A Multigene Approach for Comparing Genealogy of Betacoronavirus from Cattle and Horses

Iracema N. Barros,1,2 Sheila O. S. Silva,1,2 Francisco S. Nogueira Neto,3 Karen M. Asano,1,2 Sibele P. Souza,1,2 Leonardo J. Richtzenhain,1,2 and Paulo E. Brandao1,2

1 Department of Preventive Veterinary Medicine and Animal Health, College of Veterinary Medicine, University of São Paulo, Avenue Professor Dr. Orlando Marques de Paiva 87, Cidade Universitária, 05508-270 São Paulo, SP, Brazil
2 Coronavirus Research Group, College of Veterinary Medicine, University of São Paulo, Avenue Professor Dr. Orlando Marques de Paiva 87, Cidade Universitária, 05508-270 São Paulo, SP, Brazil
3 Jockey Club of São Paulo, Bento Frias Street 248, Group 555, 05423-050 São Paulo, SP, Brazil

Correspondence should be addressed to Iracema N. Barros; cermaivet@gmail.com

Received 6 September 2013; Accepted 3 October 2013

Copyright © 2013 Iracema N. Barros et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Gastroenteritis is one of the leading causes of morbidity and mortality among young and newborn animals and is often caused by multiple intestinal infections, with rotavirus and bovine coronavirus (BCoV) being the main viral causes in cattle. Given that BCoV is better studied than equine coronaviruses and given the possibility of interspecies transmission of these viruses, this research was designed to compare the partial sequences of the spike glycoprotein (S), hemagglutinin-esterase protein (HE), and nucleoprotein (N) genes from coronaviruses from adult cattle with winter dysentery, calves with neonatal diarrhea, and horses. To achieve this, eleven fecal samples from dairy cows with winter dysentery, three from calves, and two from horses, all from Brazil, were analysed.

It could be concluded that the enteric BCoV genealogy from newborn and adult cattle is directly associated with geographic distribution patterns, when S and HE genes are taken into account. A less-resolved genealogy exists for the HE and N genes in cattle, with a trend for an age-related segregation pattern. The coronavirus strains from horses revealed Betacoronavirus sequences indistinguishable from those found in cattle, a fact previously unknown.

1. Introduction

Currently, coronaviruses (CoVs) with genetic and antigenic proximities to bovine coronavirus (BCoV) such as human coronaviruses HCoV-OC43, porcine hemagglutinating encephalomyelitis virus (PHEV), and equine coronavirus (EqCoV) are not considered to be separate species but as belonging to the species Betacoronavirus-1 within the genus Betacoronavirus. This genus has replaced Group 2 in the order Nidovirales, family Coronaviridae, and according to the new taxonomy; this family is separated into two subfamilies: Torovirinae and Coronavirinae. The latter comprises the genera Betacoronavirus, Alphacoronavirus, and Gammacoronavirus [1, 2].

CoVs are enveloped, single-stranded positive sense RNA viruses with a genome encoding replicase polyproteins, the four structural proteins: spike (S) glycoprotein (a receptor-interacting and a target for neutralizing antibody in the envelope); nucleocapsid (N) (associated with the genomic RNA in the nucleocapsid); and the two proteins essential for virion formation, envelope (E) and membrane (M) proteins; some Betacoronaviruses also present the hemagglutinin-esterase (HE) protein, with the role as a secondary receptor-binding envelope protein and accessory proteins [3, 4].

BCoV is a major pathogen for cattle, frequently found in neonatal diarrhea, dysentery in the adult and respiratory disease [5, 6]. Similarly, in horses, coronaviruses lead to neonatal enterocolitis [7, 8], although there are very few studies on the genealogy of coronaviruses from these animals [7, 9, 10].

Given the little information available on coronaviruses of horses and the genealogic relationship of these coronaviruses
from cattle, this research was designed to perform a multi-
genomic comparison of coronaviruses from adult cattle with
winter dysentery, calves with neonatal diarrhea, and horses
based on partial sequences of the HE, S, and N genes.

2. Materials and Methods

lung (HmLu) cells, with a hemagglutination titer of 256
and DEPC-treated water, was used as positive and negative
controls, respectively.

In the nested RT-PCRs, DEPC-treated water was used as a
negative control every five samples, also added to the mix, and
placed in a thermocycler in order to monitor contamination
by DNA amplicons. Each step of the study (RNA extraction,
nested RT-PCR, electrophoresis, and DNA sequencing) was
carried out in different rooms with materials and reagents
exclusive for that specific step in order to prevent DNA
carryover.

2.2. Field Samples. Fecal samples were collected from eight
dysenteric and three healthy adult cows (named B1 to B11)
in 2009 from a farm in São Paulo State, Southeastern Brazil;
two samples came from healthy young adult horses (E17 and E19)
in 2010 from a farm in Parana State, Southern Brazil; two
dysenteric and three healthy adult cows (named B1 to B11)
and DEPC-treated water, was used as positive and negative
controls, respectively.

were used to perform a multi-

2.3. Partial HE, S, and N genes Amplification. Total RNA
was extracted from the supernatants with the TRIzol reagent
(Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized
using random primers (Invitrogen, Carlsbad, CA, USA) and
M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) as described by the manufacturer.

2.4. DNA Sequencing and Genealogy. Amplicons for each
gene (HE: 441bp; S: 488bp; and N: 306bp) were purified from
agarose gels with the GFX PCR DNA and GB Purification Kit
(GE Healthcare Bio-sciences Corp, Piscataway, NJ, USA) and
submitted to bidirectional sequencing with BigDye version
3.1 (Applied Biosystems, Carlsbad, CA, USA) according to
the manufacturer’s instructions. Sequences were resolved in
an ABI-377 sequencer.

Chromatograms were submitted to Phred analysis (http://
asparagin.cenargen.embrapa.br/phph/), and positions with
scores >20 were used to assemble sequences with CAP-
Contig in BioEdit 7.0.9.0 [15].

The sequences were then aligned with BCoV and EqCoV
homologous sequences retrieved from GenBank (accession
numbers for HE gene: AF391541, EF424619, AF058944,
EF424620, EF424615, EF424618, EF424616, GU214763,
GU214757, GU214765, GU214769, GU214767, GU214766,
GU214768, GU214761, AF058943, DQ811784, AF220295,
AB354579, U00735, AF058942, AY585229, AY316300,
NC010327, and EF446615; S gene: AY606198, AY606205,
AY606203, AY606202, AY606192, AY606204, AY606196,
AY606197, AY606200, AY606194, AY606199, AY606201,
DQ479424, U00735, AB354579, AF220295, EF446620,
AF239307, AF391541, DQ811784, U06093, AF058942,
EF424619, EF424615, AF058944, EF424616, EF424618,
FJ899737, AF058943, DQ479423, DQ479421, DQ479422,
AFL55229, AY316300, EF446615, and NC010327; and N
gene: AB354579, AF220295, U00735, AF058942, AY585229,
AF251144, NC010327, EF446615, AF058943, GU80349,
GU80341, GU80345, GU80344, EF446619, GU80343,
GU80348, EF446616, DQ811784, EF446618, EF446615,
EF446620, GU80350, AG391541, and AF058944) using
CLUSTAL/W with BioEdit 7.0.9.0 [15] and the BLOSSUM62
matrix (for the putative amino acids sequences). Distance
phylogenetic trees were calculated using the Neighbor-
Joining algorithm and Maximum Composite Likelihood
Models for nucleotides (nt.) sequences and the Poisson cor-
rection for amino acid (aa) sequences with 1,000 bootstrap
replicates with MEGA 4 [16]. Canine coronavirus (CCoV,
Alphacoronavirus) S and N genes and Murine hepatitis virus
(MHV, Betacoronavirus) HE genes (absent in Alphacoro-
navirus) were used as outgroups. MHV was not used as
outgroups for S and N in order to have an outgroup that
diverged from the ingroup (Betacoronavirus) before the
internal speciation of this group.

3. Results

The tree based on HE nt. sequences (Figure 2) showed that
both equine strains which segregated with BCoV strains from
this study and others retrieved from GenBank, are included in the same subcluster of BCoV Kakegawa strain (GenBank accession number AB354579), divergent from EqCoV strains (AY36300, NC_010327, and EF446615).

In this tree, the BCoV strains found in adult dairy cows and calves analyzed in this study segregated in a single cluster, together with other BCoV strains.

Regarding the nt. and aa for the HE sequences, the identity amongst the two equine strains E17 and E19 and groups of BCoV strains ranged from 97.35 to 98.25% and 96.81 to 97.65%, respectively. However, amongst these two equine strains and EqCoV strain, the lowest identity percentage was 71.65% for aa and the highest was 72.85% for nt. Besides, the identity amongst groups of BCoV strains studied herein and other BCoV strains ranged from 98.19 to 99.60% and 98.85 to 100% for nt. and aa, respectively.

Taking into account the nt. sequence tree for the S gene (Figure 3), equine strains E17 and E19 segregated amongst BCoV strains from calves of Southeastern region of Brazil, identified as USP and again diverged from EqCoV strains (AY36300, NC_010327, and EF446615).

The cluster with these two equine strains also contains BCoV strains described by Brandão et al. [12] in which a deletion of 18 nt./6aa in the SI subunit region of S protein was detected, also found in E17 and E19 strains. In turn, strain USP01, which did not have the aforementioned deletion, segregated with the Brazilian strain cow/WDBR-96/BRA/2003 (FJ899737), also from Southeastern region of Brazil. BCoV strains from adult dairy cows segregated in a single cluster, as described for HE gene.

Considering the nt. and aa identities for S gene, the lowest identity found amongst the two equine studied strains and groups of BCoV was 89.27% or 89.60% and the highest identities were 99.92% and 99.83%, respectively. Amongst these two equine strains and EqCoV strains, the lowest and highest identities were 47.2% (nt.) and 56.8% (aa), and the identity amongst groups of BCoV strains studied herein and other BCoV strains ranged from 89.20 to 98.17% and 86.45 to 97.89% for nt. and aa, respectively.
The tree for N gene (Figure 4) showed that both equine strains segregated in the same cluster of Kakegawa BCoV (AB354579), similar to that found in the nt. sequences tree for HE gene and also diverged from EqCoV strains (AY316300, NC_010327, and EF446615). The BCoV strains from calves from Southeastern Brazil segregated in a distinct subcluster to the BCoV strains of adult cows, similar to that found for genes HE and S, although a lower resolution was found in the N gene tree, since the subclusters described for S and HE genes were not detected, without geographical distinction.

Regarding the identities of nucleotides and amino acids for the N gene, the lowest identities found amongst the equine strains (92% (nt.) and 95.10% (aa)) and all the BCoV strains were 96.94% and 97.18%, respectively. Amongst these two strains and EqCoV strains, the lowest and highest percentage identities were 92% (nt.) and 99.29% to 99.89% for nt. and aa, respectively.

The nt. sequence of all genes studied in this study has been deposited in the GenBank under accession numbers (Table 1).

4. Discussion and Conclusions

With respect to the genealogical analysis, the coronavirus strains E17 and E19, detected in horses, did not cluster with EqCoV strains already described [7, 9]. Nonetheless the genes analysed, unexpectedly, clustered with BCoV strains. These results demonstrate that the coronaviruses found in these horses are divergent from EqCoV and similar to BCoV.

The presence of coronaviruses similar to BCoV in hosts other than cattle has already been reported in buffalos [17, 18], lamas and alpacas [19, 20], deer [21], and giraffes [22], demonstrating that this virus can adapt to other herbivores, including horses, as found in the present study, a fact not reported previously.

Furthermore, the 18 nt./ six aa deletion in S1, already described for BCoV strains [12], was also detected in strains E17 and E19, possibly allowing for changes in the spike glycoprotein that could reduce crossed immunity with other BCoV strains [23].

Regarding the HE gene, strains E17 and E19 both clustered with Kakegawa BCoV strain (Figure 2; Genbank ID: AB354579). Though this BCoV strain originated in Japan [11], it is possible that a common ancestor of the three strains has spread worldwide.

Alternatively, BCoV strains from bovine resulted in three main clusters for HE: (a) strains from adult cows studied herein; (b) strains from dairy cows with winter dysentery previously reported in Brazil (Genbank); and (c) strains from cattle from other countries retrieved from the GenBank.

This model of segregation might represent a phylogeographic pattern rather than temporal and/ or host-specific patterns, since it is known that there are no markers to differentiate strains of BCoV from calves and adult cattle.
[24, 25] and either for temporal changes [26–28]. A similar pattern of segregation for HE was maintained for S (Figure 3), strengthening the hypothesis of regional genetic signatures.

Considering the N gene tree (Figure 4), the strain E17 has diverged from E19 and BCoV strains, owing to a single nucleotide substitution, leading to aa substitution Val92Leu, increasing, for this strain, the number of nonsynonymous substitutions, which might mean that strain E17 is adapting to the equine host, and increasing its divergence from an ancestor BCoV strain.

Thus, one can speculate that successive natural passages of the strain E17 amongst horses, without the participation of cattle, led to different host-parasite relationships due to differences in receptors, in viral replication, and in the intracytoplasmic content [29], and probably, this distance has a tendency to rise over evolutionary time.

In the nucleotide tree for the N gene, two clusters of BCoV strains were formed; one cluster containing strains from calves (USP) and another with all of the remaining strains. Considering that N is the most conserved gene amongst those studied herein [4, 30], one can speculate that the strains from calves in this case have markers for the discrimination amongst strains from neonatal diarrhea and winter dysentery in cattle, in that the clustering was maintained despite the lack of geographical differentiation.

The debate on the taxonomy of coronaviruses has firstly led to the proposition of 3 groups in the genus Coronavirus [30] and became quite controversial after the description of the SARS coronavirus [31], in which taxonomy culminated with the proposition of a fourth group [32] which was then refuted and the virus was finally classified as a Group 2 member [33]. The newly proposed taxonomy for the Nidovirales, with the three coronavirus genera replacing the three groups, represents a great advance in organizing the increasing number of coronavirus "species" constantly being discovered.

In conclusion, the genealogy of enteric BCoVs from newborn and adult cattle is directly associated with geographical patterns, when the S and HE genes are taken into account, with a less-resolved genealogy for the HE and N genes, and with a trend for an age-related segregation pattern for the last, and horses might present Betacoronavirus highly similar to those found in cattle, supporting the existence of the Betacoronavirus-1 species.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgment

The authors are grateful to Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) SP, Brazil, for financial support (Grant no. 2008/51517-5).

References


