-4 Bat coronavirus HKU3-4 Bat coronavirus HKU9-2 Bat coronavirus HKU9-2 Bat coronavirus HKU9-3 -Bat coronavirus HKU9-5-1 -Human coronavirus HKU23 Equine coronavirus HKU3 Equine coronavirus HKU3 Equine coronavirus HKU3	YP_009047211 AG008008 YP_007188586 AIG13103 ASL68960 AIV61344 AWH65917 AGP04896 QC20721 AP040586 QC20721 AP040586 QLHA2485 QC20721 AP040586 QLHA2485 AIA62308 QLHA84848 AHX37566 QDF43818 AIA62285 AIA62318 QO0468 ABD75315 ADE34787	119 DGIVW/HED EIE M Y-E Y-V-W-E EI-ATE EI-ATE EATE E	3ATDAPSTF	GTRNPNNDSAIVTC		158 P GTKLPK
(B) Lineage C Beta-CoV (Merbecovirus) Lineage B Beta-CoV (Sarbecovirus) Lineage D Beta-CoV (Nobecovirus) Lineage A Beta-CoV (Embecovirus)	-Middle East respiratory syndro Human betacoronavirus 2c EMC/2 Betacoronavirus England 1 Coronavirus Neoromicia/PML-PHE Hypsugo bat coronavirus HKU25 Btvs-BetacoV/SC2013 Pipistrellus bat coronavirus Tylonycteris pachypus bat coro Hedgehog coronavirus 1 -Severe acute respiratory syndr Bat coronavirus RaTG13 Pangolin coronavirus Rhinolophus affinis coronaviru Coronavirus BtR1-BetaCoV/SC201 BtR-BetaCoV/HuB2013 Bat CoV 279/2005 Bat SARS coronavirus HKU3-4 Bat SARS coronavirus HKU3-4 Bat SARS coronavirus HKU3-4 Bat SARS coronavirus HKU3-9 = BtR-BetaCoV/HuB2013 -Rousettus bat coronavirus HKU9-2 Bat coronavirus HKU9-10-2 Bat coronavirus HKU9-2 Bat coronavirus HKU9-2 Bat coronavirus HKU9-2 Bat coronavirus HKU9-2 Bat coronavirus HKU9-2 Bat coron	YP_009047211 AG006008 YP_007188586 AIG13103 ASL68960 AIG13103 ASL68960 AIK31544 AWH65917 AGP04986 QHA24685 QC20721 AP040586 QH484848 AIA62485 AIA62318 Q00468 AIA62285 AIA62318 Q00468 ADD5315 ADE34730 ADE34787 AIA62298 AVP25399 ABN10939 ABN10939 ABN10931 ADM33562 AJ024067 ALA50084 BA553430 AIL49389 Q090X6	119 DGIVWVHEDC EIE N E 	3ATDAPSTF	GTRNPNNDSA I V TC 		158 P GTKLPK

Figure 3. Partial sequence alignments of two conserved regions from the RdRp and nucleocapsid proteins showing a number of CSIs that are specific for the subgenera Merbecovirus and Nobecovirus. (A) Partial sequence alignment of RdRp protein showing a CSI consisting of 2-aa insertion (highlighted in blue and labeled (9) which is specific for the *Merbecovirus*. (B) Partial sequence alignment of nucleocapsid protein showing two different CSIs, one of which (④) is specific for the subgenus Merbecovirus and another CSI (labeled **⑤**), which is only present in different viruses from the subgenus *Nobecovirus*.

In the sequence alignment of the N-protein shown in Figure 3B, in addition to the CSI that is specific for *Merbecovirus*, there is another 2-aa deletion (marked **9**) present, which is specific for the subgenus Nobecovirus. In Figure 4, we present sequence alignments of two other conserved regions of the N-protein, where multiple CSIs specific for different lineages of Betacoronavirus are found. In the sequence alignment shown in Figure 4A, a CSI consisting of a 1-aa deletion is present (marked **③**) that is commonly shared by all viruses from the subgenera Merbecovirus and Sarbecovirus, but not found in the other two subgenera of Betacoronavirus. Close to this CSI, there is another CSI present consisting of a 1-aa insertion (marked **⑦**), which is uniquely shared by all viruses from the subgenus *Sarbecovirus*. Both

6

17

these CSIs are separated by conserved regions indicating that they constitute reliable characteristics and are not caused by sequence alignment artifacts. Figure 4B shows two additional CSIs, one consisting of a 2-aa deletion (marked ③) and another consisting of a 2aa insertion (marked **③**), which are also specific for the CoVs from the subgenus *Nobecovirus*. Based on these CSIs, members of the subgenera Nobecovirus and Sarbecovirus can be reliably distinguished from other CoVs.

122

(A)

			120		
	SARS-CoV-2	QIZ64406	YGANKDGI IWVATEGAL	NTPKD	H IGTRNPSNNAAIVLQLPQG
	Bat coronavirus RaTG13	QHR63308			A
	Pangolin coronavirus	QIA48630	E		N
	Bat SARS-like coronavirus	AVP78049	E		A
Lineage B	SARS coronavirus ExoN1	ACZ72117	EV		NT
Bata CoV	SARS coronavirus Frankfurt	AAP33707	EV		NT
Bela-Cov	Bat SARS coronavirus Rp1	AAZ67043	EV		N
(Sarbecovirus)	Bat SARS-like coronavirus YNLF	AKZ19095	E		NT
	BtBs-BetaCoV/GX2013	AIA62328	EVI		N
	Bat coronavirus Cp/Yunnan2011	AGC74175	EVI		N
	Bat SARS-like coronavirus RsSH	AGZ48815	EV		NT
	-MERS coronavirus	AVV62533	FR-VVHET	DA-ST	FN-DT-FAP-
	Bat coronavirus HKU5-2	ABN10891	FRSVHENT	DA-SV	FA-DPT-FAP-
	Human betacoronavirus 2c EMC/2	AG006008	FB-VVHEDT	DA-ST	FT-EAP-
	Betacoronavirus England 1	YP 007188586	FB-VVHEDT	DA-ST	EN-DST-EAP-
	Betacoronavirus Erinaceus/ VMC/	YP_009513018	FRTVYWES	FA-S-	FA-DT-FAP-
Lincore C	Tylonycteris bat coronavirus H	AGP04897	FRSVVYET	DA-SI	EA-DC-EAP-
Lineage C	BtTn-BetaCoV/ GX2012	ATA62359	FRSVVYET	DA-SV	EC-EAP-
Beta-CoV	Tylonycteris pachypus bat coro	0HA24685	FRSVVYET	DA-SV	FA-DC-FAP-
(Merbecovirus)	Human betacoronavirus 2c Jordan	AGH58724	FB-VVHEDT	DA-ST	EN-DST-FAP-
12. 2023	Betacoronavirus England 1	VP 007188586	FB-VV-HED-T	DA-ST	EN-DST-FAP-
	Human betacoronavirus 2c EMC/2	AG006008	FR-VV-HED-T	DA-ST	EN-DST-FAP-
	Pinistrellus hat coronavirus H	AGP04909	FRSVHENT	DA-SV	FA-DPT-FAP-
	BtPa-BetaCoV/ GD2013	ATA62350	FRSV	DA-SV	EA-DPT-FAP-
Lingage D	Bat coronavirus HKI9-5-2	ADM33570	SKNPDGAN T	-BVG-	
Lineage D	Bousettus bat coronavirus HKIN	AVP25401	- SKNPDGAN I	- RVG -	
Beta-Cov -	Coronavirus BtBt-BetaCoV/ GY201	ODE43843		-BVG-	Mana and Na DGa a PV and HD a
(Nobecovirus)	Bat coronavirus HKI19-4	ABN10939	W-OKNPDLGEDN T	-BTG-	Manager Na DS PV - FGS -
Lineage A	-But coronavirus 0043	AGT51416			
Beta CoV	Camel coronavirus HKU23	AL 450084	TDTFSNQ-D V	A-	- VD-DSDEPTRE-P-
E l · · · ·	Bodent coronavirus	ATP66782	- FII- VV SOO-D T	S S.	SD-D-SHE-PTRESP
(Embecovirus)	Mucine coronavirus	AF011512	DDIE-VVS00-D T	B-SA-	-VE-DSHEPTREAP-
		A 011012		11 0/1	
(B)			159	8	9 198
	r RousettusbatcoronavirusHKU9	AVP25399	IGTRNPSNDAAIPVQLA	DG 🚺 II	PKGFYAEG RN SRGNSRNNSF
Lineage D	BatcoronavirusHKU9-5-1	ADM33562	MNS		·····\$-·
Beta-CoV -	BatcoronavirusHKU9-3	ABN10931	MNS		S
(Nobecovirus)	BatcoronavirusHKU9-4	ABN10939	M N A G	S	S
(4.00000011110)	- TylonycterisbatcoronavirusHKU4	YP 001039960	FAVC-F-	P-TI-	N-HI TGOSS
	BatcoronavirusHKU4-4	ABN10873	FAVC-F-	P- TL -	N-HI TGQSS
	BtTp-BetaCoV/ GX2012	AIA62359	FAVC-F-	P- TL -	N-HI TGQSS
Lineage C	PipistrellusbatcoronavirusHKU5	AWH65917	FAPVT-F-	P- TT L	N-HI TGQLS
Beta-CoV -	BetacoronavirusEngland1	YP_007188586	FNSVT-F-	P- TK L	N-HI TGQSS
(Merhecovirus)	MERS coronavirus	YP_009047211	FNSVT-F-	P- TK L	N-HI TGQSS
(merbecovirus)	BtVg_BetaCoV/ SC2013	A1013103	FNSVI-F-	P- IK L	N-HI IGQSS
	HypsugobatcoronavirusHKU25	ASI 68960	FNVT-F-	P- TO I	N-HI TG055
	L BetacoronavirusErinaceus/ VMC/ D	AGX27818	FATVT-F-	P- TQ L	NCHI TGOSS
	SARScoronavirusRs_672/ 2006	ACU31047	N-NVLP	Q- TT L	G-QAS
	BatSARScoronavirusHKU3-4	ADE34730	P	Q- TT L	G-QSS

Figure 4. Partial sequence alignments of two conserved regions of the nucleocapsid proteins showing a number of CSIs specific for different subgenera of Betacoronavirus. (A) This sequence region depicts two different CSIs. The CSI labeled **6** is commonly shared by different viruses from the subgenera *Merbecovirus* and *Sarbecovirus*, whereas the CSIs marked **@** is specific for the viruses from the subgenera Sarbecovirus. (B) This sequence region depicts two CSIs marked 3 and 9 which are specific for the subgenus Nobecovirus. Dashes (-) in the alignments indicate identity with the amino acid shown in the top sequence.

ADE34787

ATA62285

ABD75315

AP040586

QHR63308 QIA48648

AWB14620

AID16664

AIL49389

AHN64796

AID16634

ATP66753

BatSARScoronavirusHKU3-9

LongquanRlratcoronavirus

Severeacuterespiratorysyndrome

DromedarycamelcoronavirusHKU23 LongquanAamousecoronavirus

BtRf-BetaCoV/JL2012 BatSARSCoVRf1/2004

BatcoronavirusRaTG13 Pangolincoronavirus

Humancoronavirus0C43

Murinecoronavirus

Rodentcoronavirus

Lineage B

Beta-CoV

(Sarbecovirus)

Lineage A

Beta-CoV

(Embecovirus)

164

---G-QSS--

--SG-0AS--

--NG-QAS--

---G-QAS--

---G-QAS---G-QAS-

S-R-APA--

S-R-APG--

S-R-AP---

S-R-AS---

R-APS

S-R-AP---

-----N-N----VL--PQ- TT L------

-----N-N----VL--PQ- TT L------

-----N-N---VL--PQ- TT L------

-----A-N----VL--PQ- TT L------

-----A-N---VL--PQ- TT L------

-VE-D--SHE---TRF-P- TV L-Q---V--TE-D--SHE---TRF-P- TV L-Q---V--VD-D--S-E---TRFPP- TV L-Q-Y-I--

-VD-D--S-E---TRFPP- TV L-Q-Y-I--

-QD-D--SFE---TRFPP- TV L-Q-Y-I--

VPE-D-TSHP-E-TRF-P-

TV

0

0

Lastly, in Figure 5 we present partial sequence alignment of the spike protein, where a 4-aa insertion (marked $\mathbf{\Phi}$) in a conserved region is commonly shared by all CoVs from the subgenera *Merbecovirus*, *Nobecovirus*, and *Sarbecovirus*. This insert is absent in all viruses from the subgenus *Embecovirus* as well as by viruses from other CoVs genera, viz., Alpha-, Delta-, and Gamma-CoVs). Based on its distribution in different CoVs, the genetic change giving rise to this CSI is postulated to have occurred in a common ancestor of the subgenera *Merbecovirus*, *Nobecovirus*, and *Sarbecovirus* after the divergence of *Embecovirus* as well as other genera of CoVs.

			847	10	907
	SARS-CoV-2	YP_009724390	DLICAQKFNGLTVLPPLLTDEMIAQYTSALLAGTITS	GWTF	GAGAALQIPFAMQMAYRFN
	BatcoronavirusRaTG13	QHR63300			
	Pangolincoronavirus	QIA48641			
	SARScoronavirus_beta	QIA48641	VDAAVSA-A		
	Coronavirus BtRs-BetaCoV/YN2018B	QDF43815	AAVSA-A		
1990 - 1990 - 1990 - 1990 - 1990 - 1990 - 1990 - 1990 - 1990 - 1990 - 1990 - 1990 - 1990 - 1990 - 1990 - 1990 -	Bat SARS-like coronavirus	AT098181	DAAVSA-A		
Lineage B	SARS coronavirus ExoN1	AFM43867	DAVSA-A		
Beta-CoV	Bat SARS coronavirus HKU3-8	ADE34766	AAVSA-A		
(Sarbecovirus)	Bat SARS coronavirus HKU3-2	AAZ41329	V-AAVSA-A		
,	BtRs-BetaCoV/ GX2013	AIA62320	AVSA-A		
	Coronavirus BtRs-BetaCoV/YN2018D	QDF43835	DAAVSA-A		
	Pipistrellus_bat_coronavirus_HKU5	YP_001039962	YVA-YKYDPY-E-ASGSIAGA	SA	-LSSFAAQSIFL-
L' 0	BtPa-BetaCoV/ GD2013	AIA62343	YVA-YKYDPY-E-ASGSIAGA	SA	-LSSFAAQSIFL-
Lineage C	Bat_coronavirus_HKU5-2	ABN10884	VVA VK	SA	-LSSFAAQSIFL-
Beta-CoV -	Bat_coronavirus_HKU5-3	ABN10893	YVA-YKYDPY-E-ASGSTAGA	SA	-LSSFAAQSIFL-
(Merbecovirus)	MERS_COFONAVIFUS	AT039390	VVA VK NDVN E A COTACV	A	-LSSFAAUSIFL-
	Betacoronavirus_England_1	1P_00/1885/9		A	-LOSFAAQSIFL-
	=Ret appapavipus HKU0 5 1	ADM22559		A	-LSSFAAUSIFL-
	Bat_coronavirus_HKU9_3	ADW335556		- T	TEVOV TIOFI
	Bat_coronavirus_HKU9-10-1	ADM22574			
Lineage D	Bat_coronavirus_HKU9-10-2	ADM33582		.v	- TSVGVT-LQF-L-
Beta-CoV -	Bousettus bat coronavirus HKU9	YP 001039971		. Y	- TTS-GVT-LQF-L-
(Nobecovirus)	Coronavirus BtBt-BetaCoV/ GX2018	0DE43840		.v	- ITSVGVT-LOF-L-
	Bat coronavirus HKU9-4	ABN10935	T-VIAIVSPG-Q-LL-VGAVAS-	- Y	-ITSVGVT-LQF-L-
	Bousettus bat coronavirus	AVP25406	T-TISIVSPG-Q-LL-VGAVAS-	- Y	- ITSVGV T - LQF - L -
	-Murine coronavirus	AF011507	L-V-SIKV-SESQ-SGAGAT-AAMFP		PWT AGV SLNVQ I -
	Bovine coronavirus	AG098871	V-SYIKSENQ-SGL-ATSASLFP		PWSAGVYLNVQI-
	Rabbit coronavirus HKU14	AFE48827	V-SYIKSENQ-SGL-ATSASLFP		PWT AGV YLNVQF - I -
Lineage A	Human coronavirus 0C43	AXX83351	V-SYK-IKSENQ-SGL-ATSASLFP		PWT AGV YLNVQ I -
Beta-CoV -	Camel coronavirus HKU23	ATI09449	L-V-SYIKSENQ-SGL-ATSASLFP		PWT AGV YLNVQ I -
(Embecovirus)	Human_coronavirus_HKU1	ABD75513	L-V-SIKI-SETQ-SGT-ATVAAMFP		PWSAGVSLNVQI-
(Lindecovirus)	Rat_coronavirus	AFG25760	L-V-SIKV-SESQ-SGAGAT-SAMFP		PWSAGVLSVQI-
	Rat_coronavirus_Parker	YP_003029848	L-V-SIKV-SESQ-SGAGAT-SAMFP		PWSAGVLSVQI-
	Equine_coronavirus	BAM15656	L-V-TIKV-SENQ-SGL-ATSASLFP		PWT AG YLNVQ I -
	-Human_coronavirus_229E	BAL45640	AYYIMGVADA-RM-MGS-IG-IALG		-LTS-ASSLAIQS-L-
Alaha CaV	229E-related_bat_coronavirus	APD51507	AYYIMGVADA-RM-MGS-IG-MALG		-ITG-AASLALQA-L-
Alpha-Cov -	Camel_alphacoronavirus	ALA50256	AYYIMGVADA-RM-MGS-IG-IALG		-LTS-AASLALQA-L-
	_Alpaca_respiratory_coronavirus	AFI49431	AYYIMGVADA-RM-MGS-IG-IALG		-LTS-AASLALQA-L-
	Human_coronavirus_NL63	BBL54116	AYYIMGVADA-RM-MGS-IG-MVLG		-LTS-AASLALQA-L-
	C ^{Duck_coronavirus}	AKF17724	TFYIMGVVDPDLMTLGSG-MSFG		-LSS-AST-VQA-I-
Gamma-CoV -	Turkey_coronavirus	CBA13338	TYYVLGVVDPTLMGLTSG-MAFG		- IT AA T - VQA - I -
	Avian_coronavirus	ANY59000	VREYLII-AQTMS-V-SMALG		- IT GA T - LQA - I -
	-European_turkey_coronavirus	ALQ43515	TYYVLGVVDPTLMGLTSG-MAFG		- IT AA T - VQA - I -
	Falcon_coronavirus_UAE-HKU27	BBC54822	AYY IM GVADPAKM - M AS - TG - MVFG		-FTAASLAVQS-L-
	Pigeon_coronavirus_UAE-HKU29	BBC54842	AYYIMGVADPAKM-MAS-TG-MVFG		-LTS-AASLAVQS-L-
Dalta CaV	Porcine_deltacoronavirus	QIV13127	V-S-YYIMGVVDA-KM-MGS-TGAMVFG		-LIATTAVQA-L-
Dena-Cov -	Inrusn_coronavirus_HKU12-600	YP_002308497	AYYS-IMGVVDA-KM-MAS-TG-MFFG		-LISSSVAVQA-L-
	BUIDUI_COPONAVIPUS_HKU11-934	TP_002308479	v-I-YYIMGVVDAQKM-MAS-IGAMVFG		-MIAASIAVQA-L-
	Sparrow_deltacoronavirus	AWV6/125	v-5-YYIMGVVDAQKM-MGS-TGAMVFG		-LIAAIAVQA-L-
	-wnite-eye_coronavirus_HKU16	YP_005352838	VYYIMGVVDA-KM-MGS-TGAMVFG		-LIAASIAVQA-L-

Figure 5. Excerpts from the sequence alignment of the spike protein showing a 4-aa CSI that is present only in the *Betacoronavirus* subgenera *Merbecovirus*, *Nobecovirus*, and *Sarbecovirus*. This CSI (**①**) provides evidence that the CoVs from these subgenera are more closely related to each other, and they evolved after the divergence of other CoVs. No change is observed in this region in the Omicron variant.

3.3. Localizations of the CSIs in Protein Structures

We have also mapped the locations of eight of the identified CSIs in the spike and N-proteins using their 3D structural coordinates (Figure 5). For these studies, we have used the available structures of the spike protein from SARS-CoV-2 (PDB ID: 6VSB) [34] in both

pre-fusion and post-fusion state and the cryo-EM based structure of the porcine epidemic diarrhea virus (PEDV) (PDB ID: 6U7K_A) [35], which is an alphacoronavirus. The CSIs in the spike protein for which the structural localization was determined include two large 14-aa deletions (**0** and **2**) which are specific for the genus *Betacoronavirus*, and a 4-aa CSI (**0**) which is commonly shared by members of the subgenera *Merbecovirus*, *Nobecovirus*, and *Sarbecovirus* (Figure 6).



Figure 6. Mapping the surface locations of eight of the identified CSIs in the spike and nucleocapsid proteins. (**A**) Cryo-EM-based structure of the post-fusion form of the SARS-CoV spike protein (PDB ID: 6m3w) showing the structural location of CSIs **0** and **0**. The regions where these CSIs are found are circled. Of these two CSIs, (**0**) is present within the HR2 motif, whereas CSI **0** is found within the HR1 motif in the S2 subunit. (**B**) The structural location of the 4-aa CSI (**0**), which is commonly shared by the *Merbecovirus*, *Nobecovirus*, and *Sarbecovirus* subgenera, using a superimposed structure of the spike protein from SARS-CoV-2 (shown in green) and the PEDV virus (shown in cyan color). (**C**) Crystal structure of the N-terminal domain of the N-protein (PDB ID: 6LNN) from MERS-CoV in which the structural locations of two CSIs (**0** and **6**) are highlighted. (**D**) Structure of the RNA-binding domain (RBD) of the N-protein (PDB ID: 7R98) from SARS-CoV-2 depicting the structural locations of three CSIs (**6**, **6**, and **6**) in RBD.

The cartoon representations of the superimposed forms of the 3D structures of the spike proteins from CoVs containing these CSIs are presented in Figure 6. In panel A of Figure 6, a cryo-EM-based structure of the post-fusion form of the SARS-CoV spike protein (PDB ID: 6m3w) was utilized to show the structural location of two large 14-aa CSIs (**0** and **2**). Of these CSIs, CSI **0** is present within the conserved heptad repeat 2 (HR2) motifs, and CSI **②** is present within the heptad repeat 1 (HR1) motif in the S2 subunit of the spike protein. Both the HR1 and HR2 motifs, which form a six-helical bundle in the S2-subunit, play a key role in mediating fusion and entry of CoV-2 into the host cell [36,37]. In Figure 6B, the structural location of the 4-aa CSI (**0** in Figure 5), which is commonly shared by the Merbecovirus, Nobecovirus, and Sarbecovirus subgenera, is shown using a superimposed structure of the spike proteins from SARS-CoV-2 (shown in green) and the PEDV-virus (shown in cyan color). In panel C, we show a crystal structure of the N-terminal domain of the N-protein (PDB ID: 6LNN) from MERS-CoV in which the structural locations of two CSIs (**4** and **5**) are highlighted. Similarly in panel D, we show the structure of the N-protein RNA-binding domain (RBD) (PDB ID: 7R98) from SARS-CoV-2 to depict the structural locations of three CSIs (**③**, **④**, and **③** shown in Figure 4A,B). As seen from these figures, all the CSIs identified and analyzed in this study are present in the surface-exposed loop regions of the spike and N-protein structures.

The structural localization of the two other CSIs i.e., a 2-aa insert (③ shown in Figure 3A) present near the N-terminal end of the RdRp protein, and a 2-aa insert (④ shown in Figure 4B) which is present in the RBD of N-protein, were not determined in this study, as experimentally solved structural information for these sequence regions is not available.

4. Discussion

CoVs have been responsible for three major outbreaks in the past 20 years including the current COVID-19 pandemic caused by the SARS-CoV-2 virus, which has infected >307 million people worldwide leading to >5.6 million deaths (https://coronavirus.jhu.edu/ (accessed on 2 February 2022) [1,2,5,6,38–41]. The two earlier outbreaks of CoVs, known as the severe acute respiratory syndrome (SARS) and the Middle East respiratory syndrome (MERS), were caused by the SARS-CoV and MERS-CoV, respectively. Although the CoVs are comprised of four genera, all CoVs responsible for the major outbreaks/pandemics are a part of the genus *Betacoronavirus*. Of the four main *Betacoronavirus* subgenera, both SARS-CoV and SARS-CoV-2 are part of the *Sarbecovirus* subgenus, whereas the MERS-CoV belongs to the subgenus *Merbecovirus* [1,3,5,6]. Thus, in terms of the clinical significance and human health impact, members of the genus Betacoronavirus are of utmost importance. Other viruses such as OC43, and HKU1, which cause mild common cold-like symptoms, are a part of the *Embecovirus* subgenus [1]. In this study, we analyzed the sequences of four major conserved structural proteins i.e., spike, nucleocapsid, envelope, and RdRp proteins, which play central roles in cellular infection and replication [1,2], for the presence of conserved signature indels that are either specific for the genus *Betacoronavirus* or its constituent subgenera. The results of these studies have identified ten novel CSIs and information regarding the viral group specificity and some characteristics of these CSIs are summarized in Table 1.

Of these CSIs, two CSIs in the spike protein are specific for all members of the genus *Betacoronavirus*, whereas six CSIs found in the N-protein and one CSI found in RdRp protein are specific for members of the betacoronaviruses' subgenera *Sarbecovirus*, *Merbecovirus*, and *Nobecovirus*. One additional identified CSI in the spike protein (CSI ①, Figure 5) is shared explicitly by viruses from the *Merbecovirus*, *Nobecovirus*, and *Sarbecovirus* subgenera, providing insights into the branching order and evolutionary relationships among the *Betacoronavirus* lineages. The distribution pattern of this CSI provides evidence that the CoVs from these three subgenera are more closely related to each other, and they evolved after the divergence of the embecoviruses and viruses from the subgenus *Embecovirus*, which lack this CSI, have been reported to differ from the other three betacoronavirus subgenera

in that they contain an additional shorter spike-like protein, hemagglutinin esterase [42], which is not present in the three *Betacoronavirus* subgenera containing this 4-aa insert in the spike protein. Although the presence of this 4-aa insert coincides with the loss of the hemagglutinin esterase protein from these three subgenera, it is unclear whether these two genetic events are functionally correlated. The shared presence of the CSIs **0**, **2**, and **0** by a number of different subgenera of betacoronaviruses indicates that they represent important conserved properties of these viruses, while these viruses differ from each other in other regards.

Table 1. Conserved signature indels found in spike, nucleocapsid, and RNA-dependent RNA polymerase (RdRp) proteins that are specific for various members of lineages of coronaviruses.

Protein Name	Acc. No:	Indel Length	Indel Location	Indel Specificity	Figure No:
Spike	YP_009724390	14-aa del	1172-1205	β-CoV	Figure 2
Spike	YP_009724390	14-aa del	915-950	β-CoV	Figure 2
RdRp	AWH65886	2-aa ins	17–57	Merbecovirus	Figure 3A
Nucleocapsid	YP_009047211	1-aa del	119-158	Merbecovirus	Figure 3B
Nucleocapsid	YP_009047211	2-aa del	119-158	Nobecovirus	Figure 3B
Nucleocapsid	QIZ64406	2-aa del	123–169	Sarbecovirus and Merbecovirus	Figure 4A
Nucleocapsid	QIZ64406	1-aa ins	123-169	Sarbecovirus	Figure 4A
Nucleocapsid	AVP25399	2-aa del	159-198	Nobecovirus	Figure 4B
Nucleocapsid	AVP25399	2-aa ins	159-198	Nobecovirus	Figure 4B
Spike	YP_009724390	4-aa ins	847–907	Sarbecovirus, Merbecovirus and Nobecovirus	Figure 5

Earlier work on the CSIs shows that the genetic changes represented by them are functionally important for the group of organisms for which these CSIs are specific [30,43]. Furthermore, earlier studies showed that all studied CSIs are localized in surface-exposed loops of the structures of different proteins [19,28–31,44]. In accordance with the results from earlier studies, all eight CSIs whose structural localization was analyzed in this study were also found to be located within the surface-exposed loops of the spike and nucleocapsid proteins. Surface-exposed loops in proteins are known to play important functional roles by mediating novel protein–protein or protein–ligand interactions [29,43,45,46]. Of the CSIs identified in the present work, the two CSIs, which are specific for the genus Betacoronavirus, are both comprised of 14-aa deletions in the spike protein. Interestingly, one of these CSIs (2) is present within conserved heptad repeat 1 (HR1) motif, whereas the other CSI $(\mathbf{0})$ is found within the heptad repeat 2 (HR2) motif of the spike protein S2 subunit. The HR1 and HR2 motifs in the S2 subunit are known to interact with each other to form a six-helical bundle, which by bringing viral and cellular membranes in proximity, plays a crucial role in mediating membrane-fusion and entry of CoV-2 into the host cell [36,37,47]. As both these large CSIs are deletions, in other genera of CoVs, that do not contain these deletions, the lengths of the HR1 and HR2 motifs are longer than those found in the betacoronaviruses. It is of much interest to note that these two large CSIs, in addition to the members of the genus *Betacoronavirus* are also commonly shared by the avian coronavirus, which is a Gammacoronavirus. The avian CoV, also known as infectious bronchitis virus (IBV) is a highly infectious virus of major economic concern and is responsible for most of the infections caused by the Gammacoronaviruses [48,49]. It is unclear at present, how these important changes brought about by these two large CSIs may affect the cellular function of the spike protein in the betacoronaviruses and avian-CoV. However, in view of the fact that the viruses containing these large deletions (CSIs) comprise the most infectious members of the Orthocoronavirinae family, it is hypothesized that these genetic changes likely play an important role in determining the pathogenicity and infectiousness of the coronaviruses.

With regard to the functional significance of the CSIs, it should be noted that two of the CSIs in the spike protein described in our recent work, which are commonly shared by both SARS-CoV-2r cluster of viruses and the SARS viruses [7,12,50], are located within the receptor-binding domain of the spike protein and their sequences form a significant portion of the receptor-binding motif of these viruses [5,51–54]. The residues from these CSIs have been shown to play a critical role in the binding of spike protein from these viruses to the human ACE2 receptor [12,13,51,53,55,56]. These observations underscore the importance of the identified CSIs in the functioning and pathogenicity of viruses. Although the functions of the CSIs identified in the present work, which are specific for *Betacoronavirus* or its different subgenera are presently not known, based on earlier work, it is hypothesized that these CSIs will also be playing important roles in the functioning of these CSIs by experimental studies.

In addition to the CSIs identified in the present work, our recent work also identified several CSIs specific for several lineages of *Sarbecovirus*, including two CSIs specific for the SARS-CoV-2-related cluster of viruses [7]. Because of the specificities of the CSIs identified in the present study and our earlier work for different lineages of betacoronaviruses, they provide novel molecular means for distinguishing viruses from these groups/lineages from each other. Furthermore, as all CSIs identified in this study, and in our earlier work [8], are flanked by conserved regions, the sequences for these regions provide potential means for developing novel diagnostic tests to identify these specific viruses [57,58]. These tests can be based on different commonly employed experimental techniques, viz., PCR-based, q-PCR-based, immunological, or antibody-based methods, as well as in silico identification in genomic and metagenomic sequences by means of BLAST searches. It should be noted that the CSIs have been successfully used previously for development of highly specific diagnostic tests for two important bacterial pathogens [16,57,58].

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10 .3390/genes13030423/s1. Figure S1. A maximum-likelihood tree based on the sequence alignment of spike protein from representative viruses/strains from different genera/subgenera of CoVs.

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