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Short communication

Molecular characterization of a canine respiratory coronavirus strain detected in Italy☆

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ABSTRACT

Coronaviruses (CoVs) are positive-stranded, non-segmented RNA viruses generally responsible for the emergence of respiratory and enteric disease in humans, companion animals and livestock. Their aptitude to evolve by genetic recombination and/or point mutation is recognized, thus giving rise to new viral genotypes and mutants with different tissues or host tropism. In particular, a probable origin from the strictly related bovine coronavirus (BCoV) or, alternatively, from a common ancestor has been suggested for some group 2a CoVs, including canine respiratory coronavirus (CRCoV). In this study, we report the sequence analysis of the viral RNA 3′-end of an Italian CRCoV, strain 240/05, together with the sequence comparison with extant bovine-like viruses including the sole CRCoV strain 4182 previously described. Interestingly, although the structural proteins show the same features of CRCoV 4182, the genomic region between the spike and the envelope protein genes of CRCoV 240/05 encodes for three distinct products, including the equivalent bovine 4.9 kDa non-structural protein and a truncated form of the 4.8 kDa protein, whereas CRCoV 4182 has a unique 8.8 kDa protein.

Coronaviruses (CoVs) are enveloped (+) strand RNA viruses and are generally responsible for respiratory and enteric diseases affecting mammals and birds. With genome sizes ranging from 27.6 kb to 31.6 kb, they represent the largest RNA viruses known to date (Gorbalenya et al., 2006; Siddell and Snijder, 2008). After the emergence of SARS in humans, concern regarding animal CoVs has arisen in the scientific community, since animal CoVs can be assumed as models for human CoVs. In addition, as a consequence of the implemented epidemiological survey programs, new members of the family Coronaviridae have been detected in animals and humans. Currently, CoVs are organized into three antigenic groups with group 2 including bovine-like (group 2a) and SARS-like (group 2b) viruses (Gorbalenya, 2008). It has been suggested that human coronavirus (HCoV) OC43 has arisen as consequence of a trans-species infection caused by bovine coronavirus (BCoV) (Vijgen et al., 2005), and this may be also true for some animal group 2a CoVs, including porcine hemagglutinating encephalomyelitis virus (PHEV), buffalo coronavirus (BuCoV), giraffe coronavirus (GiCoV), alpaca coronavirus (ApCoV), and canine respiratory coronavirus (CRCoV) (Hasoksuz et al., 1999; Vijgen et al., 2006; Erles et al., 2007; Jin et al., 2007; Decaro et al., 2008; Genova et al., 2008). Alternatively, BCoV and bovine-like CoVs may have arisen from a common ancestor (Vijgen et al., 2006). Unlike the group 1a enteric canine coronaviruses (CCoVs) types I and II (for a review, see Decaro and Buonavoglia, 2008) CRCoV has been associated with mild respiratory signs and has been proposed as an etiological agent of canine infectious respiratory disease (CIRD) together with *Bordetella bronchiseptica*, canine adenovirus types 1 and 2, canine parainfluenza virus, canine herpesvirus, reoviruses and influenza viruses (Erles et al., 2004; Buonavoglia and Martella, 2007; Erles and Brownlie, 2008). CRCoV has been detected firstly in UK in 2003 (Erles et al., 2003) and subsequently in Italy (Decaro et al., 2007). A group 2a CoV has also been identified in dogs in Canada (Ellis et al., 2005) and Japan (Kaneshima et al., 2006), and there is serological evidence that a bovine-like CoV is circulating in the canine population in other countries, including USA, Ireland and Greece (Priestnall et al., 2006). CRCoV possesses the canonical genome organization of the group 2 CoVs (Erles et al., 2007). Briefly, proceeding from the 5′-end of the genome are the genes encoding for the replicase complex and for the structural proteins (hemagglutinin-esterase, HE; spike, S; envelope, E; membrane, M; nucleocapsid, N), expressed through a 3′-coterminal nested set of subgenomic (sg) mRNAs by a process of discontinuous transcription (de Vries et al., 1997). In addition, interspersed among the structural genes, CRCoV, analogously to BCoV, carries some accessory genes, encoding for non-structural proteins (nsp), located between the replicase and the HE protein genes and

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between the S and E protein genes that are also expressed from sg mRNAs (Narayanan et al., 2008). These sg mRNAs each contain a short leader sequence at their 5′-end, followed by a transcription regulatory sequence (TRS). TRS sequences are also present in the genome upstream of each gene and they serve as signals for the generation of the sg mRNAs (for a review, see Britton and Cavanagh, 2008). Despite the serological evidence that BCoV-like particles are circulating in dogs, few partial genomic sequences of CRCoVs are publicly available, mostly regarding the S and HE proteins, except the English isolate CRCoV 4182 whose entire genomic 3′-end has been determined and analyzed (Erles et al., 2007).

In the present study, we report the sequence and phylogenetic analyses of the entire genomic 3′-end of the CRCoV 240/05 strain previously detected in the lungs of a dog in Italy (Decaro et al., 2007).

A 9.6-kb region encompassing the complete 3′-end of the viral genome of CRCoV 240/05 was determined through RT-PCR amplifications of overlapping fragments by using primers previously published (Decaro et al., 2008; Decaro and Buonavoglia, 2008) and the kit SuperScript™ One-Step RT-PCR for Long Templates (Life Technologies, Invitrogen, Milan, Italy) which contains a high-fidelity DNA polymerase (Platinum® Taq Hi Fi). The RNA was extracted from a fragment of the original lung sample stored in RNAlater RNA Stabilization Reagent (Qiagen S.p.A., Milan, Italy) by using the QIAamp® RNeasy Mini Kit (Qiagen S.p.A.), following the manufacturer's instructions, with the template RNA being eluted in 50 μl of RNase-free water. DNA samples generated from two different RT-PCR runs were sequenced in both directions by Cogenics Europe (Meylan, France). Additional RT-PCR assays and sequencing attempts were performed to close gaps between assembled contigs using strain-specific primers. The analysis of the 240/05 3′-end genomic sequence by means of the NCBI graphical analysis ORF Finder tool (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) identified nine ORFs that can be deduced to encode the non-structural and structural proteins of the virus (Fig. 1). All the predicted ORFs but the 2.7 kDa gene were preceded by a TRS sequence (CUAAAC or CCAAAC) which is assumed to interact with the viral polymerase during the discontinuous transcription of the negative sg RNAs (for a review, see Britton and Cavanagh, 2008). The ORFs encoding structural proteins are ORF 2b (nt 1017–2291), ORF 3 (nt 2306–6397), ORF 5 (nt 7087–7341), ORF 6 (nt 7356–8048), ORF 7 (nt 8058–9404). The predicted translation products are the HE (47.7 kDa), S (151.1 kDa), E (9.2 kDa), M (26.4 kDa) and N (49.3 kDa) proteins, respectively. Analogously to other bovine-like CoVs, the genome of strain 240/05 contained three accessory genes encoding for four group-specific non-structural proteins, namely ORF 2a, located between pol1b and HE genes and encoding for the 32 kDa, and, between the S and E genes, ORF 4a encoding for the 4.9 kDa and the 2.7 kDa proteins and ORF 4b encoding for the 12.8 kDa protein. Additionally, the N protein gene displayed an accompanying internal ORF 8 encoding for the I protein of 23.3 kDa. CRCoV 240/05 and 4182 differed in 88 positions (excluding the putative 4.9 kDa and the truncated 4.8 kDa proteins), 38 of which were non-synonymous and scattered over all the proteins apart from the E protein.

The group 2a-specific accessory structural HE protein was 424 amino acids long with its TRS located nine nucleotides upstream of the AUG protein initiation codon. The HE protein gene showed eight synonymous and two non-synonymous nucleotide variations when compared with that of CRCoV 4182. Interestingly, strain 240/05 shared the same amino acid mutations with all BCoV strains and derivatives described to date, so therefore these mutations are unique to CRCoV 4182. Amino acid sequence identity was 99.5% between 240/05 and 4182, whereas it ranged from 93.4% to 97.7% between 240/05 and other BCoV/bovine-like CoVs (Table 1). By analysis with the NetNglyc server (http://www.cbs.dtu.dk/services/NetNGlyc/), the HE of 240/05 was found to contain eight potential glycosylation sites analogously to CRCoV 4182. Accordingly, a potential signal peptide was identified by SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/) at amino acids 1–18 with a potential cleavage site between amino acids 18 and 19 and the predicted site for neuraminidase-*O*-acetylesterase activity, FGDS, was detected at the N-terminus.

The S protein was long 1363 amino acids and its AUG codon was directly preceded by the TRS sequence. Amino acid identity in comparison with the S protein of CRCoV 4182 was 98.4% (Table 1) showing 22 amino acid substitutions, of which nine were found to be unique to 240/05 strain whereas 10 were present in other BCoV and derivatives. Twenty-one synonymous nucleotide variations were encountered in the S gene. Amino acid identity between 240/05 and BCoV/bovine-like viruses S protein ranged from 91.2% to 95.7% (Table 1). A potential N-terminal signal peptide was identified at amino acids 1–17 and 21 potential glycosylation sites were detected throughout the protein. No deletions or insertions were observed in strain 240/05 compared with bovine-like CoVs. Moreover, the amino acid stretch KRRSRR, responsible for the proteolytic cleavage of the S protein at residue 768 into subunits S1 and S2, was conserved.

The E protein was 84 amino acids long and identical, in terms of amino acid sequence, to that of CRCoV 4182. Only one synonymous nucleotide change differentiated the E protein encoding gene of strains 240/05 and 4182. Two residues were found to be different from the same protein of BCoV-Mebus, only one if compared with the extant BCoVs. The TRS sequence was located 123 nucleotides upstream the AUG codon as already shown in 4182 strain. SignalP assigned a signal anchor at position 1–34 and no glycosylation sites were found.

The M protein was 230 amino acids in length and the AUG translation initiation codon was separated from the TRS stretch by three nucleotides. Five synonymous and five non-synonymous nucleotide variations were found with respect to the M gene of CRCoV 4182, three of which were unique to 240/05 strain while the remaining

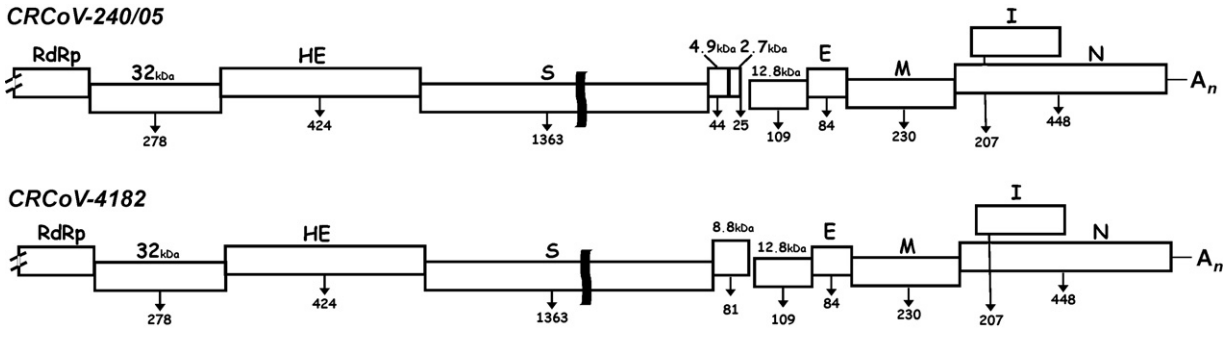

Fig. 1. Schematic comparison of the CRCoV 240/05 and 4182 genomes. Below the diagram, the length in amino acids is reported for the encoded structural and the putative non-structural proteins.

Table 1
Amino acid identity (%) of CRCoV 240/05 to group 2 CoV reference strains in non-structural and structural proteins.

CoV strain	Amino acid identity (%) to CRCoV 240/05									
	32 kDa	HE	S	4.9 kDa	2.7 kDa[a]	12.8 kDa	E	M	N	I
BCoV-Mebus	97.1	97.9	95.5	90.7	90.7	96.3	97.6	97.6	97.6	95.5
BCoV-Quebec	88.5	97.6	95.5	90.7	90.7	88.5	98.8	98.8	98.8	95.5
BCoV-DB2	96.4	97.6	95.7	90.7	90.7	96.3	98.8	98.8	98.8	95.5
BCoV-ENT	96.0	97.2	95.3	82.8	82.8	96.3	98.8	98.8	98.8	94.6
BCoV-LUN	96.0	96.7	95.3	79.3	79.3	95.4	98.8	98.8	98.8	94.6
BCoV-E-AH65	96.4	96.9	95.6	82.8	82.8	95.4	98.8	98.8	98.8	95.0
BCoV-R-AH65	96.8	96.9	95.4	82.8	82.8	96.3	98.8	98.8	98.8	95.0
BCoV-E-AH65-TC	96.8	96.7	95.3	82.8	82.8	96.3	98.8	98.8	98.8	95.0
BCoV-R-AH65-TC	96.8	96.9	95.4	82.8	82.8	96.3	98.8	98.8	98.8	95.0
BCoV-E-AH187	98.2	96.9	95.2	82.8	82.8	96.3	98.8	98.8	98.8	94.1
BCoV-R-AH187	96.0	97.2	95.2	82.8	82.8	96.3	98.8	98.8	98.8	95.0
GiCoV-US/OH3/2003	96.8	97.2	95.4	82.8	82.8	95.4	98.8	98.8	98.8	95.0
BuCoV-179/07-11	95.7	96.7	95.2	86.0	86.0	95.4	98.8	98.8	98.8	94.6
ApCoV	96.8	96.9	95.1	82.8	82.8	95.4	98.8	98.8	98.8	94.6
SACoV-US/OH1/2003	96.8	97.2	95.3	82.8	82.8	94.5	98.8	98.8	96.4	94.6
CRCoV-4182	98.9	99.5	98.4	NP	NP	97.2	100	100	100	99.5
HCoV-OC43	95.0	93.4	91.2	NP	NP	89.9	96.4	96.4	96.4	54.3
HECV-4408	NA	97.4	95.5	90.7	90.7	95.4	97.6	97.6	97.6	95.0
MHV-A59	48.9	59.8	66.8	NP	NP	NP	63.4	63.4	63.4	45.7

NA, not available; NP, not present.
[a] For this comparison, the correspondent region of the 4.8 kDa protein of the bovine-like CoVs was considered.

two changes were shared with extant BCoV strains. Moreover, four potential *O*-glycosylation sites (http://www.cbs.dtu.dk/services/NetOGlyc/) and one *N*-glycosylation site were identified at the N-terminus of the protein as previously described for all bovine-like CoVs.

The N protein of strain 240/05 had a length of 448 amino acids and was highly conserved among the bovine and bovine-like viruses. Six synonymous and two non-synonymous changes were present with respect to strain 4182. The same amino acid changes were present in extant BCoV-like viruses.

The putative 32 kDa nsp showed the same length (278 amino acids) as most other bovine-like CoVs, including GiCoV, ApCoV and sable antelope coronavirus (SACoV), as well as HCoV-OC43 and CRCoV 4182. Three amino acid changes were found in comparison with 4182 strain, of which one was unique to 240/05 whereas the remaining two substitutions were shared with other bovine-like strains. Five were the synonymous nucleotide substitutions. The corresponding TRS sequence was located seven nucleotides upstream of the AUG codon confirming the genome organization of CRCoV 4182.

In the majority of their genome, CRCoV 240/05 and 4182 possessed the same genomic arrangement, but they displayed a diverse genome structure in the accessory genes encoding for nsp located between the S and E genes. In this region, most BCoV-like CoVs display three accessory genes, namely the 4.9 kDa, 4.8 kDa and 12.8 kDa protein genes. In CRCoV 4182 only two accessory genes were detected (Erles et al., 2007), whereas the genomic arrangement of other bovine-like CoVs was partially conserved in strain 240/05. Indeed, CRCoV 4182 showed a unique 8.8 kDa protein fashioned by the fusion of the 4.9 kDa protein and a truncated form of the 4.8 kDa protein. This was the result of a two nucleotide deletion that inactivated the stop codon of the 4.9 kDa protein encoding sequence and introduced additional 12 amino acids not found in any BCoV strains (Erles et al., 2007). In contrast, in the 240/05 genome the terminating codon of the 4.9 kDa encoding sequence is conserved exactly as in most group 2a CoVs, including BCoVs-Mebus, Quebec and DB2, HECV-4408 and BuCoV. The corresponding TRS sequence is located 317 nucleotides upstream of the initiation codon according to previous observations for other ruminant CoVs. However, the 4.8 kDa protein of most BCoV and bovine-like CoVs is replaced in strain 240/05 by a 2.7 kDa protein (25 amino acids in length) due to the presence of an early stop codon. An identical truncated protein has been already described for CRCoV strain G9142 (Erles et al., 2007), whereas a slightly longer truncated protein is present in GiCoV and SACoV (Hasoksuz et al., 1999). The corresponding TRS sequence was not detected in any bovine-like viruses with the exception of CRCoV G9142 (Erles et al., 2007). The 12.8 kDa protein was 109 amino acids in length according to the extant BCoV and bovine-like CoVs except BCoV Quebec that exhibits a truncated form of the protein. The related TRS was located 73 nucleotides upstream of the AUG codon. Two synonymous and three non-synonymous nucleotide substitutions with respect to strain 4182 were detected in the coding sequence, with one change being unique if compared to other bovine-like CoVs previously described. The I protein was 207 amino acids in length according to CRCoV 4182. Only one amino acid change and two synonymous nucleotide variations differentiated the two CRCoV strains and strain 240/05 showed almost 95% average amino acid identity with most of the bovine-derivative viruses.

The rooted phylogenetic analysis (Fig. 2) performed on the S (a) and M/N (b) proteins confirmed the high genomic relatedness of CRCoV with BCoV and its derivatives, thus supporting data obtained previously (Decaro et al., 2007, 2008; Erles et al., 2007). Noteworthy, in the S and M/N phylogenetic trees, CRCoV 240/05 and 4182 formed a separate bunch among the group 2a CoVs, most likely as a consequence of the speciation of BCoV in the canine host.

This study provides useful data for the molecular comprehension of bovine-like CoVs in the canine host. The CoV ecology is intricate due to the number of apparently frequent cross-species jumps that entail its evolution. Indeed, the presence of bovine-like CoVs in dogs was clearly demonstrated in previous studies by serological methods, sequence comparison and phylogenetic analysis, but molecular data available to date is incomplete and limited in number. Sequence analysis of the 3′-end of the viral genome showed that strain 240/05 has a genomic organization similar to BCoV, including the presence of the ORFs encoding for the nsp between the S and E protein genes. Although the structural genes are highly conserved among CRCoV strains 240/05 and 4182, the location and number of the accessory genes differ between the two viruses. Moreover, their function in the viral life cycle has not been established yet. In general, as already demonstrated for other CoVs, accessory genes are not essential for replication and their expression could even decrease viral fitness *in vitro* (Schwarz et al., 1990; Yokomori et al., 1991; Curtis et al., 2002; Ortego et al., 2003; Youn et

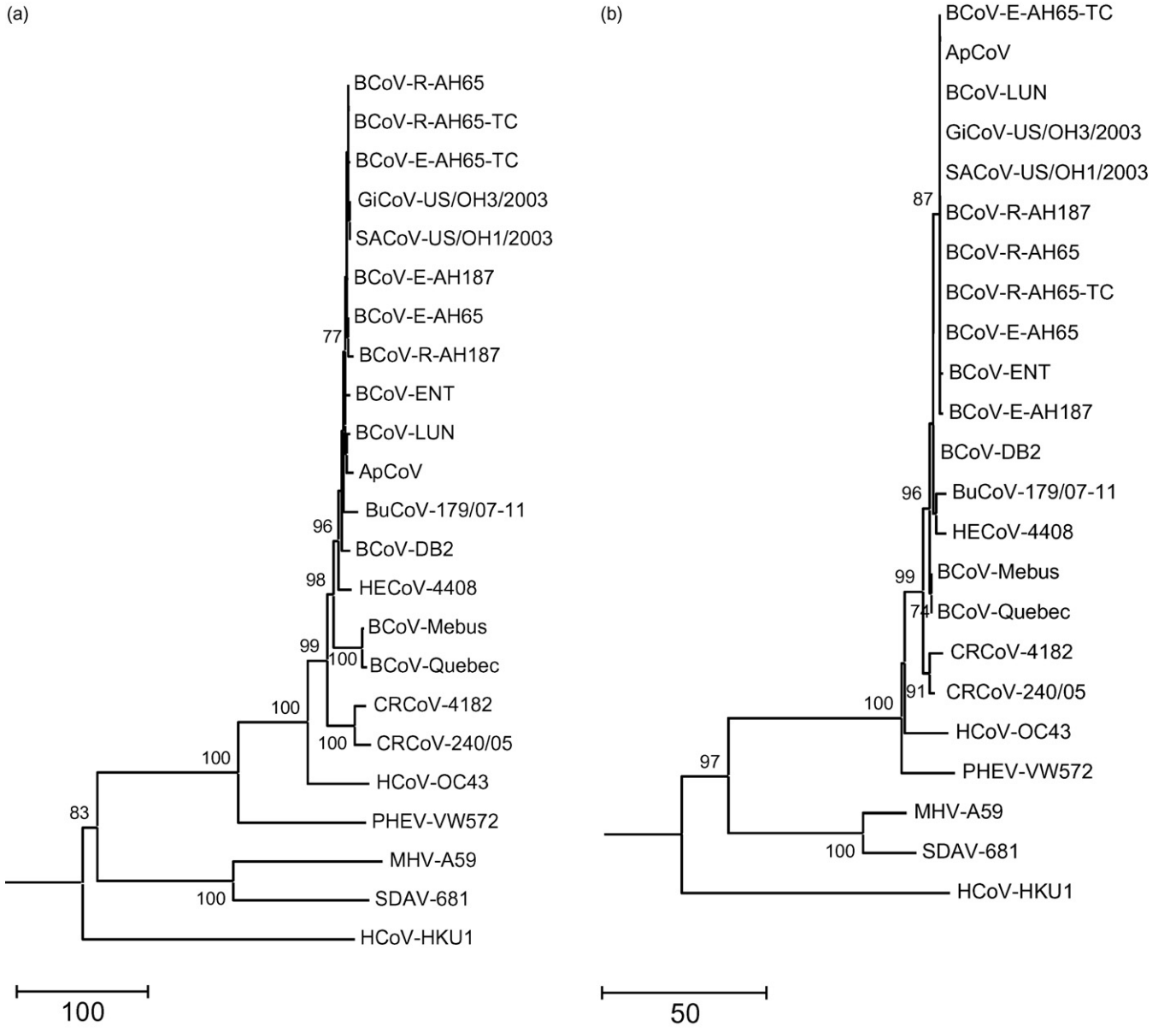

Fig. 2. Rooted neighbor-joining tree inferred from multiple amino acid sequence alignment of the S (a) and M/N (b) protein, illustrating the relationship of CRCoV in the group 2a. For the analysis, CCoV-II CB/05 (DQ112226) served as outgroup and the following CoVs strain were used: BCoV-Mebus (U00735), Quebec (AF220295), DB 2 (DQ811784), ENT (AF391541), LUN (AF391542), E-AH65 (EF424615), R-AH65 (EF424617), E-AH65-TC (EF424616), R-AH65-TC (EF424618), E-AH187 (EF424619), R-AH187 (EF424620); GiCoV (EF424623); ApCoV (DQ915164); SACoV (EF424621); CRCoV-4182 (DQ682406); HCoV-OC43 (NC_005147); PHEV-VW572 (DQ011855); MHV-A59 (AY700211); SDAV (AF207551); HCoV-HKU1 (NC_006577); BuCoV-179/07-11 (EU019216). A statistical support was provided by bootstrapping over 1000 replicates and bootstrap values >70 are indicated at the correspondent node. The scale bars indicate the estimated numbers of amino acid substitutions per site.

al., 2005; Yount et al., 2005). Nevertheless, in field conditions those genes are constantly maintained (Herrewegh et al., 1995; Smits et al., 2005; Dijkman et al., 2006) and their loss is often accompanied by the decline of virulence in the natural host (de Haan et al., 2002; Ortego et al., 2003; Haijema et al., 2004). CRCoV 240/05 was directly sequenced from the lung sample of a dog allowing the molecular characterization of viral RNA coming straight from field conditions with no adaptation to cell culture. Apparently, in natural conditions the 4.8 kDa protein, strictly maintained in other bovine-like CoVs, was truncated in CRCoV 240/05, and this may be potentially associated to the cross-species transmission and subsequent adaptation of the ancestor BCoV to a different host. However, directly downstream of the S protein gene, CRCoV 4182 possessed a unique 8.8 kDa protein gene, whereas CRCoV G9142 displayed the canonical set of BCoV accessory genes but with the equal truncated form of the 4.8 kDa protein gene as in strain 240/05. Noteworthy, the identical truncated 4.8 kDa terminating codon is present in 240/05, G9142 and in the unique 8.8 kDa corresponding nucleotide sequence of CRCoV strain 4182 (Erles et al., 2007). Accordingly, more than one BCoV strain or ancestor virus was likely involved in the origin of CRCoV, thus leading to the emergence of different canine strains with a different organizations of the accessory genes. It has been hypothesized that the 4.9 kDa and 4.8 kDa proteins of BCoV may have arisen through mutation from a bovine 11 kDa protein (Abraham et al., 1990). According to this scenario, CRCoV 4182 may descent from a mutation of an ancestral bovine-like strain that exhibited the full-length 11 kDa protein, whereas CRCoV 240/05 and G9142 presumably descended from a different ancestor that showed the two distinct non-structural proteins. Furthermore, nsp 4.9 and 4.8 are not present in the bovine-derivative HCoV-OC43 (Vijgen et al., 2005) and their function is yet unknown in BCoV itself where the 4.9 kDa protein could not been expressed due to

the absence of a start codon in its mRNA (Hofmann et al., 1993). Obviously, it cannot be ruled out that the elevated level of genomic differences among the accessory genes is due to the high frequency of mutations/deletions that occur during CoV evolution, rather than to RNA recombination events that characterize CoV ecology (Lai et al., 1985; Makino et al., 1986; Banner and Lai, 1991). Accordingly, all CRCoV strains analyzed so far constantly cluster at the phylogenetic level with extant BCoV strains (Fig. 2), reinforcing previous suggestions that CRCoV apparently originated as a host variant of BCoV or both viruses descended from a common ancestor. Nevertheless, the elevated level of amino acid similarity with extant bovine-like CoVs characterizing the 12.8 kDa protein suggests a more important role of this protein in viral pathobiology (Erles et al., 2007). Unfortunately, CRCoV 240/05 could not be propagated in tissue culture probably as a consequence of the long storage of the tissue sample, thus preventing further analysis of viral mRNAs in the context of the infected cells. However, several studies are warranted in order to investigate the effective functionality of the accessory genes in BCoV and CRCoV, thus elucidating the relationship and variations in their expression and the interconnected evolution of highly similar viruses in different hosts.

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References

Abraham, S., Kienzle, T.E., Lapps, W.E., Brian, D.A., 1990. Sequence and expression analysis of potential nonstructural proteins of 4.9, 4.8, 12.7, and 9.5 kDa encoded between the spike and membrane protein genes of the bovine coronavirus. Virology 177, 488–495.

Banner, L.R., Lai, M.M., 1991. Random nature of coronavirus RNA recombination in the absence of selection pressure. Virology 185, 441–445.

Britton, P., Cavanagh, D., 2008. Nidovirus genome organization and expression mechanisms. In: Perlman, S., Gallagher, T., Snijder, E.J. (Eds.), Nidoviruses. ASM Press, Washington, DC, pp. 29–46.

Buonavoglia, C., Martella, V., 2007. Canine respiratory viruses. Vet. Res. 38, 355–373.

Curtis, K.M., Yount, B., Baric, R.S., 2002. Heterologous gene expression from transmissible gastroenteritis virus replicon particles. J. Virol. 76, 1422–1434.

de Haan, C.A.M., Masters, P.S., Shen, X., Weiss, S., Rottier, P.J.M., 2002. The group-specific murine coronavirus genes are not essential, but their deletion, by reverse genetics, is attenuating in the natural host. Virology 296, 177–189.

de Vries, A.A.F., Horzinek, M.C., Rottier, P.J.M., de Groot, R.J., 1997. The genome organization of the nidovirales: similarities and differences between arteri-, toro-, and coronaviruses. Semin. Virol. 8, 33–47.

Decaro, N., Desario, C., Elia, G., Mari, V., Lucente, M.S., Cordioli, P., Colaianni, M.L., Martella, V., Buonavoglia, C., 2007. Serological and molecular evidence that canine respiratory coronavirus is circulating in Italy. Vet. Microbiol. 121, 225–230.

Decaro, N., Martella, V., Elia, G., Campolo, M., Mari, V., Desario, C., Lucente, M.S., Lorusso, A., Greco, G., Corrente, M., Tempesta, M., Buonavoglia, C., 2008. Biological and genetic analysis of a bovine-like coronavirus isolated from water buffalo (*Bubalus bubalis*) calves. Virology 370, 213–222.

Decaro, N., Buonavoglia, C., 2008. An update on canine coronaviruses: viral evolution and pathobiology. Vet. Microbiol. 132, 221–234.

Dijkman, R., Jebbink, M.F., Wilbrink, B., Pyrc, K., Zaaijer, H.L., Minor, P.D., Franklin, S., Berkhout, B., Thiel, V., van der Hoek, L., 2006. Human coronavirus 229E encodes a single ORF4 protein between the spike and the envelope genes. Virol. J. 3, 106.

Ellis, J.A., McLean, N., Hupaelo, R., Haines, D.M., 2005. Detection of coronavirus in cases of tracheobronchitis in dogs: a retrospective study from 1971 to 2003. Can. Vet. J. 46, 447–448.

Erles, K., Brownlie, J., 2008. Canine respiratory coronavirus: an emerging pathogen in the canine infectious respiratory disease complex. Vet. Clin. North Am. Small Anim. Pract. 38, 815–825.

Erles, K., Dubovi, E.J., Brooks, H.W., Brownlie, J., 2004. Longitudinal study of viruses associated with canine infectious respiratory disease. J. Clin. Microbiol. 42, 4524–4529.

Erles, K., Shiu, K.B., Brownlie, J., 2007. Isolation and sequence analysis of canine respiratory coronavirus. Virus Res. 124, 78–87.

Erles, K., Toomey, C., Brooks, H.W., Brownlie, J., 2003. Detection of a group 2 coronavirus in dogs with canine infectious respiratory disease. Virology 310, 216–223.

Genova, S.G., Streeter, R.N., Simpson, K.M., Kapil, S., 2008. Detection of antigenic group 2 coronavirus in an adult alpaca with enteritis. Clin. Vaccine Immunol. 15, 1629–1632.

Gorbalenya, A.E., 2008. Genomics and evolution of the Nidovirales. In: Perlman, S., Gallagher, T., Snijder, E.J. (Eds.), Nidoviruses. ASM Press, Washington, DC, pp. 15–28.

Gorbalenya, A.E., Enjuanes, L., Ziebuhr, J., Snijder, E.J., 2006. Nidovirales: evolving the largest RNA virus genome. Virus Res. 117, 17–37.

Haijema, B.J., Volders, H., Rottier, P.J., 2004. Live, attenuated coronavirus vaccines through the directed deletion of group-specific genes provide protection against feline infectious peritonitis. J. Virol. 78, 3863–3871.

Hasoksuz, M., Lathrop, S.L., Gadfield, K.L., Saif, L.J., 1999. Isolation of bovine respiratory coronaviruses from feedlot cattle and comparison of their biological and antigenic properties with bovine enteric coronaviruses. Am. J. Vet. Res. 60, 1227–1233.

Herrewegh, A.A., Vennema, H., Horzinek, M.C., Rottier, P.J., de Groot, R.J., 1995. The molecular genetics of feline coronaviruses: comparative sequence analysis of the ORF7a/7b transcription unit of different biotypes. Virology 212, 622–631.

Hofmann, M.A., Chang, R.-Y., Ku, S., Brian, D.A., 1993. Leader-mRNA junction sequences are unique for each subgenomic mRNA species in the bovine coronavirus and remain so throughout persistent infection. Virology 196, 163–171.

Jin, L., Cebra, C.K., Baker, R.J., Mattson, D.E., Cohen, S.A., Alvarado, D.E., Rohrmann, G.F., 2007. Analysis of the genome sequence of an alpaca coronavirus. Virology 365, 198–203.

Kaneshima, T., Hohdatsu, T., Satoh, K., Takano, T., Motokawa, K., Koyama, H., 2006. The prevalence of a group 2 coronavirus in dogs in Japan. J. Vet. Med. Sci. 68, 21–25.

Lai, M.M., Baric, R.S., Makino, S., Keck, J.G., Egbert, J., Leibowitz, J.L., Stohlman, S.A., 1985. Recombination between nonsegmented RNA genomes of murine coronaviruses. J. Virol. 56, 449–456.

Makino, S., Keck, J.G., Stohlman, S.A., Lai, M.M., 1986. High-frequency RNA recombination of murine coronaviruses. J. Virol. 57, 729–737.

Narayanan, K., Huang, C., Makino, S., 2008. Coronavirus accessory proteins. In: Perlman, S., Gallagher, T., Snijder, E.J. (Eds.), Nidoviruses. ASM Press, Washington, DC, pp. 235–244.

Ortego, J., Sola, I., Almazan, F., Ceriani, J.E., Riquelme, C., Balasch, M., Plana, J., Enjuanes, L., 2003. Transmissible gastroenteritis coronavirus gene 7 is not essential but influences in vivo virus replication and virulence. Virology 308, 13–22.

Priestnall, S.L., Brownlie, J., Dubovi, E.J., Erles, K., 2006. Serological prevalence of canine respiratory coronavirus. Vet. Microbiol. 115, 43–53.

Schwarz, B., Routledge, E., Siddell, S.G., 1990. Murine coronavirus nonstructural protein ns2 is not essential for virus replication in transformed cells. J. Virol. 64, 4784–4791.

Siddell, S., Snijder, E.J., 2008. An introduction to Nidovirus. In: Perlman, S., Gallagher, T., Snijder, E.J. (Eds.), Nidoviruses. ASM Press, Washington, DC, pp. 1–13.

Smits, S.L., Gerwig, G.J., van Vliet, A.L., Lissenberg, A., Briza, P., Kamerling, J.P., Vlasak, R., de Groot, R.J., 2005. Nidovirus sialate-O-acetylesterases: evolution and substrate specificity of coronaviral and toroviral receptor-destroying enzymes. J. Biol. Chem. 280, 6933–6941.

Vijgen, L., Keyaerts, E., Lemey, P., Maes, P., Van Reeth, K., Nauwynck, H., Pensaert, M., Van Ranst, M., 2006. Evolutionary history of the closely related group 2 coronaviruses: porcine hemagglutinating encephalomyelitis virus, bovine coronavirus, and human coronavirus OC43. J. Virol. 80, 7270–7274.

Vijgen, L., Keyaerts, E., Moes, E., Thoelen, I., Wollants, E., Lemey, P., Vandamme, A.M., Van Ranst, M., 2005. Complete genomic sequence of human coronavirus OC43: molecular clock analysis suggests a relatively recent zoonotic coronavirus transmission event. J. Virol. 79, 1595–1604.

Yokomori, K., Banner, L.R., Lai, M.M., 1991. Heterogeneity of gene expression of the hemagglutinin-esterase (HE) protein of murine coronaviruses. Virology 183, 647–657.

Youn, S., Leibowitz, J.L., Collisson, E.W., 2005. In vitro assembled, recombinant infectious bronchitis viruses demonstrate that the 5a open reading frame is not essential for replication. Virology 332, 206–215.

Yount, B., Roberts, R.S., Sims, A.C., Deming, D., Frieman, M.B., Sparks, J., Denison, M.R., Davis, N., Baric, R.S., 2005. Severe acute respiratory syndrome coronavirus group-specific open reading frames encode nonessential functions for replication in cell cultures and mice. J. Virol. 79, 14909–14922.