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Original Article

Molecular epidemiology of coronavirus in faeces of Brazilian calves and Peruvian camelid herds

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Abstract

Introduction: The enteric disorders represent a serious hazard for bovine and camelid breeding. The aim of this study was to examine the frequency of detection and molecular characteristics of enteric coronavirus (CoV) infections in cattle, alpaca, and llama herds bred in family-based farms in Brazil and Peru.

Methodology: Stool samples were collected from calves from Brazil and camelids from Peru for detection and characterization of CoV by reverse transcription polymerase chain reaction (RT-PCR) and sequence analysis.

Results: 46.5% (47/101) samples from calves and 26.8% (70/261) from alpaca tested positive for CoV. All strains belong to lineage A1 of the *Betacoronavirus* genus. Phylogenetic analysis showed high identity between CoV strains detected in calves and alpacas.

Conclusions: This study characterised CoV strains from dairy cattle herds in the state of Rio de Janeiro, Brazil, and indicated that this virus is spread among the state herds. The results also indicate widespread circulation of CoV among the alpacas of Cuzco, Peru.

Key words: coronavirus; bovine; alpaca; diarrhoea; epidemiology.

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Introduction

The enteric disorders represent a serious hazard for bovine and camelid breeding [1-3]. Small producers have limited investment capacity and often operate with very low returns insufficient to enable capitalization of their production units. This implies that owners of these small businesses do not have autonomy in the production process and face limitations on the prospects of their investment in farm livestock. One of the main diseases that can endanger the young ruminant population and ultimately lead to major economic losses is diarrhoea. The aetiology is complex and multifactorial, involving infectious, nutritional, immunological, and environmental factors [4]. Coronaviruses (CoVs) are responsible for enteric and respiratory infections in bovine and camelid flocks throughout the world and are associated with significant economic losses [1,5-8].

Bovine coronaviruses (BCoVs; *Betacoronavirus* 1) are enveloped viruses with a single-stranded, positive-sense, non-segmented RNA genome that belong to the *Coronavirus* genus of the *Coronaviridae* family [9]. BCoVs can be associated with many infections in both beef and dairy cattle and are responsible for diarrhea, respiratory disease in calves and winter dysentery in adult animals [10]. Infection is primarily via feco-oral and to a lesser extent, the respiratory (aerosol) route [3]. Most often, transmission of enteric BCoV is horizontal, and transmission to young animals occurs from a carrier dam to their offspring postpartum or from clinically or chronically infected calves housed in proximity to immunologically naïve animals [3].

The first description of CoV associated with outbreaks of diarrhea in camelids occurred in Oregon, USA, in 1998 [1,5]. The viruses isolated were very similar to strains that caused diarrhea and pneumonia in cattle, and the symptoms described were similar to coronavirus infection in cattle. The sick camelids presented varying degrees of disease severity, with the

death of some animals and others requiring intensive medical care [5].

BCoVs are responsible for outbreaks of diarrhea with an impact on animal health, so prophylactic measures are necessary [1,3,6,10]. These measures include: isolation of infected animals with the objective of reducing virus transmission to susceptible animals; rearing animals of uniform age groups; cleaning and disinfection of pens; rotating pickets in herds; and passive immunity through colostrum.

Two types of vaccines are commercially available for BCoV control programs. One is a killed vaccine administered to late pregnant cows for passive maternal immunization of their calves. The other is a modified-live BCoV administered orally to calves at birth to provide active immunization or used in pregnant cows. Cows vaccinated with the latter may transfer BCoV antibodies in the colostrum or milk secretions, reducing BoCV after calving [10].

The aim of this study was to examine the detection, frequency and molecular characteristics of enteric CoV infections in cattle, alpaca, and llama herds bred in family-based farms in Brazil and Peru.

Methodology

Bovine faecal specimens from 101 calves up to age 10 months were collected between October 2013 to September 2014. The calves were from nine family-based small dairy farms located in the following municipalities within the state of Rio de Janeiro, Brazil: Northwest Aperibé and Itaocara, North Campos de Goytacazes, and Southeast Itaboraí and Sampaio Corrêa.

Faecal samples were also obtained from 261 alpacas and 42 llamas at 1–5 weeks of age. These animals came from the following three locations within the Department of Cuzco, Peru: the Instituto Veterinario de Investigaciones Tropicales y de Altura of the Universidad Nacional Mayor de San Marcos (IVITA-UNMSM), La Raya Research Center of the Universidad Nacional de San Antonio Abab del Cuzco (UNSAAC), and the rural community of Silli, located in the province of Canchis, Maragani district.

Bovine and camelid samples were collected either directly from the rectum of animals gathered in the handling corral or with the aid of swabs when needed. Stool suspensions were prepared in 10% (w/v) phosphate-buffered saline (pH 7.2) and centrifuged at 2,500 × *g* for 5 min. Nucleic acid was extracted from 300 μL of each supernatant with a total RNA extraction kit (TALLY RNA, Thermo Fisher-Ambion, Waltham, USA) according to the manufacturer's instructions. Specimens were screened for BCoV by amplifying a 251 bp fragment of the polymerase gene using a pancoronavirus conventional reverse transcription polymerase chain reaction (RT-PCR) protocol previously described [11].

For phylogenetic analysis, a fragment of the CoV gene, encoding the S1 domain of spike (S) protein, was used – PCR primers SE-1 (5'-GACCTACAATTGGGTAATTTGGG-3') and AS (5'-TTGTAAATCAGTAGAACAAGTAG-3'), gaving a predicted product of 651 bp. The S1 domain contains a hypervariable region prone to frequent mutations [12]. Semi-nested PCR using primers SE (5'-TGTCAGTTGTATTATAATTTACCTG-3') with primer AS, giving a predicted product of 581 bp, was then performed. Amplified products were sent to Hellixa (genomics service provider) using the nested PCR primers for sequencing. Overlapping sequences were assembled and edited using SeqMan, EditSeq, and MegAlign, which are all available in the Lasergene software package (version 7.0, DNASTAR, Madison, USA). Phylogenetic analyses were performed with

Table 1. Distribution of BCoV among cattle in the state of Rio de Janeiro, Brazil.

City	Location	Samples tested	Positive samples *n* (%)
	PF[a]	4	4
Aperibé	PG	10	1
	PH	11	1
Total		25	6 (24)
	PC	14	1
Itaocara	PD	12	9
	PE	9	8
Total		35	18 (51.4)
Campos de Goytacazes	PI	15	5 33.3
Itaboraí	PB	15	9 (60)
Sampaio Corrêa	PA	11	9 (81.8)
Total		101	47 (46.5)

[a] Two-letter codes used to identify each farm within each city.

MEGA software, version 7.014 (Pennsylvania State University, State College, PA, USA) [13]. Distances were corrected using the Kimura 2-parameter model, and dendrograms were constructed using the neighbour-joining method. Statistical significance was estimated by bootstrap analysis with 1000 pseudoreplicates. Sequences were compared to those of CoV strains obtained from GenBank (National Center for Biotechnology Information). Sequences generated for strains analysed in this study were deposited into GenBank under accession numbers KT381468–KT381482 and KX266939–KX266950 (Supplementary Table).

Results

BCoV was detected in the stool of 47 (46.5%) of the 101 calves tested from all studied farms, with a frequency ranging from 24% to 81.8%, (Table 1). Among the individual municipalities, the lowest BCoV detection frequency was observed in Aperibé, and the highest was observed in Sampaio Corrêa. Similar detection frequencies of 51.4% and 60% were observed in Itaocara and Itaboraí, respectively, and animals in Campos de Goytacazes had a BCoV frequency of 33.3% (Table 1). Partial sequence of the BCoV gene encoding the S1 domain was obtained for 15 BCoV strains. Phylogenetic analysis (Figure 1) showed that all strains belong to lineage A1 of the Betacoronavirus genus [14]. When compared to the reference strains, the BCoV strains from Rio de Janeiro were closely related to the Brazilian strains, BR-UEL-2 and WDBR-B1, detected in the states of Paraná and São Paulo, respectively.

261 stool samples from alpacas, 70 (26.8%) tested positive for CoV (Table 2). CoV was detected at all Peruvian collection sites, with a frequency of 10.3–46.3%. However, the virus was not detected in llamas. Among the individual locations, UNSAAC had the lowest CoV frequency (10.3%). Alpacas in the community of Silli had a larger infection frequency of 40.4%, and the highest frequency of CoV (46.3%) was observed at IVITA-UNMSM (Table 2). Sequencing of the CoV gene S1 domain was obtained for 12 alpaca CoV strains. As observed for BCoV, phylogenetic analysis revealed that all detected alpaca CoV strains

Figure 1. Dendrogram constructed from partial sequences of the gene encoding the S protein S1 domain of CoV strains detected among cattle in Brazil and alpacas in Peru. Bootstrap values above 70% are shown at branch nodes. Distance scale is in substitutions/site. Open triangle, BCoV strains from state of Rio de Janeiro; open circles, BCoV strains from other Brazilian states; black triangles, alpaca CoV strains from Peru; black circles, alpaca CoV strain from USA.

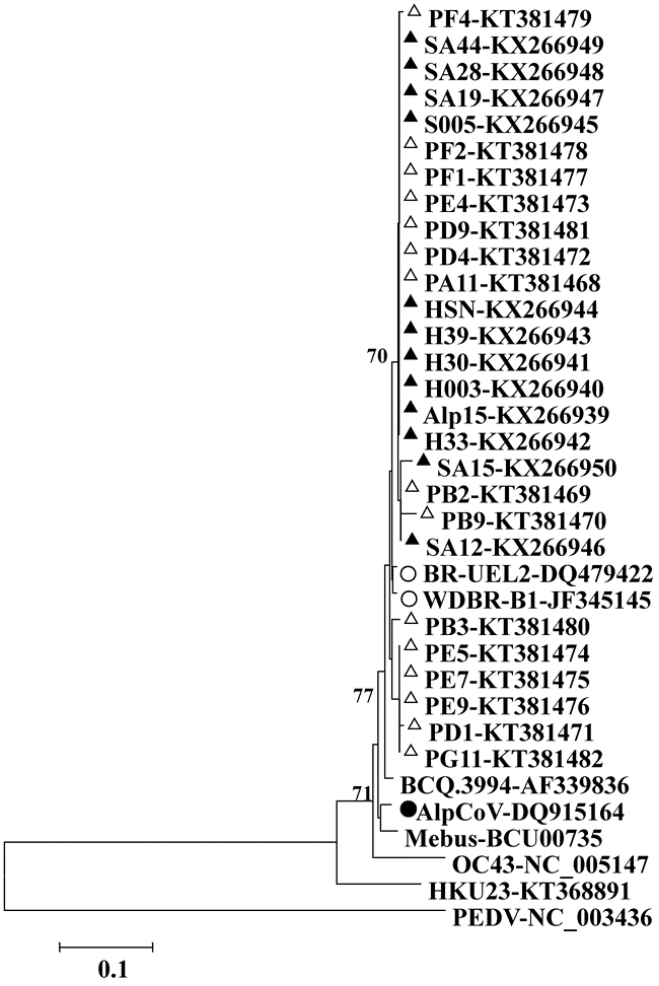

Table 2. Distribution of CoV among camelids in the Department of Cuzco, Peru.

Species	Location	Samples tested	Positive samples *n* (%)
Alpaca	Silli	94	38 (40.4)
	IVITA[a]-UNMSM[b]	41	19 (46.3)
	UNSAAC[c]	126	13 (10.3)
Total		261	70 (26.8)
Llamas	Silli	11	0
	IVITA-UNMSM	31	0
Total		42	0

[a] Instituto Veterinario de Investigaciones Tropicales y de Altura; [b] Universidad Nacional Mayor de San Marcos; [c] Universidad Nacional de San Antonio Abad del Cuzco.

belong to lineage A1 of the Betacoronavirus genus (Figure 1). When compared to the reference strains, the alpaca CoV strains were closely related to the Brazilian BCoV strain, WDBR-B1.

Comparison of the CoV sequences obtained from cattle and alpacas revealed that the Peruvian alpaca CoV strains are phylogenetically closer to the Brazilian bovine strains than they are to the ApCoV-00-1381 strain detected in an alpaca with severe diarrhoea at Oregon State University, USA, with which they share 97.3–97.7% identity. As shown in Figure 1, the bovine and alpaca CoV strains clustered into two separate branches. The clustered strains share 98.7–100% identity among themselves and 96.9–98.5% identity with strains in the different cluster. When compared to the reference strains, bovine and alpaca CoV were more closely related to the Brazilian bovine CoV strains than they were to the classical bovine strains, such as Mebus and BCQ.3994.

Discussion

Many studies have contributed to the understanding of BCoV pathogenesis; however, there are few reports about the distribution of this agent among Brazilian cattle herds. Nearly half of the cattle in our study (46.5%) were infected with BCoV. Previous studies conducted at Brazilian dairy and beef farms have reported BCoV infection frequencies ranging from 15.6–68.6% [4,12,15-20]. These studies predominantly focused on medium and large farms with good nutritional and health management that were located in states considered as large producers of bovine meat and milk for export. By contrast, our study focused on dairy calves reared in small family-based farms located in the state of Rio de Janeiro, which ranks 13th in the country for milk production and contribution to the milk supply in the domestic market. It is common for owners of small farms, with a reduced number of animals in each herd, to sell and/or exchange calves as well as adult animals among themselves, depending on the scarcity of available pasture for feeding and the financial needs of the owners. In addition, the introduction of cattle into herds acquired at auctions, as well as the transit of animals to venues during the exhibition season, often occur without compliance with the Guide of Animal Transit [21] and usually without fulfilling quarantine requirements prior to their introduction into the herd.

Our phylogenetic analysis of BCoV strains, from cattle, suggests that sequences sharing ≥ 98.7% identity represent the same variant, whereas sequences sharing < 98.5% identity represent a different variant strain. Therefore, it is highly likely that at least two different strains circulated among the cattle herds in the state of Rio de Janeiro between 2013 and 2014. These different strains circulated simultaneously in the same herd, and the same viral strain circulated among four different farms in 2014, suggesting widespread transmission of the virus among different herds in the state. Due to a lack of information on the movement of animals between these locations, it is difficult to suggest the form of viral spread. However, in the case of the cattle haerds in Aperibé and Itaocara, it is known that there is a constant movement of animals between these municipalities. Interherd contact during the purchase of calves can be an important factor in the transmission of BCoV, as demonstrated in studies conducted in Sweden [22,23].

We also investigated the frequency of CoV infection among economically important Peruvian alpacas, in which the CoV frequency was just over a quarter of the study population. The occurrence of natural CoV infection among camelids has been previously demonstrated [1, 5-8]. The observed detection of CoV at all Peruvian collection sites indicates widespread circulation of the virus. It is noteworthy that the camelids we studied were bred under different sanitary conditions. Two of the collection sites (IVITA-UNMSM and UNSAAC) are separated by a distance of 25 km, and the community of Silli is approximately 70 km away from these sites. Camelids belonging to herds at IVITA-UNMSM and UNSAAC are bred under controlled conditions: There is no exchange of animals between these sites. However, animal workers do commute between these universities without taking the sanitary control measures necessary to prevent contamination of the clothing and footwear with pathogens. In the community of Silli, animals are bred by small producers who are not trained or not able to implement proper handling conditions due to extreme poverty. Despite these circumstances, these producers usually take the animals to the veterinary medical research centre at IVITA-UNMSM for treatment during disease outbreaks.

Our analysis of alpaca CoV frequency at each individual Peruvian location revealed that the samples collected at La Raya Research Center (UNSAAC) exhibited the lowest CoV frequency (10.3%). The healthcare provided to these animals is of relatively high quality and includes prophylactic programs and hygienic services vital to the control of infections. This frequency of CoV infection is markedly lower than that (35.7%) reported in a previous study conducted at the same facility [7]. Another study of neonatal alpacas at

the La Raya experimental research station farms in Melgar Province, Puno Department, reported a CoV frequency of 18.3% [8], which was closer to our finding. By contrast, alpacas in Silli exhibited a substantially higher CoV frequency (40.4%). The camelids in this community suffer greater health problems due to improper handling and lack of prophylactic programs and hygienic services. Samples collected from alpacas at IVITA-UNMSM exhibited the highest CoV frequency of 46.3%. These high frequencies of CoV infection at Silli and IVITA-UNMSM could be explained by eventual contact between IVITA-UNMSM veterinarians and animals from Silli, because the producers of this community take their alpacas to this research centre for treatment during the disease outbreaks.

Although the occurrence of natural CoV infection among camelids has been previously reported, little is known about the characteristics of the viral strains that infect these animals. Nucleotide sequences of alpaca CoV strains exhibited high identity with circulating bovine strains, suggesting a possible bovine origin of these viruses [5,8]. This also indicates the possibility that identical CoV strains were circulating among Brazilian cattle and Peruvian alpacas. Given the fact that the hypervariable S1 domain of S protein can undergo a high number of mutations, it is believed that the CoV can adapt to new hosts and ecological niches [24]. In the colder seasons of the year, the camelids that usually are kept at high altitude are brought to lower locations due to the scarcity of pasture in the high altitudes of the Andes plateau. This relocation allows direct contact of camelids with cattle and sheep. Therefore, it is possible that this breeding scheme favours the occurrence of interspecies CoV transmission. Unfortunately, it has not been possible to obtain samples of Peruvian BCoV to prove this theory. By contrast, the llamas were not positive for CoV. To date, only one study has reported the detection of enteric CoV in llamas, with a frequency of 42% for alpacas and llamas [1].

Conclusions

The characterisation of BCoV strains detected in dairy cattle herds in the state of Rio de Janeiro, Brazil, demonstrates that this pathogen is widely spread among the state herds. Widespread circulation of CoV also exists among the alpacas of Cuzco, Peru. Our results further show high identity between bovine and alpaca CoV strains.

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Ethical approval

The collection of bovine samples was approved by the ethics committee on animal research of the Empresa de Pesquisa Agropecuária do Estado do Rio de Janeiro (PESAGRO, Rio de Janeiro, RJ, Brazil), process number 1/2014. Importation of the alpaca samples was approved by the Brazilian Institute of Environment (IBAMA, Brasília, DF, Brazil), license number 14BR012948/DF 02/20/2014.

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Conflict of interests: No conflict of interests is declared.

Annex – Supplementary Items

Supplementary Table 1. Demographic information on CoV isolates.

Municipalities	Collection date	Isolate	Accession number
Sampaio Corrêa	22-Oct-2013	PA11	KT381468
Itaboraí	03-Dec-2013	PB2	KT381469
Itaboraí	03-Dec-2013	PB3	KT381480
Itaboraí	03-Dec-2013	PB9	KT381470
Itaocara	07-May-2014	PD1	KT381471
Itaocara	07-May-2014	PD4	KT381472
Itaocara	22-Oct-2013	PD9	KT381481
Itaocara	04-Aug-2014	PE4	KT381473
Itaocara	04-Aug-2014	PE5	KT381474
Itaocara	04-Aug-2014	PE7	KT381475
Itaocara	04-Aug-2014	PE9	KT381476
Aperibé	30-Sep-2014	PF1	KT381477
Aperibé	30-Sep-2014	PF2	KT381478
Aperibé	30-Sep-2014	PF4	KT381479
Aperibé	30-Sep-2014	PG11	KT381482
Silli	10-Feb-2010	Alp15	KX266939
IVITA	10-Feb-2012	H003	KX266940
IVITA	10-Feb-2014	H30	KX266941
IVITA	10-Feb-2014	H33	KX266942
IVITA	10-Feb-2014	H39	KX266943
IVITA	10-Feb-2014	HSN	KX266944
IVITA	10-Feb-2012	S005	KX266945
Silli	10-Feb-2014	SA12	KX266946
Silli	10-Feb-2014	SA19	KX266947
Silli	10-Feb-2014	SA28	KX266948
Silli	10-Feb-2014	SA44	KX266949
Silli	10-Feb-2014	SA15	KX266950