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COMMENTARY

Genetic relatedness of the novel human group C betacoronavirus to *Tylonycteris* bat coronavirus HKU4 and *Pipistrellus* bat coronavirus HKU5

Patrick CY Woo[1,2,3,4,*], Susanna KP Lau[1,2,3,4,*], Kenneth SM Li[1], Alan KL Tsang[1] and Kwok-Yung Yuen[1,2,3,4]

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The recent outbreak of severe respiratory infections associated with a novel group C betacoronavirus (HCoV-EMC) from Saudi Arabia has drawn global attention to another highly probable "SARS-like" animal-to-human interspecies jumping event in coronavirus (CoV). The genome of HCoV-EMC is most closely related to *Tylonycteris* bat coronavirus HKU4 (Ty-BatCoV HKU4) and *Pipistrellus* bat coronavirus HKU5 (Pi-BatCoV HKU5) we discovered in 2006. Phylogenetically, HCoV-EMC is clustered with Ty-BatCoV HKU4/Pi-BatCoV HKU5 with high bootstrap supports, indicating that HCoV-EMC is a group C betaCoV. The major difference between HCoV-EMC and Ty-BatCoV HKU4/Pi-BatCoV HKU5 is in the region between S and E, where HCoV-EMC possesses five ORFs (NS3a-NS3e) instead of four, with low (31%–62%) amino acid identities to Ty-BatCoV HKU4/Pi-BatCoV HKU5. Comparison of the seven conserved replicase domains for species demarcation shows that HCoV-EMC is a novel CoV species. More intensive surveillance studies in bats and other animals may reveal the natural host of HCoV-EMC.

The recent outbreak of severe respiratory tract infections associated with a novel human group C betacoronavirus originating from Saudi Arabia has drawn global attention to another highly probable "SARS-like" interspecies jumping event of coronavirus (CoV) from animal to human. In June 2012, a novel CoV was isolated using Vero cells from the lung tissue of a 60-year-old resident of Saudi Arabia with fatal acute pneumonia and renal failure. In September 2012, another 49-year-old male resident of Qatar with severe acute pneumonia and renal failure and recent travel history to Saudi Arabia was admitted to an intensive care unit in Qatar. RT-PCR and sequencing of a short fragment of RNA-dependent RNA polymerase (RdRp) confirmed the presence of the same CoV as detected in the first Saudi Arabian case.[1] Complete genome sequencing of the virus isolated from the first patient was performed by Fouchier *et al.* at the Erasmus University Medical Centre, the Netherlands, and the sequence was released on September 28, 2012 (GenBank accession NO JX869059 and named as human betacoronavirus 2c EMC/2012). So far, there is no evidence of human-to-human transmission. The source of the virus remains obscure. In this article, this novel human group C betaCoV is abbreviated as HCoV-EMC.

After the SARS epidemic, we started to focus on CoV biodiversity, genomics and phylogeny and built up an evolutionary map of CoV evolution. Before 2003, there were less than 10 CoVs with complete genomes available, which include two human CoVs, human coronavirus 229E (HCoV-229E) and human coronavirus OC43 (HCoV-OC43). By September 2012, the number of CoVs with complete genomes sequenced had tripled. It includes two additional human CoVs, human coronavirus NL63 (HCoV-NL63) and human coronavirus HKU1 (HCoV-HKU1).[2,3] Traditionally, CoVs were classified into groups 1, 2 and 3. In 2011, the Coronavirus Study Group of the International Committee for Taxonomy of Viruses has re-classified these three groups of CoVs as three genera, *Alphacoronavirus*, *Betacoronavirus* and *Gammacoronavirus*; and we have discovered a fourth genus of CoV, *Deltacoronavirus*, which includes at least nine avian CoVs and a porcine coronavirus HKU15.[4,5] Within the betaCoVs, they are further subclassified into group A, including HCoV-HKU1, HCoV-OC43, bovine coronavirus (BCoV), sable antelope coronavirus, giraffe coronavirus, equine coronavirus, porcine hemagglutinating encephalomyelitis virus, murine hepatitis virus, rat coronavirus and rabbit coronavirus HKU14 (RbCoV HKU14);[6] group B, including the human and civet SARS-related CoVs (SARSr-CoV) and SARS-related *Rhinolophus* bat coronavirus (SARSr-Rh-BatCoV);[7,8] group C, including *Tylonycteris* bat coronavirus HKU4 (Ty-BatCoV HKU4) and *Pipistrellus* bat coronavirus HKU5 (Pi-BatCoV HKU5) we discovered in 2006;[9,10] and group D, including *Rousettus* bat coronavirus HKU9 (Ro-BatCoV HKU9).[10,11] In addition to Ty-BatCoV HKU4 and Pi-BatCoV HKU5, other group C bat betaCoVs should also be present, but their complete genome sequences are not available.[12,13] Based on the CoVs discovered, we have constructed a model of CoV evolution, with evidence supporting that bat CoVs are the gene source of alphaCoVs and betaCoVs and avian CoVs are the gene source of gammaCoVs and deltaCoVs.[5] All these works have laid down an evolutionary map for rapid phylogenetic and bioinformatics analyses of HCoV-EMC. The diversity of CoVs is a result

[1]Department of Microbiology, The University of Hong Kong, Hong Kong, China [2]State Key Laboratory of Emerging Infectious Diseases, The University of Hong Kong, Hong Kong, China [3]Research Centre of Infection and Immunology, The University of Hong Kong, Hong Kong, China and [4]Carol Yu Centre for Infection, The University of Hong Kong, Hong Kong, China
*PCY Woo and SKP Lau contributed equally to the manuscript.
Correspondence: KY Yuen
E-mail: kyyuen@hkucc.hku.hk
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of the infidelity of RdRp which make CoV genomes especially plastic, a high frequency of homologous RNA recombination due to their unique random template switching during RNA replication, and their large genomes. In addition to biodiversity, a number of natural recombination and possible interspecies jumping events has also been documented in betaCoVs.[6,11,14–18] For group A betaCoVs, molecular clock analysis has shown that HCoV-OC43 is a relatively recent zoonotic virus of bovine origin that emerged in around 1890 likely from bovine-to-human transmission.[17] We have also recently discovered RbCoV HKU14, closely related to other members of the species *Betacoronavirus 1* including HCoV-OC43 and BCoV, with recombination events that may have played a role in interspecies transmission of these HCoV-OC43-related viruses between human, cattle, rabbits, swine and horses.[6] Despite having circulated in humans for more than a century, HCoV-OC43 is also found to be continuously evolving, with the recent emergence of a novel genotype due to natural recombination.[15] For group B betaCoVs, SARSr-CoV is believed to be transmitted from civet to humans, although it is the horseshoe bat that was likely the primary host.[7,8] Civet SARSr-CoV was also likely a recombinant virus arising from different strains of SARSr-Rh-BatCoV from different geographical locations in China.[14,16] Although no interspecies transmission events have been documented in group D betaCoVs, we have also identified recombination events between different Ro-BatCoV HKU9 strains from different bat individuals, which may have allowed for the generation of different genotypes.[11] While these findings supported that betaCoVs have the propensity to recombine and cause interspecies transmission, such events were unknown in group C betaCoVs. As HCoV-EMC is most closely related to Ty-BatCoV HKU4 and Pi-BatCoV HKU5, it would be important to study their genetic relatedness, which may provide clues on whether bats are the possible origin as in SARSr-CoV.

The genome characteristics and organization of HCoV-EMC are similar to those of Ty-BatCoV HKU4 and Pi-BatCoV HKU5. Ty-BatCoV HKU4 was discovered from lesser bamboo bats (*Tylonycteris pachypus*) and Pi-BatCoV HKU5 was discovered from Japanese pipistrelles (*Pipistrellus abramus*) in Hong Kong.[9] Both lesser bamboo bats and Japanese pipistrelles are insectivorous microbats found in China and some other parts of Asia. The size of the genome of HCoV-EMC is 30 106 bases, slightly smaller than those of Ty-BatCoV HKU4 (30 286 to 30 316 bases) and Pi-BatCoV HKU5 (30 482 to 30 488 bases); and the G+C content is 41%, in between those of Ty-BatCoV HKU4 (38%) and Pi-BatCoV HKU5 (43%). The replicase ORF1ab occupies 21.5 kb of the genome. This ORF encodes 16 putative non-structural proteins, including nsp3 (which contains the putative papain-like protease (PL^{pro})), nsp5 (putative chymotrypsin-like protease ($3CL^{pro}$)), nsp12 (putative RdRp), nsp13 (putative helicase (Hel)) and other proteins of unknown functions. These proteins are produced by proteolytic cleavage of the large replicase polyprotein by PL^{pro} and $3CL^{pro}$ at specific sites which are conserved with those in Ty-BatCoV HKU4 and/or Pi-BatCoV HKU5 (Table 1).

HCoV-EMC has the same basic genome structure as Ty-BatCoV HKU4 and Pi-BatCoV HKU5 (Figure 1). It also possesses the same putative transcription regulatory sequence (TRS) motif, 5'-ACGAAC-3', as Ty-BatCoV HKU4 and Pi-BatCoV HKU5, at the 3' end of the leader sequence and precedes each ORF except NS3c, NS3e and N. This TRS has also been shown to be the TRS for other group B, C and D betaCoVs. The TRS for N is 5'-ACGAAU-3'. Similar to other group B, C and D betaCoVs, the genome of HCoV-EMC has a putative PL^{pro}, which is homologous to $PL2^{pro}$ of alphaCoVs and group A betaCoVs and PL^{pro} of gammaCoVs and deltaCoVs. Similar to Ty-BatCoV HKU4 and Pi-BatCoV HKU5, no proteolytic cleavage site is present in S of HCoV-EMC. All cysteine residues in S of HCoV-EMC, Ty-BatCoV HKU4 and Pi-BatCoV HKU5 are conserved. In contrast to the genomes of Ty-BatCoV HKU4 and Pi-BatCoV HKU5 which contain four ORFs that encode putative non-structural proteins (NS3a, NS3b, NS3c and NS3d) between S and E, this region of HCoV-EMC contains five ORFs that encode putative non-structural proteins NS3a, NS3b, NS3c, NS3d and NS3e (Figure 1). This is the region of HCoV-EMC that possesses the lowest amino acid identities to those in Ty-BatCoV HKU4 and Pi-BatCoV HKU5. NS3a, NS3b and NS3c of HCoV-EMC possess 42%–43%, 41%–47% and 31% amino acid identities to NS3a, NS3b and NS3c of Ty-BatCoV HKU4 and Pi-BatCoV HKU5, respectively. NS3d of HCoV-EMC is homologous to amino acids 1 to 110/

Table 1 Characteristics of putative non-structural proteins of ORF1ab in Ty-BatCoV HUK4, Pi-BatCoV HKU5 and HCoV-EMC

nsp	Putative function/domain[a]	Amino acids (first residue^{position} - last residue^{position})		
		Ty-BatCoV HKU4	Pi-BatCoV HKU5	HCoV-EMC
nsp1	Unknown	M^{1}–G^{195}	M^{1}–G^{195}	M^{1}–G^{193}
nsp2	Unknown	D^{196}–G^{847}	D^{196}–G^{851}	D^{194}–G^{853}
nsp3	Putative PL^{pro} domain	M^{848}–G^{2784}	A^{852}–G^{2829}	A^{854}–G^{2739}
nsp4	Hydrophobic domain	G^{2785}–Q^{3291}	G^{2830}–Q^{3337}	G^{2740}–Q^{3247}
nsp5	$3CL^{pro}$	S^{3292}–Q^{3597}	S^{3338}–Q^{3643}	S^{3248}–Q^{3553}
nsp6	Hydrophobic domain	S^{3598}–Q^{3889}	S^{3644}–Q^{3935}	S^{3554}–Q^{3845}
nsp7	Unknown	S^{3890}–Q^{3972}	S^{3936}–Q^{4018}	S^{3846}–Q^{3928}
nsp8	Unknown	A^{3973}–Q^{4171}	A^{4019}–Q^{4217}	A^{3929}–Q^{4127}
nsp9	Unknown	N^{4172}–Q^{4281}	N^{4218}–Q^{4327}	N^{4128}–Q^{4237}
nsp10	Unknown	A^{4282}–Q^{4420}	A^{4328}–Q^{4466}	A^{4238}–Q^{4377}
nsp11	Unknown (short peptide at the end of ORF1a)	S^{4421}–V^{4434}	S^{4467}–L^{4480}	S^{4378}–L^{4391}
nsp12	RdRp	S^{4421}–Q^{5354}	S^{4467}–Q^{5400}	S^{4378}–Q^{5310}
nsp13	Hel	A^{5355}–Q^{5952}	A^{5401}–Q^{5998}	A^{5311}–Q^{5908}
nsp14	ExoN	S^{5953}–Q^{6475}	S^{5999}–Q^{6522}	S^{5909}–Q^{6432}
nsp15	XendoU	G^{6476}–Q^{6817}	G^{6523}–Q^{6871}	G^{6433}–Q^{6775}
nsp16	2'-O-MT	A^{6818}–L^{7119}	A^{6872}–R^{7179}	A^{6776}–R^{7078}

Abbreviations: [a]PL^{pro}, papain-like protease ; $3CL^{pro}$, chymotrypsin-like protease; RdRp, RNA-dependent RNA polymerase; Hel, helicase; ExoN, 3'-to-5' exonuclease; XendoU, poly(U)-specific endoribonuclease; 2'-O-MT, S-adenosylmethionine-dependent 2'-O-ribose methyltransferase.

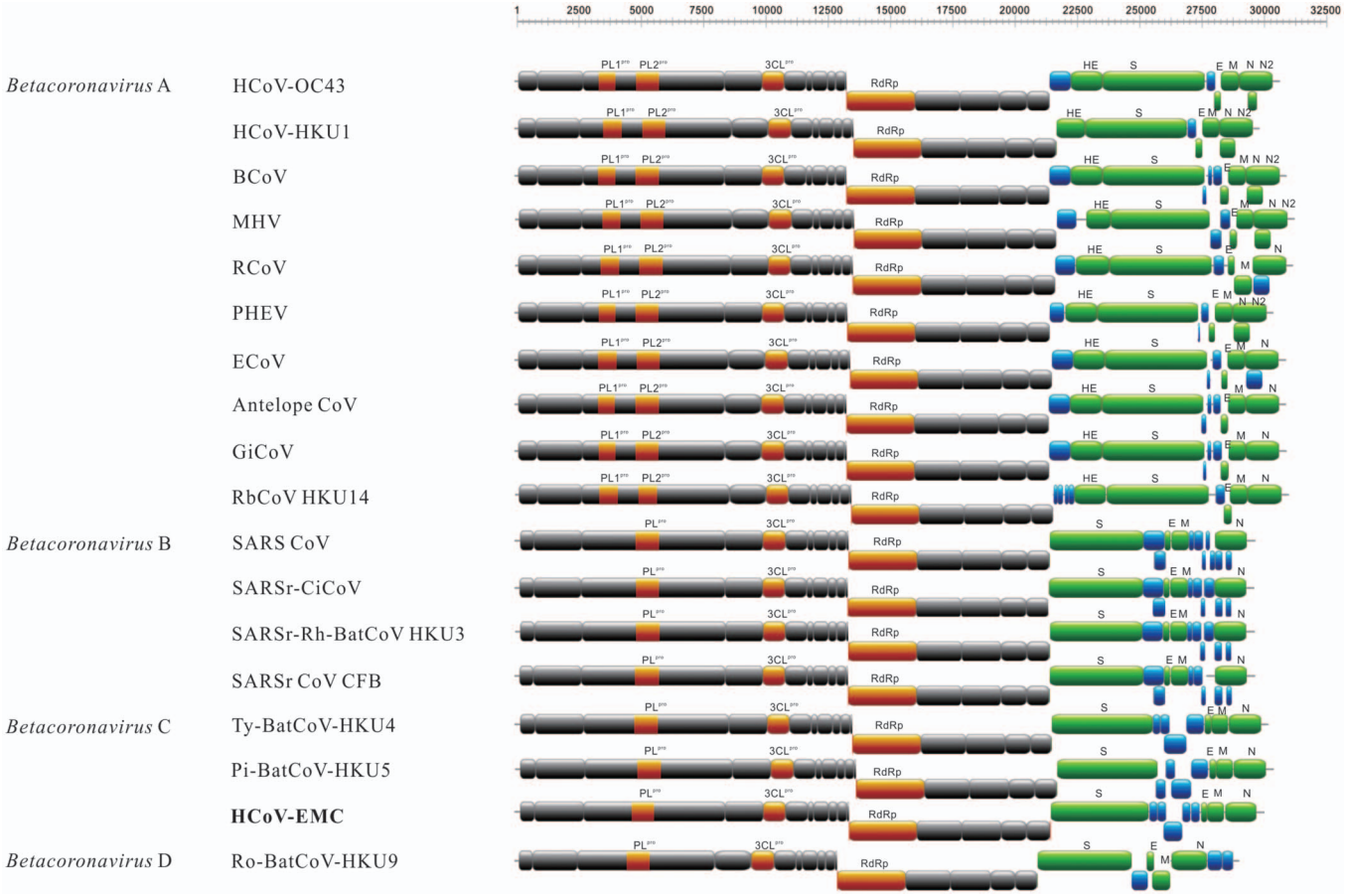
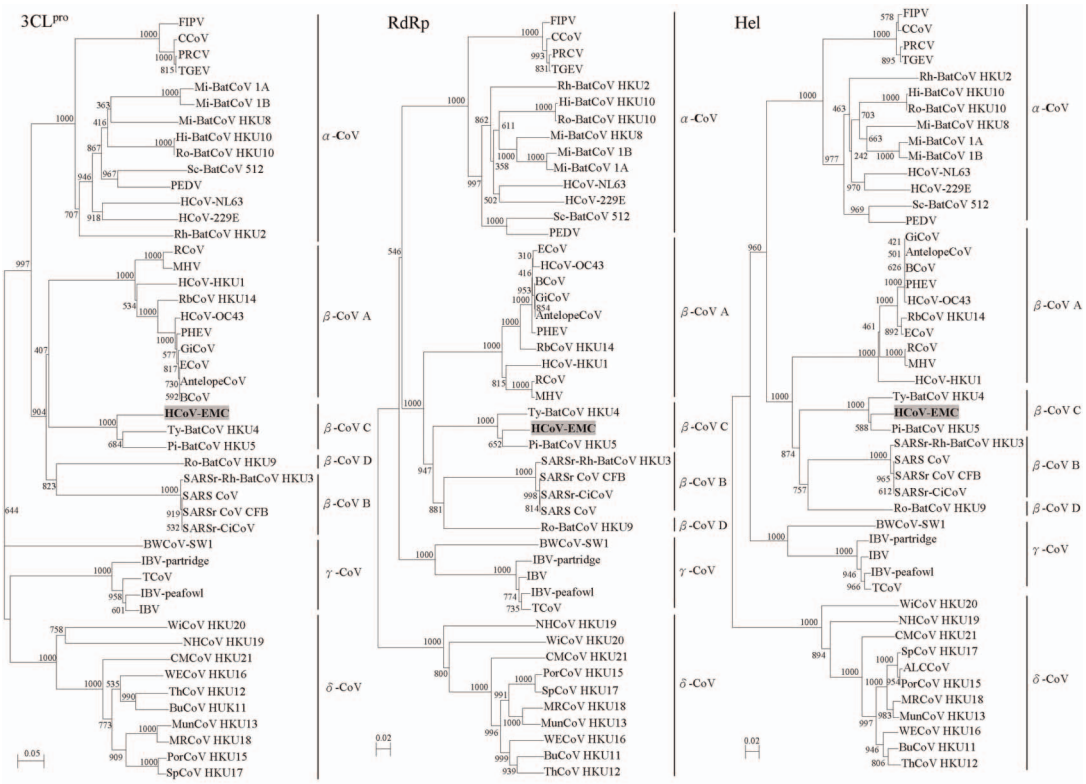


Figure 1 Genome organizations of HCoV-EMC and other betaCoVs. Papain-like proteases ($PL1^{pro}$, $PL2^{pro}$ and PL^{pro}), chymotrypsin-like protease ($3CL^{pro}$) and RNA-dependent RNA polymerase (RdRp) are represented by orange boxes. Haemagglutinin esterase (HE), spike (S), envelope (E), membrane (M) and nucleocapsid (N) are represented by green boxes. Putative accessory proteins are represented by blue boxes. HCoV-EMC is shown in bold.

103 of NS3d in Ty-BatCoV HKU4 and Pi-BatCoV HKU5 (35%–49% amino acid identities), with a stop codon UAG present at nucleotide position 27 160, leading to premature termination. NS3e of HCoV-EMC is homologous to amino acids 116/122 to 223/227 of NS3d in Ty-BatCoV HKU4 and Pi-BatCoV HKU5 (60%–62% amino acid identities). NS3c and NS3e do not possess any TRS or internal ribosomal entry site. BLAST search revealed no amino acid similarities between these putative non-structural proteins and other known proteins and no functional domains were identified by PFAM and InterProScan. TMHMM and TMpred analyses show one and two putative transmembrane domains in NS3a (residues 9 to 29) and NS3d (residues 36 to 56 and 71 to 91), respectively. Similar to Ty-BatCoV HKU4 and Pi-BatCoV HKU5, the 3' untranslated region of the genome of HCoV-EMC contains predicted bulged stem-loop structures 16 to 76 nucleotides downstream of the N genes. Downstream to the bulged stem-loop structure, 97 to 121 nucleotides downstream of the N genes, a pseudoknot structure is present. Bootscan analysis did not show any recombination between HCoV-EMC, Ty-BatCoV HKU4 and Pi-BatCoV HKU5.

The phylogenetic trees constructed using the amino acid sequences of the $3CL^{pro}$, RdRp, Hel, S and N of HCoV-EMC and other CoVs are shown in Figure 2. For all the five genes, HCoV-EMC is clustered with Ty-BatCoV HKU4 and Pi-BatCoV HKU5, with high bootstrap supports in all cases, indicating that HCoV-EMC is a group C betaCoV (Figure 2). Although it seems that HCoV-EMC is clustered with Pi-BatCoV HKU5 in the phylogenetic trees constructed using RdRp and Hel, the bootstrap supports were only 652 and 588, respectively, suggesting that there is no obvious difference between the relatedness of HCoV-EMC to Ty-BatCoV HKU4 and Pi-BatCoV HKU5. Comparison of the amino acid identities of the seven conserved replicase domains for species demarcation (ADRP, nsp5 ($3CL^{pro}$), nsp12 (RdRp), nsp13 (Hel), nsp14 (ExoN), nsp15 (NendoU) and nsp16 (2'-O-MT)) between HCoV-EMC, Ty-BatCoV HKU4 and Pi-BatCoV HKU5 showed that there is less than 90% identity in four of the seven domains (ADRP 68%–69% identity, nsp5 81%–83% identity, nsp15 76%–80% identity and nsp16 84%–85% identity), indicating that HCoV-EMC is a novel CoV species. For nsp12, nsp13 and nsp14, there are 90%–92%, 92%–94% and 86%–92% amino acid identities between HCoV-EMC and Ty-BatCoV HKU4/Pi-BatCoV HKU5.

Using the sequences available at the moment and Yule process speciation under a relaxed clock model with an uncorrelated lognormal distribution, the mean evolutionary rate of betaCoVs was estimated at 2.37×10^{-4} nucleotide substitutions per site per year for the RdRp gene. Molecular clock analysis using the RdRp gene showed that HCoV-EMC diverged from the most recent common ancestor of group C betaCoVs at ~year 941 (HPDs, 529 BC to 1878). Compared to the human and civet SARSr-CoV and SARSr-Rh-BatCoV cluster, the human/civet SARSr-CoV diverged from the


3CL^pro
RdRp
Hel
FIPV
CCoV
PRCV
TGEV
Mi-BatCoV 1A
Mi-BatCoV 1B
Mi-BatCoV HKU8
Hi-BatCoV HKU10
Ro-BatCoV HKU10
Sc-BatCoV 512
PEDV
HCoV-NL63
HCoV-229E
Rh-BatCoV HKU2
RCoV
MHV
HCoV-HKU1
RbCoV HKU14
HCoV-OC43
PHEV
GiCoV
ECoV
AntelopeCoV
BCoV
HCoV-EMC
Ty-BatCoV HKU4
Pi-BatCoV HKU5
Ro-BatCoV HKU9
SARSr-Rh-BatCoV HKU3
SARS CoV
SARSr CoV CFB
SARSr-CiCoV
BWCoV-SW1
IBV-partridge
TCoV
IBV-peafowl
IBV
WiCoV HKU20
NHCoV HKU19
CMCoV HKU21
WECoV HKU16
ThCoV HKU12
BuCoV HUK11
MunCoV HKU13
MRCoV HKU18
PorCoV HKU15
SpCoV HKU17
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β-CoV A
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β-CoV D
β-CoV B
γ-CoV
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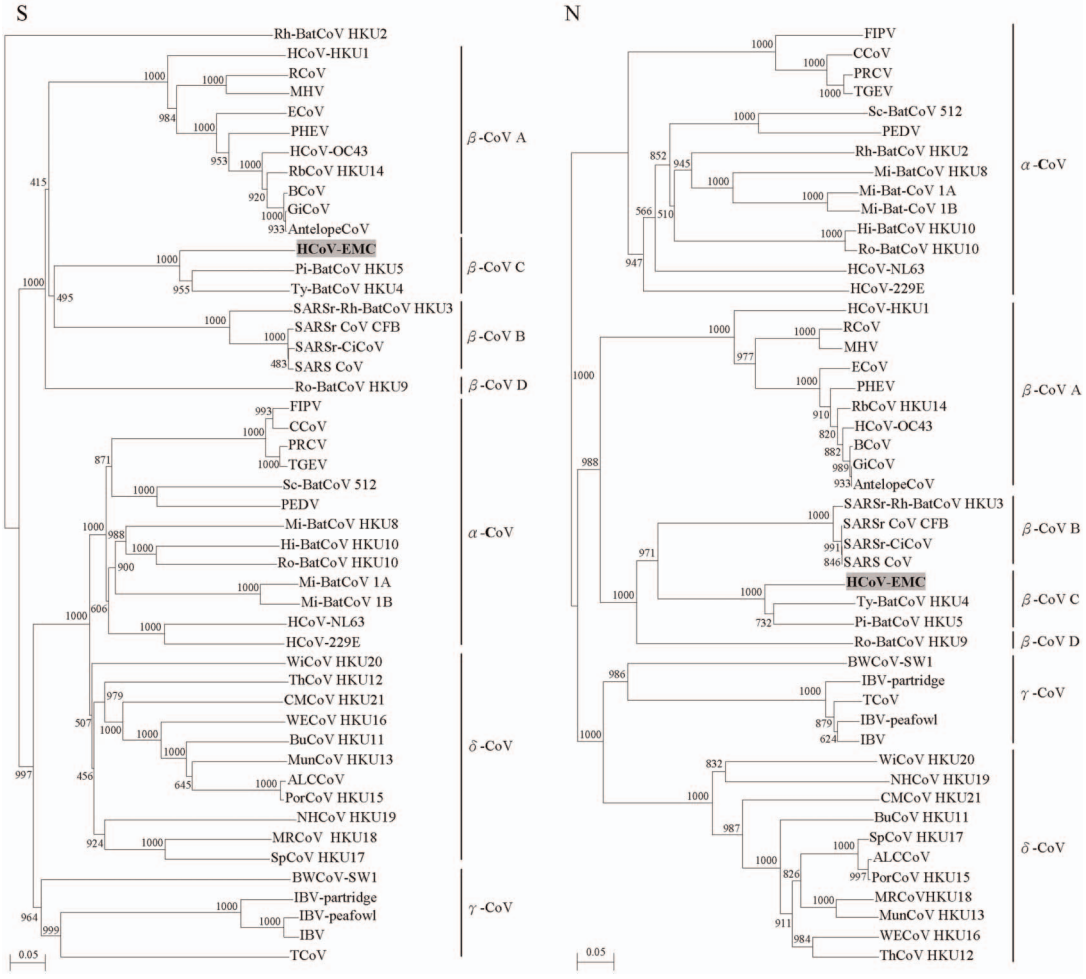
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GiCoV
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HCoV-EMC
Pi-BatCoV HKU5
Ty-BatCoV HKU4
SARSr-Rh-BatCoV HKU3
SARSr CoV CFB
SARSr-CiCoV
SARS CoV
Ro-BatCoV HKU9
FIPV
CCoV
PRCV
TGEV
Sc-BatCoV 512
PEDV
Mi-BatCoV HKU8
Hi-BatCoV HKU10
Ro-BatCoV HKU10
Mi-BatCoV 1A
Mi-BatCoV 1B
HCoV-NL63
HCoV-229E
WiCoV HKU20
ThCoV HKU12
CMCoV HKU21
WECoV HKU16
BuCoV HKU11
MunCoV HKU13
ALCCoV
PorCoV HKU15
NHCoV HKU19
MRCoV HKU18
SpCoV HKU17
BWCoV-SW1
IBV-partridge
IBV-peafowl
IBV
TCoV
β-CoV A
β-CoV C
β-CoV B
β-CoV D
α-CoV
δ-CoV
γ-CoV
0.05

Figure 2 Phylogenetic analysis of HCoV-EMC. The trees were constructed by the neighbor-joining method using Kimura correction and bootstrap values calculated from 1000 trees. 318, 951, 600, 1491 and 510 amino acid positions in chymotrypsin-like protease ($3CL^{pro}$), RNA-dependent RNA polymerase (RdRp), helicase (Hel), spike (S) and nucleocapsid (N) respectively were included in the analysis. For $3CL^{pro}$, S and N, the scale bars indicate the estimated number of substitutions per 20 amino acids. For RdRp and Hel, the scale bars indicate the estimated number of substitutions per 50 amino acids. PEDV, porcine epidemic diarrhea virus (NC_003436); Sc-BatCoV-512, *Scotophilus* bat coronavirus 512 (NC_009657); TGEV, transmissible gastroenteritis virus (NC_002306); FIPV, feline infectious peritonitis virus (AY994055); CCoV, canine coronavirus (GQ477367); PRCV, porcine respiratory coronavirus (DQ811787); Rh-BatCoV-HKU2, *Rhinolophus* bat coronavirus HKU2 (EF203064); Mi-BatCoV 1A, *Miniopterus* bat coronavirus 1A (NC_010437); Mi-BatCoV 1B, *Miniopterus* bat coronavirus 1B (NC_010436); Mi-BatCoV-HKU8, *Miniopterus* bat coronavirus HKU8 (NC_010438); Hi-BatCoV HKU10, *Hipposideros* bat coronavirus HKU10 (JQ989269); Ro-BatCoV HKU10, *Rousettus* bat coronavirus HKU10 (JQ989270); HCoV-229E, human coronavirus 229E (NC_002645); HCoV-NL63, human coronavirus NL63 (NC_005831); HCoV OC43, human coronavirus OC43 (NC_005147); BCoV, bovine coronavirus (NC_003045); AntelopeCoV, sable antelope coronavirus (EF424621); GiCoV, giraffe coronavirus (EF424622); ECoV, equine coronavirus (NC_010327); PHEV, porcine hemagglutinating encephalomyelitis virus (NC_007732); MHV, murine hepatitis virus (NC_001846); RCoV, rat coronavirus (NC_012936); RbCoV HKU14, rabbit coronavirus HKU14 (NC_017083); HCoV-HKU1, human coronavirus HKU1 (NC_006577); Ty-BatCoV-HKU4, *Tylonycteris* bat coronavirus HKU4 (NC_009019); Pi-BatCoV-HKU5, *Pipistrellus* bat coronavirus HKU5 (NC_009020); SARS CoV, SARS-related human coronavirus (NC_004718); SARSr-Rh-BatCoV HKU3, SARS-related *Rhinolophus* bat coronavirus HKU3 (DQ022305); SARSr CoV CFB, SARS-related Chinese ferret badger coronavirus (AY545919); SARSr-CiCoV, SARS-related palm civet coronavirus (AY304488); Ro-BatCoV-HKU9, *Rousettus* bat coronavirus HKU9 (NC_009021); IBV, infectious bronchitis virus (NC_001451); IBV-partridge, partridge coronavirus (AY646283); TCoV, turkey coronavirus (NC_010800); IBV-peafowl, peafowl coronavirus (AY641576); BWCoV-SW1, beluga whale coronavirus SW1 (NC_010646); ALCCoV, Asian leopard cat coronavirus (EF584908); BuCoV HKU11, bulbul coronavirus HKU11 (FJ376619); ThCoV HKU12, thrush coronavirus HKU12 (FJ376621); MunCoV HKU13, munia coronavirus HKU13 (FJ376622); PorCoV HKU15, porcine coronavirus HKU15 (NC_016990); WECoV HKU16, white-eye coronavirus HKU16 (NC_016991); SpCoV HKU17, sparrow coronavirus HKU17 (NC_016992); MRCoV HKU18, magpie robin coronavirus HKU18 (NC_016993); NHCoV HKU19, night heron coronavirus HKU19 (NC_016994); WiCoV HKU20, wigeon coronavirus HKU20 (NC_016995); CMCoV HKU21, common moorhen coronavirus HKU21 (NC_016996).

most recent common ancestor of the human/civet SARSr-CoV and SARSr-Rh-BatCoV at ~year 1653 (HPDs, 1150 to 1968). By definition, the human and civet SARSr-CoV and SARSr-Rh-BatCoV are the same CoV species. These observations suggest that there should be one or more intermediate hosts between Ty-BatCoV HKU4, Pi-BatCoV HKU5 and HCoV-EMC. Sequencing more strains of Ty-BatCoV HKU4, Pi-BatCoV HKU5 and HCoV-EMC, as well as other group C betaCoVs collected at different time points, should be performed to achieve a more accurate estimation of the divergence time.

In the last decade, we have already witnessed the discovery of two novel human CoVs and an animal-to-human CoV interspecies jumping event on SARSr-CoVs. In contrast to HCoV-229E, HCoV-OC43, HCoV-NL63 and HCoV-HKU1, which are notoriously difficult to culture, HCoV-EMC and human SARS-CoV are both readily cultivable using primate cell lines. This may suggest a possible correlation between cultivability and virulence/recent interspecies jumping. Sequencing more genomes and performing evolutionary analysis will help us understand whether HCoV-EMC represent another recent interspecies jumping event from animal to human or another human CoV that has stably infected human. Our most recent findings showed that CoVs can be transmitted between two bat species of different suborders, suggesting that different degrees of interspecies jumping can occur in nature.[19] More intensive surveillance studies for group C betaCoVs in bats and other animals may reveal the natural host of this novel human group C betaCoV. As coronaviruses are prone to recombination and mutation and it has been documented that different levels of interspecies jumping can indeed occur in nature, we should not underestimate the potential of coronaviruses being the cause of another major "SARS-like" pandemic.

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