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# Molecular and antigenic characterization of bovine Coronavirus circulating in Argentinean cattle during 1994–2010

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ABSTRACT

Bovine coronavirus (BCoV) is an important viral pathogen associated with neonatal calf diarrhea. Our aim was to investigate the incidence of BCoV in diarrhea outbreaks in beef and dairy herds from Argentina during 1994–2010. A total of 5.365 fecal samples from diarrheic calves were screened for BCoV diagnosis by ELISA. The virus was detected in 1.71% (92/5365) of the samples corresponding to 5.95% (63/1058) of the diarrhea cases in 239 beef and 324 dairy farms. The detection rate of BCoV was significantly higher in dairy than in beef herds: 12.13% (29/239) *vs.* 4.32% (14/324) respectively. Phylogenetic analysis of the hypervariable S1 region of seven representative samples (from different husbandry systems, farm locations and years of sampling) indicated that BCoV strains circulating in Argentinean beef and dairy herds formed a cluster distinct from other geographical regions. Interestingly, Argentinean strains are distantly related (at both the nucleotide and amino acid levels) with the Mebus historic reference BCoV strain included in the vaccines currently available in Argentina. However, Mebus-induced antibodies were capable of neutralizing the BCoV Arg95, a field strain adapted to grow *in vitro,* and *vice versa*, indicating that both strains belong to the same CoV serotype reported in cattle. This work represents the first large survey describing BCoV circulation in Argentinean cattle.



## 1. Introduction

Bovine coronavirus (BCoV) is a major viral pathogen associated with neonatal calf diarrhea (NCD) (Mebus et al., 1973), winter dysentery in adult cattle (Saif et al., 1988) and respiratory tract disorders in cattle of all ages (Cho et al., 2001b; Decaro et al., 2008a). It causes important economic losses to the beef and dairy industry worldwide (Boileau and Kapil, 2010; Vlasova and Saif, 2014). Serological surveys indicate that approximately 90% of the worldwide cattle population has antibodies (Abs) against BCoV (Lin et al., 1996). Also, detection of similar CoV strains among wild ruminants, dogs and horses, with evidence for experimental interspecies transmission to calves, suggests that these species could harbor CoVs transmissible to cattle or *vice versa* (Barros et al., 2013; Saif, 2010).

Three antigenic groups of coronaviruses have been established and all BCoV strains characterized worldwide belonged to the subgroup initially designated as 2a (Hasoksuz et al., 2008). The International Commitee for Taxonomy Viruses (ICTV) has proposed a revision of the family Coronaviridae to create a new subfamily Coronavirinae that includes the Alpha, Beta and Gammacoronavirus genera. Following this new suggested taxonomy, BCoV belongs to the *Betacoronavirus* genus, cluster within the Coronavirinae subfamily, Coronaviridae family and the order Nidovirales (http://ictvonline.org/virusTaxonomy.asp).

The virus genome is comprised of single stranded non-segmented positive-sense RNA (32 kb) associated to the nucleoprotein (N) and forming a nucleocapsid with helical symmetry (Clark, 1993). Viral particles are large (100–150 nm), pleomorphic and enveloped with four major structural proteins comprising a

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membrane (M) glycoprotein, an envelope (E) protein, a spike (S) glycoprotein and the hemagglutinin-esterase (HE) glycoprotein (Lai, 2001). It is interesting to note that the hemagglutinating activity of the HE from BCoVs strains is lower than the hemagglutinating activity of the S glycoprotein, which forms large spike-like projections in the viral envelope (Schultze et al., 1991). Moreover, the S glycoprotein harbors domains responsible for receptor binding and induction of neutralizing antibodies, and is the most polymorphic viral protein among CoV species and also among strains of the same species. It is utilized for the molecular characterization of the isolates (Collins et al., 1982). The S glycoprotein consists of two subunits, S1 (N-terminal half) and S2 (C-terminal half). The S1 hypervariable region is useful to study the variability and evolution of this virus (Brandao et al., 2006; Hasoksuz et al., 2002).

Most of the studies assessing BCoV relevance as a primary pathogen in neonatal calf diarrhea (NCD) were conducted in the northern hemisphere (Ammar et al., 2014; Bidokhti et al., 2013; Decaro et al., 2008b; Hasoksuz et al., 2002; Jeong et al., 2005; Lu et al., 1991; Mawatari et al., 2014; Ohlson et al., 2013). In contrast, little epidemiological information is available regarding BCoV detection, incidence and characterization in cattle from Central and South American countries. In Cuba, BCoV sequences clustered with BCoV strains from USA, suggesting a common origin for these viruses (Martinez et al., 2012). In South America, most of the information comes from studies conducted in Brazil (Asano et al., 2010; Barros et al., 2013; Brandao et al., 2008, 2006; Takiuchi et al., 2008). Stipp et al. (2009) reported a 15.6% detection rate of BCoV in diarrheic calves from dairy and beef farms during a survey conducted in four states of Brazil. Phylogenetic studies based on the hypervariable region of the S glycoprotein gene indicated that Brazilian BCoV strains belong to two different clusters, suggesting that at least two different BCoV strains are circulating in Brazil (Brandao et al., 2006; Takiuchi et al., 2008). Interestingly, some BCoV strains detected in Brazil showed a gap of 18 nucleotides (nt 1577–1594; aa 461–570) in the hypervariable region within the S1 encoding gene, giving rise to a paraphyletic group in the evolution of BCoV circulating in Brazil. Similar gaps were also reported in porcine and human CoV strains causing respiratory disease. The presence of this gap in the S protein from swine CoVs has been associated with a change from enteric to respiratory tropism (Saif and Sestak, 2006; St-Jean et al., 2004).

For the entire S1 encoding gene, Takiuchi et al. (2008) showed that the Brazilian BCoV strains were distant from the Mebus strain (97.8% identity for nucleotides and 96.8% identity for amino acids) and more similar to the American BCoV-ENT strain 182NS and other Canadian strains (98.7% for nucleotides and 98.7% for amino acids).

The aim of the present study was to determine the infection rates of BCoV in diarrheic calves from Argentinean farms. Additionally, to conduct a phylogenetic study with the Argentinean BCoV strains in comparison with the BCoV strains, characterized worldwide, we focused our analysis on the hypervariable region of the BCoV S1 encoding gene (330 bp -nucleotide 1381–1711 of the Mebus strain S gene U00735.2-). To confirm the results, a phylogenetic analysis using a 1555 bp (nucleotide 1066–2621 of the Mebus strain S gene U00735.2) fragment of the S glycoprotein gene from two Argentinean strains detected 18 years apart (Arg95 and 5324-2013), was also performed. Finally, to evaluate the cross-reactivity between the Arg95 isolate and the Mebus reference strain, an *in vitro* virus neutralization assay was conducted.

## 2. Materials and methods

### 2.1. Fecal samples and viral detection

A total of 5.365 fecal samples were collected from diarrheic calves during 1994–2010, corresponding to a total of 1.058 outbreaks or cases of NCD in dairy farms ($n = 239$), beef herds ($n = 324$) and cattle from non-specified farm types ($n = 495$). The survey included farms located in 10 different provinces from Argentina (Buenos Aires, Corrientes, Entre Rios, Santiago del Estero, Santa Fe, Córdoba, La Pampa, San Luis, Río Negro and Neuquén).

The detection of BCoV antigens in fecal samples was performed by an indirect antigen-capture ELISA as described elsewhere (Smith et al., 1996). Briefly, 96 well ELISA plates (Maxisorp, NUNC, Denmark) were coated with four monoclonal antibodies (MAbs BC 21 F63C, BC 22 F83C directed to HE, BC 28H1.2C directed to N and BC 29 G72C directed to S) developed against CD DB2 strain and then incubated with 10% nonfat milk in PBS-Tween 0.05% for blocking of non-specific activity. Then, samples were added and incubated for 1 h at 37 °C. The plates were later incubated with polyclonal guinea pig anti-serum to BCoV at a 1:4000 dilution, and finally with commercial HRP-labeled goat polyclonal Abs to guinea pig IgG at a 1:3000 dilution (KPL, USA) for 1 h at 37 °C. Hydrogen peroxide and ABTS were used as substrate/chromogenic system (KPL, USA).

A total of seven ELISA-positive samples were selected for molecular characterization. The selection criteria included samples representing different husbandry systems, farm locations and years of sampling. Five of these fecal samples collected from three dairy herds and two beef herds were stored at −70 °C before sequencing and one sample collected in 2013 (5324) was sequenced from fresh stool. In addition, both original and the eight passage of the tissue culture adapted Argentinean strain (Arg95) were analyzed. The studied samples were identified as CoV/Bovine-B/Argentina/1617/2001, CoV/Bovine-B/Argentina/2026/2002, CoV/Bovine D/Argentina/4041/2009, CoV/Bovine-D/Argentina/4733/2009, CoV/Bovine-D/Argentina/4583/2010, CoV/Bovine-D/Argentina/5324/2013, and both the original and the 8th passage Arg95/1995. Accession numbers were deposited in GenBank database as KP033205–KP033210 and KP059126, KP059127.

**Table 1**
Primers pairs used to amplify the S1 gene hypervariable region and S gene fragment.

| Name | Primer sequence (5′–3′) | Location (BCoV complete genome) | Amplicon size (basepairs) | References |
|---|---|---|---|---|
| S1HS | CTATACCCAATGGTAGGA | 24827–24844 | 885 bp | Brandao et al. (2006) |
| S1HA | CTGAAACACGACCGCTAT | 25694–25711 | | |
| S1NS | GTTTCTGTTAGCAGGTTTAA | 24952–24971 | 488 bp | Brandao et al. (2006) |
| S1NA | ATATTACACCTATC CCCTTG | 25420–25439 | | |
| S1For | TTGTAATTTTAATATGAGCAGCC | 24808–24830 | 908 pb | In this study |
| S1Rev | TTCTGCCAACTATTATAATAAG | 25695–25716 | | |
| S2For | TTATAATAGTTGGCAGAACC | 25699–25718 | 672 pb | In this study |
| S2Rev | ACCATTCATTAAACTATTAGC | 26351–26371 | | |

Primers pairs used for amplification of S1 hypervariable region: S1HS, S1HA, S1NS, S1NA, primers pairs used for amplification of S gene fragment: S1For, S1Rev, S2for, S2 Rev.