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Severe enteritis in Italian Mediterranean buffalo calves associated with a novel bovine-like coronavirus

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ABSTRACT - An outbreak of severe enteritis in Italian Mediterranean buffalo calves is reported, which was associated to infection by a novel bovine-like coronavirus (CoV). By conventional and real-time RT-PCR assays for bovine-like CoVs, the virus was demonstrated in the intestinal contents of two 20-day-old buffalo calves that died of a severe form of enteritis, as well as in the fecal specimens of additional 17 buffalo calves with diarrhea. Biological and genetic characterization showed that the bubaline strain can be considered as prototype of a novel group 2 CoV, namely bubaline CoV (BuCoV).

Key words: Enteritis, Buffalo calves, Novel coronavirus.

INTRODUCTION - Coronaviruses (CoVs) (order *Nidovirales*, family *Coronaviridae*) are enveloped, positive-sense single-stranded RNA particles that are responsible for enteric and/or respiratory disease in mammals and birds. Bovine coronavirus (BCoV) is a group 2 CoV which causes enteric disease and/or respiratory distress in calves and adults (Decaro *et al.*, 2007). Recently, bovine-like CoVs have been identified in wild or domesticated ruminants, including giraffe, alpaca, sable antelope and several species of deer. To date, CoVs have never been isolated from buffaloes, although there is a single report on the detection of BCoV antibodies and antigens in Bulgarian buffaloes (Muniiappa *et al.*, 1985). Here, we describe an outbreak of enteritis in buffalo calves which was associated with infection with a bovine-like CoV.

MATERIAL AND METHODS - The outbreak occurred between October 2006 and April 2007 in a herd of Italian Mediterranean buffalos (*Bubalus bubalis*) in Campania (southern Italy). At that time the herd consisted of 460 buffaloes, including 215 lactating cows, all vaccinated against colibacillosis, clostridiosis and salmonellosis. Buffalo calves were removed from their dams shortly after their birth and placed in separate hutches according to the gender where they were hand-fed fresh colustrum for 5 days. Neonatal mortality was firstly observed in October 2006 in 30 5-20-day-old calves (out of 40 newborns) that displayed severe diarrhea and died despite treatment with antibiotics (oxy-

tetracyclin and amoxicillin). Simultaneously, gastroenteric disease was also observed in older calves (1-3 months of age). On April 2007, neonatal mortality and enteric signs in calves persisted and two carcasses of 20-day-old dead calves together with fecal samples from additional 17 diseased calves were submitted to our laboratory for routine analysis. Nucleic acids extracted with commercial kits were subjected to (RT-)PCR assays for detection of the most common viral pathogens of ruminants, including BCoV, toroviruses, rotaviruses, caliciviruses, bovine viral diarrhea virus, bovine respiratory syncytial virus, bovine herpesvirus types 1 and 4. The samples were also examined for bacterial and parasitic pathogens by standardized methods. A recently developed real-time RT-PCR assay (G. Elia *et al.*, manuscript in preparation) was used to quantify the viral load in samples tested positive for bovine-like CoVs by conventional RT-PCR (Erles *et al.*, 2003). The buffalo fecal sample containing the highest RNA titer of BCoV-like coronavirus (strain 179/07-11) was used for virus isolation attempts on human rectal tumor (HRT-18) and Madin Darby Bovine Kidney (MDBK) cells as previously described (Decaro *et al.*, 2007). Hemagglutination (HA) and receptor-destroying enzyme (RDE) activities of the isolated strain were assessed in the presence of mouse and chicken erythrocytes (Hasoksuz *et al.*, 1999). The sequence of the genomic 3' end of strain 179/07-11 was determined by PCR amplifications of 13 overlapping fragments using primer pairs designed on conserved regions among bovine-like CoVs. The PCR-amplified products were sequenced by Genome Express (Meylan, France) and the obtained sequences were assembled and analyzed using the BioEdit software package (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and the NCBI's (htttp://www.ncbi.nlm.nih.gov) and EMBL's (http://www.ebi.ac.uk) analysis tools. Phylogenetic and molecular evolutionary analyses were conducted using Mega3 (http://www.megasoftware.net/) on the main nonstructural and structural proteins encoded by ORFs contained in the sequenced region.

RESULTS AND CONCLUSIONS - At necropsy, the carcasses of the two dead buffalo calves showed severe gastroenteritis, with enlargement of the mesenteric lymph nodes and glad bladder. By conventional RT-PCR, bovine-like CoV RNA was detected in the intestinal content of the dead animals as well as in all 17 fecal samples from calves with diarrhea. Using real-time RT-PCR, bovine-like CoV RNA was detected at low titers in the intestinal contents of the dead animals, whereas higher viral loads were found in the fecal samples of calves with diarrhea, with a peak of 5.23×10^7 RNA copies/µl of template in calf 179/07-11. Other viral, bacterial and parasitic pathogens of ruminants were not detected by molecular and/or traditional methods. A bovine-like CoV was isolated from the fecal sample of calf 179/07-11, as showed by the appearance of cytopathic effect on HRT-18 cells (Fig. 1a), where the growth of a bovine-like CoV was confirmed by the cytoplasmic fluorescence detected by the IF assay using a BCoV-specific serum (Fig. 1b). In comparison to BCoV reference strains, the bubaline strain grew very poorly on MDBK cells and did not display any HA activity in the presence of chicken erythrocytes. A 9.6-kb region encompassing the entire 3' end of the viral genome (from the 32-kDa to nucleocapsid protein genes) was determined. At the 3' end of viral RNA, bubaline CoV strain 179/07-11 had the same genomic organization of other group 2 CoVs related to BCoV. Sequence analysis showed that strain 179/07-11 possesses a high genetic relatedness to BCoV, although it is less related to BCoV in comparison with other ruminant CoVs in major structural and

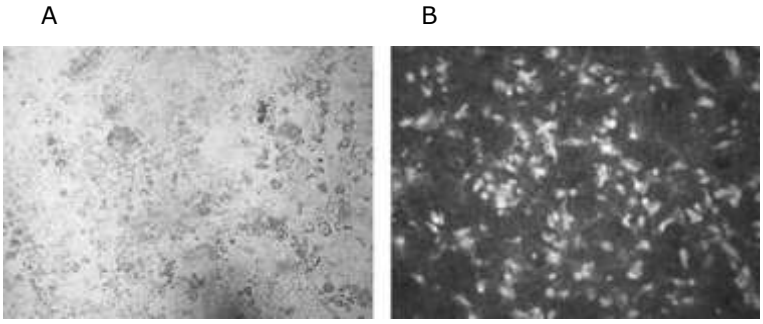
nonstructural proteins (Table 1). Unique amino acid (aa) changes were identified in the encoded proteins of strain 179/07-11 with respect to other ruminant CoVs, mostly accumulated in the N-terminus of the S protein. Phylogenetic analysis with nsp 32kDa and structural proteins S, E, M and N showed that the bubaline strain 179/07-11 clusters together with the bovine-like CoVs, being more related to ruminant viruses in all proteins but the M protein, where it forms a separate cluster with human enteric coronavirus 4408 (HECV-4448) into the bovine subgroup.

Based on the unique biological properties and the more distant relatedness of strain 179/07-11 to BCoV with respect to other ruminant CoVs, we propose to designate this strain as prototype of a novel bovine-like CoV, namely bubaline CoV (BuCoV). With regards to the its possible origin, BuCoV has likely arisen through interspecies transmission of a BCoV strain from cattle to water buffaloes. This hypothesis is supported by the high genetic relatedness between BuCoV and BCoV. Moreover, it should be also considered that the bovine origin has been strongly suggested for other group 2 CoVs less genetically related to BCoV, such as human coronavirus OC43 (HCoV-OC43), porcine hemagglutinating encephalomyelitis virus (PHEV) and canine respiratory coronavirus (CRCoV). Although this study has detected a CoV strain in buffalo calves with severe diarrhea, the pathogenicity of this virus and its etiologic role in enteric disease of water buffalo have to be studied more extensively. In addition, epidemiological studies will assess whether BuCoV is widespread among water buffalo herds as well as whether cross-species transmission between buffalo and cattle occurs mainly in areas where both closely related ruminant species are raised intensively.

Table 1: Amino acid identity (%) of ruminant CoVs to reference BCoV Mebus in nonstructural and structural proteins.

	Amino acid identity (%) to BCoV Mebus			
CoV strain	BuCoV 179/07-11	GiCoV US/OH3/2003	ACoV	SACoV US/OH1/2003
32 kDa	97.1	97.4	98.2	97.4
HE	98.3	98.8	98.5	98.8
S	96.6	96.8	97.3	97.2
4.9 kDa	93.0	58.1	58.1	58.1
4.8 kDa	82.2	71.1	82.2	71.1
12.7 kDa	99.0	98.1	98.1	97.2
E	98.8	98.8	97.6	96.4
M	98.6	99.1	99.1	99.1
N	98.6	98.8	98.6	98.8
I	96.1	96.6	96.1	96.1

Figure 1.



Virus isolation of the bubaline CoV on human rectal tumor cells. (A) Cytopathic effect (syncytia) caused by CoV strain 179/07-11. (B) Cytoplasmic fluorescence detected by the immunofluorescence assay using a BCoV-specific serum.

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