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Characterization of a Novel Betacoronavirus Related to Middle East Respiratory Syndrome Coronavirus in European Hedgehogs

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Bats are known to host viruses closely related to important human coronaviruses (HCoVs), such as HCoV-229E, severe-acute respiratory syndrome coronavirus (SARS-CoV), and Middle East respiratory syndrome CoV (MERS-CoV). As RNA viruses may coevolve with their hosts, we sought to investigate the closest sister taxon to bats, the *Eulipotyphla*, and screened European hedgehogs (*Erinaceus europaeus*) from Germany for CoV by nested reverse transcriptase PCR. A novel betacoronavirus species in a phylogenetic sister relationship to MERS-CoV and clade c bat CoVs was detected and characterized on the whole-genome level. A total of 58.9% of hedgehog fecal specimens were positive for the novel CoV (EriCoV) at 7.9 $\log_{10}$ mean RNA copies per ml. EriCoV RNA concentrations were higher in the intestine than in other solid organs, blood, or urine. Detailed analyses of the full hedgehog intestine showed the highest EriCoV concentrations in lower gastrointestinal tract specimens, compatible with viral replication in the lower intestine and fecal-oral transmission. Thirteen of 27 (48.2%) hedgehog sera contained non-neutralizing antibodies against MERS-CoV. The animal origins of this betacoronavirus clade that includes MERS-CoV may thus include both bat and nonbat hosts.

The *Coronaviridae* subfamily *Coronavirinae* contains the four genera *Alpha-*, *Beta-*, *Gamma-* and *Deltacoronavirus* (1, 2). Betacoronaviruses are further discriminated into clades a to d. Until recently, five human coronaviruses (HCoVs) were known, namely, the alphacoronaviruses HCoV-229E and HCoV-NL63 and the betacoronaviruses HCoV-OC43, HCoV-HKU1 (both clade a), and *Severe acute respiratory syndrome coronavirus* (SARS-CoV, clade b) (3–8). In 2012, a highly pathogenic novel HCoV termed *Middle East respiratory syndrome coronavirus* (MERS-CoV) emerged (9–11). MERS-CoV belongs to the *Betacoronavirus* clade c, which previously contained only bat CoVs (BtCovs) (12–17). Because of the high number of bat CoVs newly described in the aftermath of SARS, it was assumed that all mammalian CoVs originated in the order *Chiroptera* (18). The majority of these novel bat CoVs were found in insectivorous bats (18). Therefore, we speculated that other insectivorous mammals could also harbor CoVs. This might specifically apply to the animal order *Eulipotyphla*, which includes hedgehogs, moles, solenodons, and shrews, because this and the order *Chiroptera* are phylogenetically related (19). For this reason, we analyzed fecal samples from 248 European hedgehogs (*Erinaceus europaeus*) for CoVs. A novel betacoronavirus clade c species was found and described using molecular and immunologic tools.

MATERIALS AND METHODS

Sample collection, processing, and screening for coronavirus RNA. Fecal samples from European hedgehogs (*Erinaceus europaeus*) kept in an animal shelter in northern Germany because of poor physical condition or injuries were sampled noninvasively and stored in RNAlater (Qiagen, Hilden, Germany) at −20°C until further investigation. For the initial CoV screening, fecal samples of 10 individual animals were pooled. RNA purification and CoV detection using two different nested reverse transcription-PCR (RT-PCR) assays targeting the *RNA-dependent RNA polymerase* (*RdRp*) gene were done as described previously (16, 20, 21). Individual specimens in positive pools were identified using a strain-specific real-time RT-PCR (oligonucleotide sequences available upon request) based on the nucleotide sequences obtained from sequencing of initial PCR amplicons. For phylogenetic analyses, sequences from the PCR screening assays were extended to an 816-nucleotide (nt) *RdRp* fragment (22). In addition, carcasses from 27 hedgehogs that died in the animal shelter during their stay were collected and stored at −20°C until dissection. Samples from the brain, heart, lung, liver, kidney, spleen, and intestine were taken. The intestines of five additional CoV-positive animals were cleaned and dissected in 10 portions taken in equal intervals immediately after the stomach and until the anal orifice.

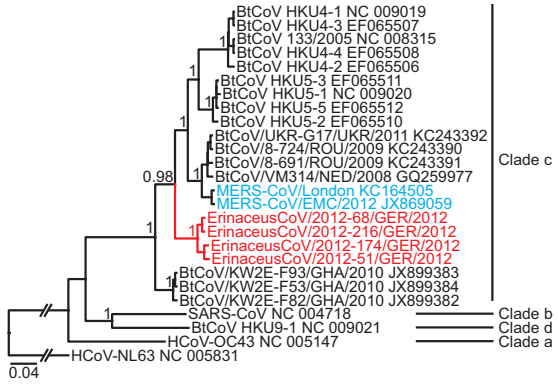

FIG 1 Betacoronavirus phylogeny, including the novel viruses from European hedgehogs. Bayesian phylogeny of an 816-nucleotide RdRp gene sequence fragment corresponding to positions 14822 to 15637 in MERS-CoV strain EMC/2012 (GenBank accession no. JX869059). The novel *Erinaceus* viruses are shown in red, and MERS-CoVs in blue.

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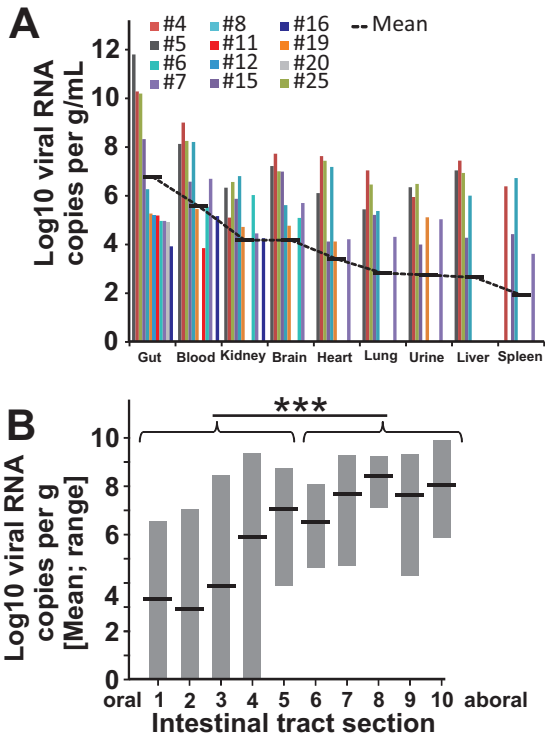

FIG 2 *Erinaceus* CoV RNA concentrations in solid organs, urine, and blood, and virus distribution within the intestine. (A) Virus concentrations in solid organs, urine, and blood of 12 EriCoV-positive animals are given in $\log_{10}$ RNA copies per milliliter or gram of tissue. Horizontal bars represent mean virus concentrations per organ category. Missing bars represent negative test results. For all organs, specimens from 12 individual animals were available, except urine, where no specimen was available from animal 12. Colors represent individual animals as identified in the key. (B) Ranges of EriCoV concentrations in 10 different intestinal sections of five RNA-positive individuals are given in $\log_{10}$ RNA copies per gram of tissue. Black horizontal bars represent mean virus concentrations; ***, $P < 0.005$ according to Mann-Whitney U test.

Blood was sampled from inside the heart and urine from inside the bladder by puncture of these organs before removal. Quantification of viral RNA was done using strain-specific assays and photometrically quantified *in vitro* cRNA transcripts as described previously (10, 23).

Whole-genome sequencing. RNA extracts of two positive samples were determined and prepared for 454 next-generation sequencing (NGS) as described previously (24, 25). Sequences obtained from 454-NGS were reproduced on individual samples and connected by long-range reverse transcription-PCR using specific oligonucleotide primers (available upon request). Determination of the 5′ and 3′ genome ends was done using a rapid amplification of cDNA ends kit (Roche, Penzberg, Germany). PCR products were sequenced by dye terminator chemistry (Seqlab, Goettingen, Germany).

Genome analyses. The nucleotide sequences of the genomes and the amino acid sequences of the presumed open reading frames (ORFs) were compared to other c clade betacoronaviruses for which full-length genome sequences were available. Nucleic acid alignments were done based on the amino acid coding using the MAFFT algorithm (26) in the geneious software package (Biomatters, Auckland, New Zealand). Phylogenetic analyses of the extended screening fragments, as well as the presumed ORFs, were done using MrBayes version 3.1 (27) using a WAG amino acid substitution model and 4,000,000 generations sampled every 100 steps. Trees were annotated using a burn-in of 10,000 in TreeAnnotator version 1.5 and visualized with FigTree version 1.4 from the BEAST package (28). The pairwise identities of all ORFs and predicted proteins of the two *Erinaceus* CoVs (EriCoV) were calculated using MEGA5 (29). Similarity plots were generated using SSE version 1.0 (30) using a sliding window of 400 and a step size of 40 nucleotides.

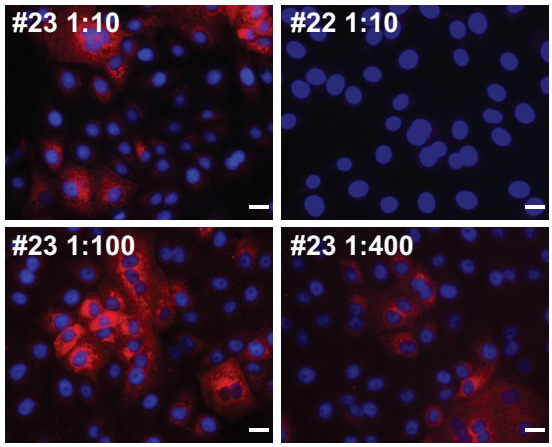

FIG 3 Serologic testing of hedgehog blood. (Top) Reaction patterns of a reactive (23) and a nonreactive (22) hedgehog serum with MERS-CoV-infected Vero cells at 1:10 screening dilution. (Bottom) Endpoint dilution of serum sample 23. Scale bar, 20 μm.

Virus isolation attempts. Isolation of virus from those specimens containing the highest RNA concentrations was attempted on Vero E6 cells, which are known to support MERS-CoV infection (31). In addition, immortalized kidney cells of a *Pipistrellus* bat and immortalized lung cells from *Crocidura suaveolens* from the animal order *Eulipotyphla* were used for isolation attempts (our own unpublished cell lines).

Serology. Blood samples obtained during dissection of the 27 hedgehog carcasses were tested for antibodies against MERS-CoV using a commercially available indirect immunofluorescence assay (IFA; Euroimmun AG, Lübeck, Germany) with slight modifications. A rabbit anti-suncus immunoglobulin G (IgG) adapted for cross-recognition of hedgehog Ig was used as a secondary antibody at a 1:200 dilution. Detection was done with a cyanine 3-conjugated goat anti-rabbit IgG (Dianova, Hamburg, Germany). Virus neutralization tests against MERS-CoV were done as described previously (32). Briefly, blood samples were serially diluted from 1:20 to 1:2,560 in serum-free medium, mixed with 100 PFU, and preincubated for 1 h at 37°C before being added to a Vero B4 cell monolayer. After adsorption for 1 h at 37°C, the serum-virus mixture was discarded and fresh medium (Dulbecco's modified Eagle's medium) was added to the cells. Cytopathogenic effects were visualized 3 days postinfection by fixation and staining with crystal violet solution.

Nucleotide sequence accession numbers. The four virus sequences obtained from European hedgehog fecal samples were deposited in GenBank with accession numbers KC545383 to KC545386.

RESULTS AND DISCUSSION

Fecal specimens from 248 European hedgehogs (*Erinaceus europaeus*) were tested for CoVs by broad-range nested RT-PCRs. This approach yielded four different virus sequences. These sequences (GenBank accession numbers KC545383 to KC545386) were classifiable as clade c betacoronaviruses in initial BLAST comparisons and were named *Erinaceus* CoV (EriCoV). Within EriCoVs, two different clades separated by 3.1 to 3.4% nucleotide distance in an 816-nt *RdRp* fragment were identified. Figure 1 shows a Bayesian phylogeny of this *RdRp* fragment. All EriCoVs grouped phylogenetically within the *Betacoronavirus* clade c. The EriCoVs clustered in sister relationship to a clade defined by the bat CoVs HKU4 and HKU5, the MERS-CoV-related viruses, and a clade of *Nycteris* bat CoVs. The amino acid distances to the clade c proto-

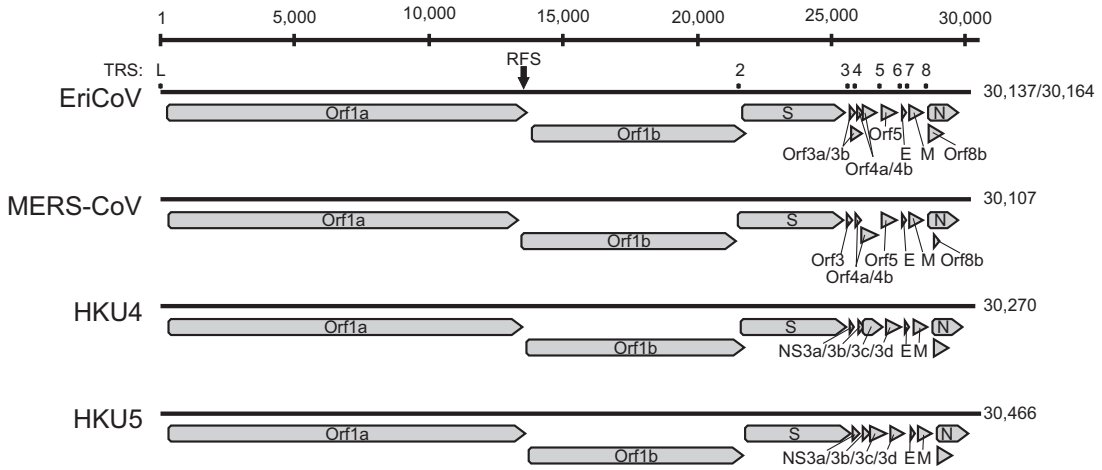

FIG 4 Genome organization of EriCoV and other clade c betacoronaviruses. Genomes are represented by black lines, and ORFs are indicated by gray arrows. The ribosomal frameshift site (RFS) at nucleotide positions 13578 to 13584 (EriCoV/2012-174) and 13605 to 13611 (EriCoV/2012-216) is marked with a black arrow. The locations of transcription regulatory core sequences (TRS) are marked by labeled dots.

TABLE 1 Coding of potential and putative transcription regulatory sequences of the EriCoV genome sequences

Strain, protein/ORF	Nucleotide position	Length (aa)	TRS nucleotide position in genome	No. of matching base pairs compared to leader TRS (body/leader)	Sequence comparison of leader TRS region and each putative body TRS[a]
EriCoV/2012-174/GER/2012					
ORF1ab	241-21692	7,150	52		UCUUGUUUUAACGAACUUAA(169)**AUG**
S	21610-25602	1,330	21549	11/20	AG.CAGA.....CGU(41)**AUG**
ORF3a	25620-25937	105	25597	10/20	CA..AAAA.....GCCU.(3)**AUG**
ORF3b	25714-26136	140			
ORF4a	25891-26136	82	25872	13/20	AG.GAG...U.....G**AUG**
ORF4b	26123-26794	223			
ORF5	26804-27505	233	26787	10/17	.A.AU.AAGG.....**AUG**
E (ORF6)	27579-27827	82	27562	10/17	CA..UGGAA.....**AUG**
M (ORF7)	27842-28498	218	27817	15/20	AU.G.....AU.(5)**AUG**
N (ORF8a)	28552-29826	424	28522	14/20	.A.AUCA.....U....(10)**AUG**
ORF8b	28598-29197	199			
EriCoV/2012-216/GER/2012					
ORF1ab	241-21719	7,159	52		UCUUGUUUUAACGAACUUAA(169)**AUG**
S	21637-25629	1,330	21576	11/20	AG.CAGA.....CGU(41)**AUG**
ORF3a	25647-25961	104	25624	10/20	CAC.AAAA.....UC.U.(3)**AUG**
ORF3b	25741-26163	140			
ORF4a	25918-26163	82	25899	15/20	AG.G.....U.....G**AUG**
ORF4b	26150-26821	223			
ORF5	26831-27532	233	26814	10/17	.A.AU.AAGG.....**AUG**
E (ORF6)	27606-27854	82	27589	10/17	CA..UGGAA.....**AUG**
M (ORF7)	27869-28525	218	27844	15/20	AU.G.....AU.(5)**AUG**
N (ORF8a)	28580-29854	424	28550	14/20	AUA.CA.....U....(10)**AUG**
ORF8b	28626-29225	199			

[a] Dots represent identical nucleotides in comparison to the leader TRS. Numbers in parentheses represent number of nucleotides to the putative start codon, indicated by underlining. The conserved TRS core sequence of clade c betacoronaviruses (40, 52), ACGAA, is highlighted in gray.

TABLE 2 Prediction of the putative polyprotein pp1a/pp1ab cleavage sites of hedgehog coronaviruses based on sequence comparison with MERS-CoV strain EMC/2012

NSP	First–last amino acid residues[a] of EriCoV/2012-174[b]	Protein size (aa)	First–last amino acid residues of EriCoV/2012-216[c]	Protein size (aa)	Putative functional domain(s)[d]
1	Met^{1}–Gly^{200}	200	Met^{1}–Gly^{200}	200	
2	Asp^{201}–Gly^{859}	659	Asp^{201}–Gly^{859}	659	
3	Ala^{860}–Gly^{2805}	1,946	Ala^{860}–Gly^{2814}	1,955	ADRP, PL2pro
4	Ser^{2806}–Gln^{3310}	505	Ser^{2815}–Gln^{3319}	505	
5	Ser^{3311}–Gln^{3616}	306	Ser^{3320}–Gln^{3625}	306	3CLpro
6	Ser^{3617}–Gln^{3908}	292	Ser^{3626}–Gln^{3917}	292	
7	Ser^{3909}–Gln^{3991}	83	Ser^{3918}–Gln^{4000}	83	
8	Ser^{3992}–Gln^{4190}	199	Ser^{4001}–Gln^{4199}	199	Primase
9	Asn^{4191}–Gln^{4300}	110	Asn^{4200}–Gln^{4309}	110	
10	Ala^{4301}–His^{4440}	140	Ala^{4310}–His^{4449}	140	
11	Ser^{4441}–Leu^{4454}	14	Ser^{4450}–Leu^{4463}	14	Short peptide at the end of ORF1a
12	Ser^{4441}–Gln^{5374}	934	Ser^{4450}–Gln^{5383}	934	RdRp
13	Ala^{5375}–Gln^{5972}	598	Ala^{5384}–Gln^{5981}	598	Hel, NTPase
14	Ser^{5973}–Gln^{6496}	524	Ser^{5982}–Gln^{6505}	524	ExoN, NMT
15	Gly^{6497}–Gln^{6839}	343	Gly^{6506}–Gln^{6848}	343	NendoU
16	Ala^{6840}–Cys^{7150}	311	Ala^{6849}–Cys^{7159}	311	OMT

[a] Superscript numbers indicate positions in polyprotein pp1a/pp1ab, with the supposition of a ribosomal frameshift resulting in a peptide bond between Asn4448/Arg4449 (EriCoV/2012-174/GER/2012) and Asn4457/Arg4458 (EriCoV/2012-216/GER/2012) for the expression of ORF1ab.
[b] GenBank accession number KC545383.
[c] GenBank accession number KC545386.
[d] ADRP, ADP-ribose 1″-phosphatase; PL2pro, papain-like protease 2; 3CLpro, coronavirus nsp5 protease; Hel, helicase; NTPase, nucleoside triphosphatase; ExoN, exoribonuclease; NMT, N7 methyltransferase; NendoU, endoribonuclease; OMT, 2′ *O*-methyltransferase.

type viruses HKU4, HKU5, and MERS-CoV were 7.7 to 8.8% in the translated 816-nt *RdRp* fragment. The distance from the *Nycteris* CoV was 8.5 to 9.2%. In our previous proposal to tentatively classify CoVs into *RdRp*-grouping units (RGU), which are predictive of species classification, betacoronavirus species were at least 6.3% different on the amino acid level in this sequence fragment (22). EriCoVs therefore represented a novel tentative betacoronavirus clade c species.

For an estimate of EriCoV prevalence and an assessment of viral RNA concentrations, all specimens were retested using strain-specific real-time RT-PCR assays. EriCoVs were now detected in 146 of 248 individual specimens (58.9%). Viral RNA concentrations were high, with a mean of 7.9 $\log_{10}$ copies per ml of fecal suspension (range, 4.2 to 11). Isolation of virus was attempted unsuccessfully on three different cell lines for those specimens containing the highest RNA concentrations.

The high detection rate and virus concentrations in feces appeared compatible with replication in the intestine. Therefore, 27 hedgehog carcasses were dissected and the intestines were tested using specific real-time RT-PCR assays. EriCoV RNA was found in intestinal specimens from 12 of these 27 animals (44.4%). The difference in detection rates between feces and intestinal specimens was not statistically significant (corrected $\chi^2 = 1.5$, $P = 0.2$). The data in Fig. 2A show that intestinal virus concentrations in these 12 animals were high, 6.78 $\log_{10}$ mean RNA copies per gram of tissue (range, 3.9 to 11.8). The mean virus concentrations in all other solid organs, urine, and blood from these 12 animals were at least 10-fold lower. This and the high EriCoV detection rate in feces might be compatible with a fecal-oral route of transmission. Furthermore, the high EriCoV concentrations were compatible with virus replication in the intestinal tract. This was comparable to viruses replicating in the human gut, such as Aichi, rota- and noroviruses (33). We could not determine if infection was associated with clinical disease in hedgehogs. While gastroenteritis caused by CoVs is not rare in animals (34), intestinal virus replication could also be asymptomatic or associated with nonenteric disease, similar to, e.g., human enteroviruses (35).

Because only small fragments of the intestine of the initial 27 animals had been collected upon dissection, it was impossible to determine in which portion of the gastrointestinal tract the virus might replicate. Therefore, five additional EriCoV-positive intestines were identified by testing of 10 additional hedgehog carcasses. The entire intestines of these animals were cut into 10 equal-sized pieces starting from the stomach, and EriCoV RNA concentrations were determined in each portion. The data in

TABLE 3 Comparison of amino acid identities of seven conserved replicase domains of the hedgehog coronavirus and prototype clade c betacoronaviruses for species delineation

Domain	% amino acid sequence identity (range): Within EriCoV[a]	Of EriCoV compared to: MERS-CoV[b]	HKU4[c]	HKU5[d]
ADRP	96.9–100	71.3	57.5–59.4	61.3–61.9
NSP5 (3CLpro)	99.0–100	79.1–79.7	72.9–73.5	76.8–77.1
NSP12 (RdRp)	99.0–100	88.9–89.4	87.2–88.1	88.1–88.7
NSP13 (Hel, NTPase)	99.6–100	91.1–91.5	89.5–90.0	90.3–90.8
NSP14 (ExoN, NMT)	99.6–100	89.5–89.9	83.2–84.3	88.0–88.4
NSP15 (NendoU)	97.1–100	82.5–83.1	74.3–75.4	78.4–79.3
NSP16 (OMT)	99.0–100	86.5–87.5	81.5–82.1	83.8–85.1
Concatenated domains	98.9–100	86.9	82.4–82.8	84.8–85

[a] Including sequences with accession numbers KC545383 and KC545386.
[b] Including sequences with accession numbers JX869059 and KC164505.
[c] Including sequences with accession numbers EF065505, EF065506, EF065507, EF065508, and DQ648794.
[d] Including sequences with accession numbers EF065509, EF065510, EF065511, and EF065512.

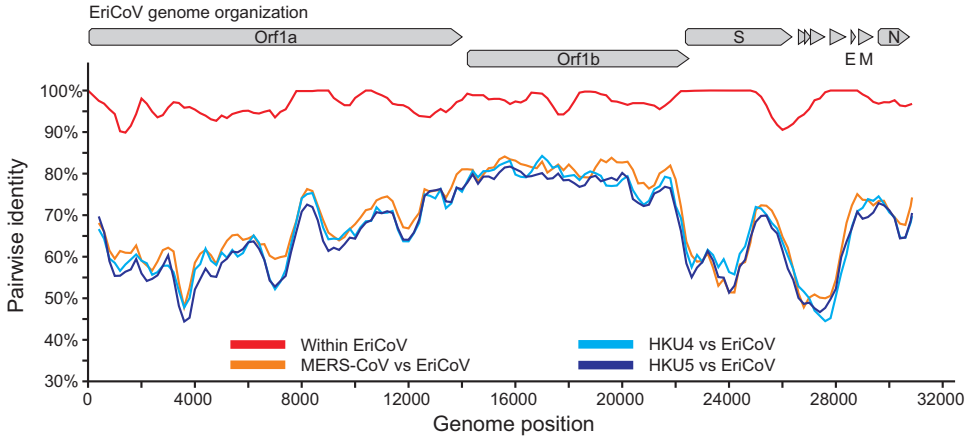

FIG 5 Genomic sequence identity between EriCoVs and other clade c betacoronaviruses. Similarity plots were generated using SSE version 1.0 (38) using a sliding window of 400 and a step size of 40 nucleotides.

Fig. 2B show that the highest concentrations were detected in distal (aboral) parts. The virus concentrations in these sections were significantly higher than those toward the stomach (Mann-Whitney U test, $P < 0.005$). This was comparable to the higher PCR positivity rates in aboral than in oral intestine sections in a previous study on Leschenault's rousette bats fed CoV-positive tissue from other bats (36).

To determine antigenic relatedness to MERS-CoV, hedgehog sera and secondary anti-suncus immunoglobulin (Ig) were adapted for use with a commercially available MERS-CoV IFA. At a serum dilution of 1:10, 13 of 27 (48.2%) blood specimens were reactive. The median IFA endpoint titers were 1:100 (range, 1:10 to 1:400). The images in Fig. 3 exemplify the IFA reaction patterns of positive and negative sera. To analyze whether the observed antibodies were indeed directed against MERS-CoV, all IFA-positive sera were tested in a MERS-CoV neutralization assay. None of the IFA-positive sera contained MERS-CoV neutralizing antibodies in dilutions higher than 1:20. This was compatible with cross-reactivity of anti-EriCoV antibodies with MERS-CoV, which they bound but did not neutralize, similar to cross-reactive anti-HCoV antibodies in humans (37, 38). The high frequency of EriCoV infection and the absence of other CoV sequences in hedgehogs in our study could indicate that all detected antibody titers were indeed specific for EriCoV. Six of the 13 IFA-positive animals (46.2%) showed concomitant detection of EriCoV RNA, implying that some hedgehogs may have cleared EriCoV infection or that a different CoV hypothetically elicited the observed antibody response. On the other hand, 6 of 12 PCR-positive animals (50%) showed no detectable antibodies, compatible with sampling before seroconversion.

To confirm the RGU-based tentative species classification and to investigate the genetic relatedness between EriCoV and MERS-CoV, one whole-genome sequence was generated for each of the two EriCoV subclades (represented by viruses EriCoV/2012-174 and EriCoV/2012-216). The sizes of the two EriCoV genomes were 30,137 and 30,164 nt, with a G+C content of 37%. The numbers and locations of EriCoV ORFs, as well as seven transcription regulatory sequences (TRS) preceding them, were characteristic for clade c betacoronaviruses (2, 39). Figure 4 shows the genome organization of EriCoV and other clade c betacoronaviruses. Table 1 provides

TABLE 4 Identities between open reading frames of the novel hedgehog coronavirus and prototype clade c betacoronaviruses

Annotation in EriCoV	Annotation in MERS-CoV/EMC	Annotation in BtCoV-HKU4 and BtCoV-HKU5	% amino acid identity[a]: Within EriCoV	Of MERS-CoV[b] to EriCoV	Of HKU4-1[c] to EriCoV	Of HKU5-1[d] to EriCoV
ORF1ab	ORF1ab	ORF1ab	97.4	73.9–74.0	68.8–69.1	71.0–71.1
S	S	S	98.5	57.9–58.2	58.4–58.6	58.2–58.3
ORF3a	ORF3	NS3a	92.3	26.7–28.9	28.2	27.8–28.9
ORF3b	ORF4a	NS3b	93.6	39.5	39.5	44.3
ORF4a	ORF4a	NS3b	97.5	39.5	39.5	44.3
ORF4b	ORF4b	NS3c	96.0	39.3–39.7	29.3	27.8
ORF5	ORF5	NS3d	100.0	52.2	38.3	45.1
E (ORF6)	E	E	100.0	72.0	62.2	59.8
M (ORF7)	M	M	99.5	78.9–79.4	78.0–78.4	78.9–79.4
N (ORF8)	N	N	98.6	71.9–72.1	71.1	69.4
ORF8b	ORF8b	Undescribed	93.5	53.6	41.2–42.8	46.6–47.6

[a] Pairwise identities of all ORFs and predicted proteins of the two EriCoVs were calculated using alignments based on amino acid coding by the MAFFT algorithm (20) in the geneious software package (Biomatters) and MEGA5 (29).
[b] Accession number JX869059.
[c] Accession number EF065505.
[d] Accession number EF065509.

details on the TRS and their genomic localizations. The predicted leader TRS of EriCoV (AACUCUUGUUUUAACGAACUUAA) differed by only three nucleotides from those of the betacoronavirus clade c prototype viruses HKU4, HKU5, and MERS-CoV [AACUUUG(U/A)UUUUAACGAACUUAA] (40, 41). The predicted AUG codons of ORF3b, ORF4b, and ORF8b were not preceded by separate body TRS elements and may be translated from bicistronic mRNAs, as discussed for other CoVs, including MERS-CoV (39, 42, 43). In ORF1a/ORF1ab, a ribosomal frameshift was predicted based on the tentative slippery sequence UUU AAAC (indicated by an arrow in Fig. 4) (39, 44). Table 2 provides details on the sizes and genomic locations of the 16 predicted ORF1ab nonstructural proteins (nsp).

A separate comparison of the amino acid sequences of seven conserved ORF1ab domains, as suggested by the International Committee on Taxonomy of Viruses (ICTV) for formal CoV species delineation, is shown in Table 3. The sequence identities of the seven concatenated domains compared to those of other clade c viruses (82.4 to 86.9%) were well below the 90% threshold proposed by the ICTV (41), confirming the presence of a separate new CoV species.

Figure 5 shows a comparison of the complete genomic nucleotide sequences of EriCoVs and HKU4, HKU5, and MERS-CoV. The EriCoVs shared 96.9% overall nucleotide identity across the two whole genomes (red line). They were almost equidistant from the prototype clade c betacoronaviruses (orange and pale and dark blue lines). Table 4 summarizes the amino acid sequence identities between the predicted proteins of EriCoV and other clade c betacoronaviruses. The highest amino acid sequence identity between EriCoV and the other clade c CoVs was observed for the membrane protein, with 78.0 to 79.4% identity, and the lowest identity was observed within ORF3a, with 26.7 to 28.9% identity. These values were similar to values found in sequence comparisons between MERS-CoV and the prototype clade c betacoronaviruses HKU4 and HKU5 (41).

Bayesian phylogenies of all EriCoV ORFs are shown in Fig. 6. EriCoVs clustered as a sister clade to all previously known bat-associated clade c CoVs and MERS-CoV. As in previous sequence comparisons between MERS-CoV and HKU4 and HKU5 (45), different topologies were observed for individual ORFs, in particular the *envelope* and *nucleocapsid* genes. While this might indicate ancient recombination, it could as well result from differential selective pressures acting on different genome portions, causing slightly deviating inferences of apical phylogenies in those closely related viruses. Analysis by Bootscans on nearly complete genome alignments identified no obvious signs of recombination.

The failure to isolate EriCoV might predict a low potential to replicate in heterologous host cells, unlike MERS-CoV, which in cell culture shows relatively little host restriction (31). An analysis of the *spike* gene sequence indicated that EriCoV cellular entry may differ from that of MERS-CoV, because the receptor binding domain (RBD) of MERS-CoV (46–48) and the corresponding region of EriCoV showed only 36.7% amino acid identity (88 of 240 residues). Whether the corresponding region in the EriCoV Spike protein can interact with the MERS-CoV receptor dipeptidyl peptidase 4 or its hedgehog-specific homologue remains to be determined (49). The detailed genomic analysis of EriCoV may facilitate the generation of recombinant clade c viruses once reverse genetic systems for this betacoronavirus clade become available to clarify the zoonotic potential of EriCoV and identify potential virulence factors.

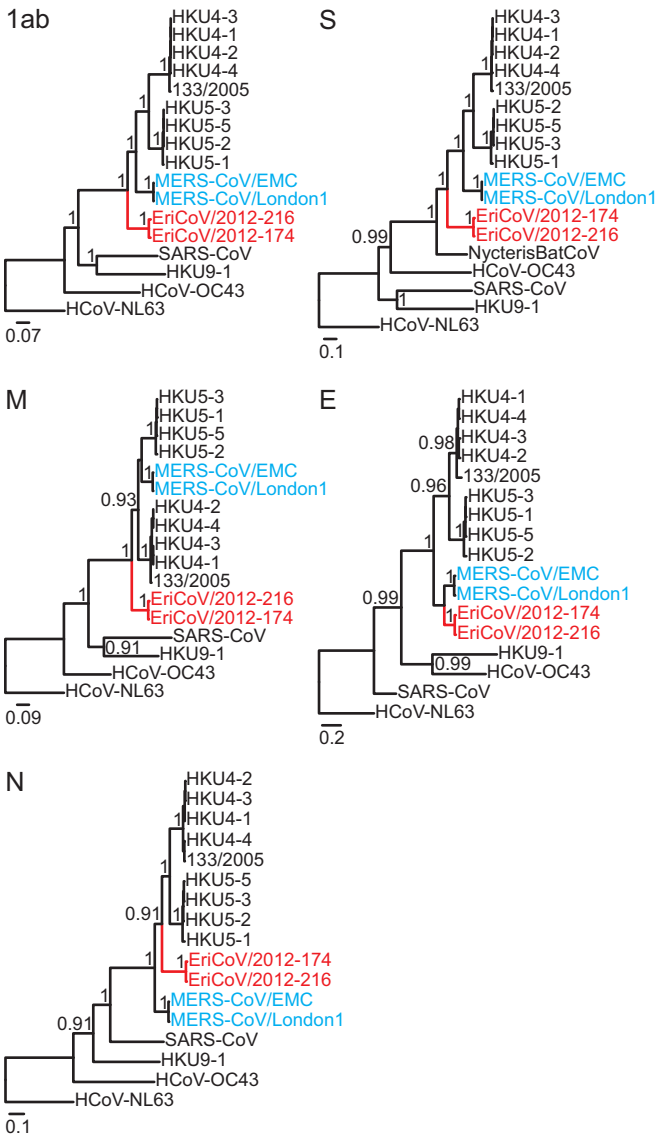

FIG 6 Phylogenies of all ORFs, including the novel hedgehog betacoronaviruses. Statistical support of grouping from Bayesian posterior probabilities is shown at deep nodes. For graphical reasons, only values above 0.7 are shown. Scale bar represents genetic distance. The novel *Erinaceus* viruses are shown in red, and MERS-CoVs in blue.

There is close phylogenetic relatedness between the mammalian orders *Chiroptera*, which includes bats, and *Eulipotyphla*, which includes hedgehogs (19, 50, 51). The phylogenetic clustering of EriCoV inside the bat-dominated clade c might hint at an exchange of viruses between bats and hedgehogs in the past, and yet, a divergence of EriCoV from bat CoVs during the formation of the eulipotyphlan stem lineage cannot be excluded. Further studies on putative CoVs in other insectivorous mammals, e.g., shrews or moles, in addition to CoVs in bats and an expansion of the nidovirus diversity in insects may therefore allow further hints at the evolutionary origins of CoVs. The detection of clade c betacoronaviruses in *Chiroptera*, *Eulipotyphla*, and primate hosts (hu-

mans) may be compatible with the high replicative capacity of MERS-CoV on different mammalian cell lines (31) and could imply that both bat and nonbat hosts should be investigated to elucidate the origins of MERS-CoV.

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