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Evolutionary History of the Closely Related Group 2 Coronaviruses: Porcine Hemagglutinating Encephalomyelitis Virus, Bovine Coronavirus, and Human Coronavirus OC43

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The close genetic and antigenic relatedness among the group 2 coronaviruses human coronavirus OC43 (HCoV-OC43), bovine coronavirus (BCoV), and porcine hemagglutinating encephalomyelitis virus (PHEV) suggests that these three viruses with different host specificities diverged fairly recently. In this study, we determined the complete genomic sequence of PHEV (strain PHEV-VW572), revealing the presence of a truncated group 2-specific ns2 gene in PHEV in comparison to other group 2 coronaviruses. Using a relaxed molecular clock approach, we reconstructed the evolutionary relationships between PHEV, BCoV, and HCoV-OC43 in real-time units, which indicated relatively recent common ancestors for these species-specific coronaviruses.

Coronaviruses (family *Coronaviridae*, order *Nidovirales*) are large, enveloped, positive-stranded RNA viruses with a typical crown-like appearance. Their viral genomes (27 to 32 kb) are some of the largest known among all RNA viruses (12). Based on genetic and serological relationships, coronaviruses can be classified into three groups (8). Group 2 coronaviruses include murine hepatitis virus (MHV), bovine coronavirus (BCoV), human coronavirus OC43 (HCoV-OC43), rat sialodacryoadenitis virus, porcine hemagglutinating encephalomyelitis virus (PHEV), canine respiratory coronavirus, and equine coronavirus.

PHEV was first isolated in 1962 in Canada from suckling piglets with encephalomyelitis (9, 18) and is now found to be widespread among swine worldwide, with frequent subclinical infections among swine. The virus has a strong tropism for epithelial cells of the upper respiratory tract and for the central nervous system (CNS) and is transmitted through nasal secretions (1). In addition to clinical signs of encephalomyelitis, vomiting and wasting disease can be another manifestation of PHEV infection in piglets (22). The clinical symptoms of vomiting and wasting are assumed to be centrally induced by infection of the vagus nerve, but a possible further dissemination of the virus into the CNS may lead to centrally induced motoric disorders.

In this study, we determined the full-length genome sequence of the PHEV-VW572 strain and we reconstructed the common evolutionary history of PHEV and the closely related BCoV and HCoV-OC43. The PHEV-VW572 strain was isolated in Belgium in 1972 from the tonsils of two diseased pigs obtained from a litter in which an outbreak of vomiting and wasting disease occurred without further progression towards CNS motoric disorders (23). The isolate was propagated in a primary porcine kidney cell line. To determine the full-length genome sequence, primers developed for sequencing of group 2 coronaviruses, as described previously, were used (33).

Multiple sequence alignments were prepared using ClustalX version 1.82 (30) and manually edited in GeneDoc (21). Maximum-likelihood phylogenetic analyses were conducted in Tree-Puzzle 5.1 using the VT (Mueller-Vingron 2000) model of amino acid substitution and a gamma distribution to model among-site rate heterogeneity (29). The SimPlot program (version 3.2) was used to analyze the genetic distance of the complete genomes of PHEV-VW572, two BCoV strains (BCoV-LUN and BCoV-Mebus), and an HCoV-OC43 contemporary strain (HCoV-OC43 BE03) in reference to the complete genome of the HCoV-OC43 ATCC strain, and this genetic distance was plotted versus nucleotide (nt) positions (14). Divergence times were estimated using a Bayesian coalescent approach implemented with BEAST version 1.2 (6). A novel relaxed molecular clock model that allows rates to change among branches in an uncorrelated fashion was applied (5). In this approach, rates are sampled identically and independently from an underlying distribution, in this case an exponential distribution. Markov Chain Monte Carlo analysis chains were run for 35×10^6 generations using a Hasegawa-Kishino-Yano model of nucleotide substitutions with gamma-distributed rates among sites and using a constant population size as a tree prior. Mean estimates and credibility intervals for the continuous parameters were obtained using Tracer (Rambaut and Drummond, 2003, available from http://evolve.zoo.ox.ac.uk/); the burn in was set at 10% of the sampled states. Instantaneous nonsynonymous substitution (dN) and synonymous substitution (dS) rates were estimated using a maximum-likelihood sliding window approach

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as previously described (13). A window size of 600 nt and a step size of 60 nt were used in the analysis.

The complete PHEV genome comprises 30,480 nucleotides, excluding the 3′ terminal polyadenylation tail, and has a GC content of 37.2%. The nucleotide sequence data were deposited in GenBank under accession number DQ011855. An analysis of these data revealed a significant truncation of the group 2-specific ns2 gene in PHEV in comparison with the ns2 gene in BCoV, HCoV-OC43, and MHV. The PHEV ns2 gene is only 585 nt in length, coding for a 194-amino-acid nonstructural protein. The carboxy-terminal truncation of the PHEV ns2 protein is caused by a deletion of 211 nucleotides, present in the BCoV and HCoV-OC43 genes encoding this protein, at the 3′ end of the gene. In the amino-terminal part of the ns2 protein of group 2 coronaviruses, a cyclic phosphodiesterase activity has been predicted (16, 27). These viral cyclic phosphodiesterase domains, which have also been predicted in toroviruses and rotaviruses, are, like their cellular counterparts, believed to mediate the conversion of ADP ribose 1″-2″ cyclic phosphate to ADP ribose 1″-phosphate, which is part of the processing of tRNA-splicing products (36). Although ns2 has been shown to be nonessential for in vitro coronavirus replication, a role for ns2 in viral pathogenicity can be suggested, as has been demonstrated by the observation that the deletion of MHV ns2 significantly attenuates the virus when it is inoculated into mice (4, 26). Potential nucleotide binding domains have been identified in the amino-terminal part of the ns2 protein of MHV-A59 and BCoV, and similar domains can also be found in the PHEV ns2 protein (3, 15).

A difference in length between the PHEV-VW572 ns4.9 open reading frame (ORF) and those of two other PHEV strains (67N and IAF404; GenBank accession no. AY078417 and AF481863) was found. The ns4.9 and ns4.8 ORFs are two ORFs present in the BCoV genome, located between the spike gene and the ns12.9 ORF. In PHEV, a nucleotide deletion similar to the 290-nt deletion in HCoV-OC43 can be demonstrated (20, 33), leading to the absence of ns4.8 and a truncated ns4.9, encoding a 20-amino-acid protein in PHEV strains 67N and IAF404. In PHEV-VW572, this ns4.9 ORF codes for a protein of 24 amino acids and this has also been demonstrated for PHEV strain NT9 (31). A potential functional consequence of this observation, if there is one, is not yet known. Interestingly, a truncation of the BCoV ns4.9 protein represents a significant difference between bovine respiratory and enteric coronavirus isolates, suggesting a possible role of ns4.9 in tissue tropism preference (7, 24). Of the PHEV strains compared in this study, the PHEV-IAF404 isolate may be more invasive for the CNS, as it was reported to cause encephalomyelitis associated with paralysis in addition to the manifestations of vomiting and wasting disease (25). Therefore, it would be possible that the ns4.9 protein plays a role in the ability or inability of a PHEV strain to disseminate into the CNS. In PHEV-67N, however, a ns4.9 protein of the same length as that in PHEV-IAF404 was found and this strain was isolated from subclinically infected older pigs. In experimental infections with PHEV-67N, this strain was shown to be pathogenic for the CNS of neonatal pigs (19). This could suggest that the ability of PHEV strains to cause motoric disease may be age dependent and that motoric disease can occur in only very young pigs. Whether there are true strain differences in the invasive capacity of PHEV strains for the CNS and whether the ns4.9 protein plays a role in this remain to be investigated.

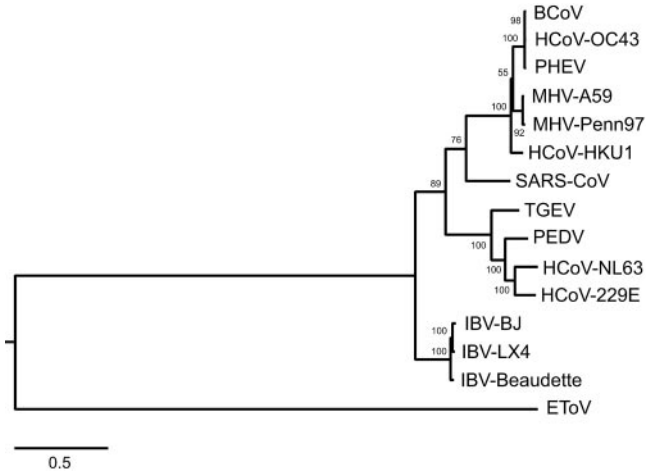

FIG. 1. Maximum-likelihood tree of coronavirus ORF1b amino acid sequences. PHEV ORF1b (GenBank accession no. DQ011855) was compared to other coronaviruses on the amino acid level. Group 1 includes human coronavirus 229E (HCoV-229E, AF304460), human coronavirus NL63 (HCoV-NL63, AY567487), porcine epidemic diarrhea virus strain CV777 (PEDV, AF353511), and porcine transmissible gastroenteritis virus Purdue strain (TGEV, AJ271965). Group 2 includes human coronavirus OC43 (HCoV-OC43, AY391777), bovine coronavirus Mebus strain (BCoV, U00735), murine hepatitis virus Penn 97-1 strain (MHV-Penn97-1, AF208066), and murine hepatitis virus A59 (MHV-A59, NC_001846). Group 3 includes avian infectious bronchitis virus Beaudette strain (IBV-Beaudette, M95169), avian infectious bronchitis virus BJ strain (IBV-BJ, AY319651) and avian infectious bronchitis virus LX4 strain (IBV-LX4, AY338732). Human coronavirus HKU1 (HCoV-HKU1, NC_006577) and SARS coronavirus Frankfurt-1 strain (SARS-CoV, AY291315) are shown. The out group includes equine Berne torovirus (EToV, X52374). The scale bar represents the genetic distance (nucleotide substitutions per site).

In a maximum-likelihood phylogenetic tree, the close genetic relatedness between PHEV, BCoV, and HCoV-OC43 is evident from the well-supported monophyletic cluster of the three viruses (Fig. 1). Using a SimPlot analysis, we demonstrated that in more than two-thirds of the genome, the genetic distance between PHEV and HCoV-OC43 is similar to the distance between BCoV and HCoV-OC43 (Fig. 2). However, in the genome region containing the spike gene, the genetic distance of PHEV to HCoV-OC43 is significantly higher than the distance of BCoV to HCoV-OC43.

Based on the spike gene sequence data and on nucleocapsid gene sequence data, we performed a relaxed molecular clock analysis of PHEV, BCoV, and HCoV-OC43 strains for which the date of sampling could be obtained (Table 1). In this analysis, we did not use sequence data from the ORF1ab region, as these data are available for only a limited number of PHEV, BCoV, and HCoV-OC43 strains with known sampling dates. The mean evolutionary rate estimate of the spike gene in PHEV, BCoV, and HCoV-OC43 is 6.1×10^{-4} nucleotide substitutions per site per year, with a 95% highest posterior density (HPD) interval of 2.1×10^{-4} to 1.0×10^{-3}. For the nucleocapsid gene in PHEV, BCoV, and HCoV-OC43, the mean evolutionary rate is estimated to be 3.6×10^{-4} nucleotide substitutions per site per year, with a 95% HPD interval of

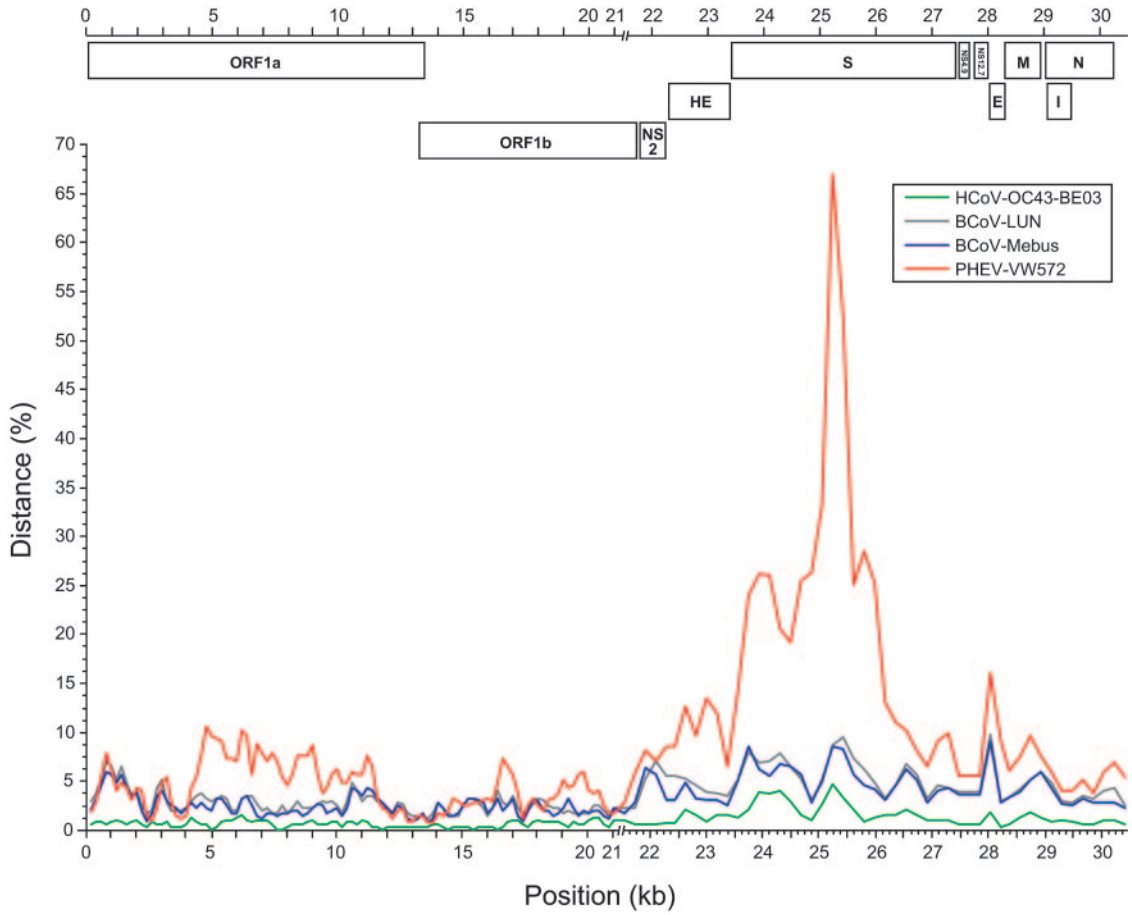

FIG. 2. Linear representation of the PHEV-VW572 genome (GenBank accession no. DQ011855) and SimPlot analysis of complete genome sequence data of PHEV-VW572, BCoV-LUN (AF391542), BCoV-Mebus (U00735), HCoV-OC43 BE03 (AY903459), and HCoV-OC43 ATCC (American Type Culture Collection, AY391777). Each point plotted is the percent genetic distance within a sliding window of 400 nt wide, centered on the position plotted, with a step size of 200 nt. Each curve represents a comparison of the sequence data of PHEV-VW572, the BCoV strains, and HCoV-OC43 BE03 to the reference sequence data of the ATCC HCoV-OC43 strain. HE, hemagglutinin-esterase gene; S, spike gene; E, envelope protein gene; M, membrane protein gene; N, nucleocapsid protein gene; I, internal ORF.

1.1×10^{-4} to 6.3×10^{-4}. The ancestral PHEV strain diverged from the common ancestor of BCoV and HCoV-OC43, and this event could be dated back to around 1878 (95% HPD interval, 1747 to 1954) based on nucleocapsid gene sequence data. When spike gene sequence data were used in this analysis, this event was dated approximately 100 years earlier (1777; 95% HPD interval, 1558 to 1919). This reflects the higher genetic distance for PHEV relative to HCoV-OC43 in this gene (Fig. 2), which implies an elevated evolutionary rate for the porcine coronavirus lineage in the spike genomic region. A maximum-likelihood sliding window approach was used to estimate dN and dS across the genome (data not shown). In the region containing the spike gene, dS is significantly higher than dN, indicating that mostly synonymous substitutions are responsible for the higher spike evolutionary rate in the PHEV lineage. The possibility of positive selection is therefore less likely, unless the synonymous substitutions would have been selected for their role in the secondary RNA structure of this genomic region. Another explanation might be a recombination event between an ancestral strain of PHEV and another hitherto unknown coronavirus. However, this hypothesis would not explain why an excess of synonymous substitutions is responsible for the high genetic distance in the PHEV spike gene region, and thus we cannot provide conclusive evidence for these speculations.

Whether the most recent common ancestor (MRCA) of PHEV, BCoV, and HCoV-OC43 was a virus replicating in a porcine, bovine, or human host, in all three species, or even in another species cannot be inferred from the present data, but we can speculate that interspecies transmission events have occurred prior to the emergence of PHEV, BCoV, and HCoV-OC43. The divergence of BCoV and HCoV-OC43 strains could be dated back to the end of the 19th to the beginning of the 20th centuries, in correspondence with our previous study (33). The time to the most recent common ancestor (TMRCA) estimates were relatively consistent when spike gene (1902; 95% HPD interval, 1802 to 1956) or nucleocapsid gene (1910; 95% HPD interval, 1812 to 1961) sequence data were used. Interestingly, the MRCAs of each of the species-specific strains, i.e., of the PHEV strains, the BCoV strains, and the HCoV-OC43 strains individually, were all estimated to have

TABLE 1. Date and area of sampling of porcine, bovine, and human coronaviruses to calculate the TMRCA

Strain	Sampling date	Sampling area	Reference
PHEV-VW572	1972	Belgium	23
PHEV-67N	1970	Iowa	18
PHEV-IAF404	1998	Quebec, Canada	24
BCoV-LY138	1965	Utah	35
BCoV Mebus	1972	Quebec, Canada	7
BCoV Quebec	1972	Quebec, Canada	11
BCoV-F15-BECS	1979	France	35
BCoV M80844[a]	1989	Giessen, Germany	34
BCoV-OK05143	1996	Kansas	7
BCoV-LSU94	1994	Louisiana	2
BCoV-ENT	1998	Texas	28
BCoV-LUN	1998	Texas	28
HCoV-OC43 ATCC	1967	Salisbury, United Kingdom	17
HCoV-OC43 BE03 isolate 37767[a]	2003	Belgium	32
HCoV-OC43 BE03 isolate 84020[a]	2003	Belgium	32
HCoV-OC43 BE03 isolate 87309	2003	Belgium	32
HCoV-OC43 BE03 isolate 89996[a]	2003	Belgium	32
HCoV-OC43 BE04 isolate 19572	2004	Belgium	32
HCoV-OC43 BE04 isolate 34364[a]	2004	Belgium	32
HCoV-OC43 BE04 isolate 36638[a]	2004	Belgium	32

[a] Strain for which the nucleocapsid gene sequence data are unavailable.

existed in a recent past, i.e., only 50 to 60 years ago. These TMRCA estimates were relatively consistent when the analysis was based on spike gene data (for PHEV strains, 1942; 95% HPD interval, 1894 to 1968; for BCoV strains, 1944; 95% HPD interval, 1910 to 1963; and for HCoV-OC43 strains, 1944; 95% HPD interval, 1910 to 1963) or when nucleocapsid gene sequence data were used (for PHEV strains, 1945; 95% HPD interval, 1894 to 1968; for BCoV strains, 1951; 95% HPD interval, 1921 to 1965; and for HCoV-OC43 strains, 1957; 95% HPD interval, 1936 to 1961). The isolation areas of the PHEV, BCoV, and HCoV-OC43 strains used in this analysis are distributed across the North American and European continents, indicating that these coronaviruses might have spread, in their natural hosts, over a large geographical region in a relatively short period of time. Our analysis does not imply that the origin of this coronavirus lineage cannot be earlier than the MRCA, which relates to only the coronaviruses that are currently circulating. Continual extinction events might have replaced earlier lineages in these species (10).

This study provides insights in the evolutionary relationships among the closely related group 2 coronaviruses PHEV, BCoV, and HCoV-OC43. The reconstruction of the evolutionary histories of closely related viruses with different host specificities might be useful for elucidating the processes of viral emergence as a result of interspecies transmission events, such as the emergence of the Severe Acute Respiratory Syndrome (SARS) coronavirus.

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