



**Molecular detection of bovine coronavirus associated with the bovine respiratory complex in beef cattle in the Mexicali Valley, Baja California, Mexico**



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**Abstract:**

The bovine respiratory complex (BRC) is the leading cause of disease and death in beef cattle worldwide. It is a multifactorial infectious syndrome caused by different viruses and bacteria that reduce the productive efficiency and cause economic losses. In Mexico, BRC has been reported in all regions where cattle are fattened; however, these reports lack information on the presence of bovine respiratory coronavirus (BCV). This makes it necessary to have reliable and accurate diagnostic tools for detecting the presence of BCV in beef cattle fattened in Mexico, in order to propose appropriate sanitary measures for their clinical management. In this work, a real-time-PCR molecular diagnostic platform (rt-PCR) was developed to amplify a fragment of the BCV S protein in nasal exudate samples. When applying the rt-PCR platform for BCV in seemingly healthy beef cattle with signs of respiratory disease associated to BRC, 19/50 (38 %) were found to be positive, confirming the presence of this virus in the cattle of the region. The results of this work constitute the first report on the presence of the BCV associated to the BRC in the cattle region of northwestern Mexico and establish the bases for future research about the role that this virus plays in the presentation of the pathology of the BRC in beef cattle exploitation systems in this region and across the country.

**Key words:** Bovine Respiratory Coronavirus, Bovine Respiratory Complex, PCR, Protein S, Beef cattle.

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## Introduction

Bovine Respiratory Complex (BRC) is considered to be the main cause of clinical disease and death in feedlot cattle worldwide, and thereby, of economic losses for the farmers<sup>(1)</sup>. Traditionally, BRC is associated with single or combined disease of bovine respiratory syncytial virus (RSV), type 1 bovine herpes virus (BHV), bovine parainfluenza virus 3 (BPI3) and bovine viral diarrhea virus (BVDV), which produce a primary infection with mild clinical signs<sup>(2)</sup>. The BRC is also associated with the presence of the bacteria *Pasteurella multocida*, *Mannheimia haemolytica*, *Histophilus somni*, and *Mycoplasma bovis*, which act as opportunistic pathogens during conditions of stress or primary viral infection<sup>(3,4)</sup>. Besides the mentioned viruses, the bovine coronavirus (BCV) has also been reported as a viral agent associated to BRC, causing respiratory disease and reduction in weight gain in beef cattle<sup>(5-9)</sup>. The infection produced by BVC has been reported all over the world and is

considered an endemic disease in dairy and beef cattle farms<sup>(7)</sup>. BVC is transmitted mainly through the fecal-oral route, although it has also been shown to be transmitted through the respiratory route, by inhalation of aerosols containing the viral particles. When BCV enters the gastrointestinal tract, it triggers a clinical picture of diarrhea, dehydration, acidosis and hypoglycemia in young animals<sup>(10)</sup>. When BVC is admitted through inhalation, it infects the respiratory epithelium of the nasal turbinates, trachea and lungs. Replication leads to the elimination of the virus in nasal secretions, and the disease produces a picture with clinical signs ranging from absent to severe, including depression, fever, conjunctivitis, respiratory distress, and mild to severe cough<sup>(11)</sup>.

In Mexico, the BRC has been reported in all regions where cattle are fattened<sup>(12,13,14)</sup>. However, these reports lack information regarding the presence of the BVC in beef cattle farms, being of the greatest importance for developing diagnostic tools that allow to confirm the presence of the BVC in the cattle. The diagnosis of BVC is achieved using different serological techniques<sup>(10,15)</sup> or viral isolation from nasal exudate and biopsies from different tissues<sup>(10,16,17)</sup>. The application of molecular techniques for the detection of BVC associated with BRC—including conventional PCR (PCR) and real-time (rt-PCR)—has recently been reported<sup>(6,10,14)</sup>. Prominent among these are the platforms that amplify the gene which encodes for the BCV S protein, the most important viral structure for the production of neutralizing antibodies<sup>(7,18)</sup>. The S-protein gene is highly conserved among the BCV strains and has been widely used as a target gene for molecular tests for diagnosing this disease in livestock and other animal species, including humans<sup>(6,19,20)</sup>.

The aim of this work was the development and use of a platform for molecular diagnosis by rt-PCR to detect a fragment of the gene that codes for the BCV S protein in samples of bovine nasal exudate. The results indicate that the rt-PCR system is highly sensitive and specific for detecting the BVC and can be used in beef cattle exploitation systems in the region and across the country.

## **Material and methods**

This study was conducted at the Diagnostic Laboratories Unit (ULADI) of the Institute of Research in Veterinary Sciences of the Autonomous University of Baja California, Mexicali Campus.

### **Nasal exudate samples**

Fifty (50) nasal exudate samples were collected from beef cattle in stables, belonging to a technified bovine exploitation system located in the Mexicali Valley, Baja California. The samples were obtained from newly admitted animals aged 18 months on average, after less than 30 d of their arrival at the farm. Thirty samples were collected from animals with nasal

discharge, cough, depression, or body temperature greater than 38.5°C (Group 1), which were classified as sick, and 20 samples, from apparently healthy animals that showed none of the above<sup>(21)</sup>. The nasal exudate samples were collected by deep intranasal route, using broom-type Dacron swabs. Once the samples were taken, each swab was immersed in a tube containing sterile phosphate-base saline (PBS), pH 7.4, and the handle was cut so that the tube could be closed to protect the sample from possible contamination; the corresponding group was identified with a progressive number. Once collected, the samples were transported to the laboratory for processing.

### **Removal of RNA from nasal exudate**

For RNA extraction, Aurum Total RNA Fibrous Tissue Reagent Kits (Bio Rad, Hercules, California, USA) were used according to the manufacturer's instructions. RNA was recovered from each extraction in a volume of 50 µL of the elution solution provided in the reagent kits. The extracted RNA was stored frozen at -20 °C until the time of the rt-PCR.

### **Oligonucleotide design for BCV**

Oligonucleotides were designed from the sequence of the gene that encodes for the S protein of the bovine coronavirus strain R-AH187 (BCV-S), with access number *GenBank* EF424620.1. The gene corresponds to a 4,090 base pair molecule published in July 2016. The Primer3Plus oligonucleotide design software, version 2006-2007, was used; it is available at: <http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>. From the sequence of the BCV-S gene, the positive band oligonucleotide called BCVf with 5'-CTACTTGGAATAGGAGATTG-3' sequence was generated, while for the negative band oligonucleotide called BCVr, the selected sequence was 3'-TACACGGAGAAATTGG-5'; the amplification of these oligonucleotides by rt-PCR generates a product of 132 base pairs and a 36 % GC content, with a dissociation temperature (T<sub>m</sub>) of 77.0 °C for this PCR product. Table 1 shows the characteristics of oligonucleotides. Oligonucleotides were synthesized by GenScript LTD (Piscataway, New Jersey, USA) and were reconstituted with molecular biology grade water equivalent to 10 times the nano molar (nM) concentration value referred to by the manufacturer, to obtain a standard concentration of 100 micro molar (µM). For the rt-PCR tests, the working concentration of the oligonucleotides was set at 10 µM.

**Table 1.** Sequences and properties of oligonucleotides designed from the BCV-S gene with the reference *GenBank* EF424620.1

Oligonucleotide:	BCVf		
Sequence:	CTACTTGGAATAGGAGATTTG		
Start: nucleotide 1337	Length: 21 pb	Tm: 55.4 °C	GC: 38%
Oligonucleotide:	BCVr		
Sequence:	TACACGGACAGAAATTTGTG		
End: nucleotide 1469	Length: 20 pb	Tm: 54.3 °C	GC: 40%
Product:	132 pb	Tm: 77.0 °C	

### Master mix

In this work it was used the One-Step RT-PCR Script masterbatch I (Bio Rad, Hercules, California, USA) formulated with SYBR Green I fluorophore in a masterbatch solution using both oligonucleotides at a concentration of 400 nM, 2 µl RNA quench and molecular biology grade water for a total reaction volume optimized to 10µL.

### Positive RNA controls for BCV (o)

As positive RNA control extracted from the liquid fraction of the Scourgard 4 K7C vaccine (Zoetis, New Jersey, USA), which contains inactivated Hansen strain bovine coronavirus and inactivated G6 and G10 bovine rotavirus strains, enterotoxigenic *E. coli* K 99 and *Clostridium perfringens* toxoid type C, was utilized. The procedure for RNA extraction was performed according to the protocol of the Bio Rad Aurum Total RNA Fibrous Tissue set of reagents, using 300 µl of the vaccine. The extracted RNA was divided into 10 µl aliquots and stored in a freezer at -20° C until the RT-PCR tests were performed.

### RT-PCR test protocols

The rt-PCR tests were performed on a Bio Rad CFX96 thermal cycler. Denaturation, hybridization and extension parameters were calculated using the Protocol Autowriter tool of the CFX96 package integrated to the thermal cycler, taking into consideration the size of the PCR product, the oligonucleotide sequence and the type of enzyme in the master mix, resulting in an initial step at 50 °C during 10 min for reverse transcription, followed by a denaturation cycle of 95 °C during 3 min, and continuing with 39 denaturation cycles at

95 °C during 10 sec, 20 sec at 51.0 °C for oligonucleotide hybridization, and 15 sec at 72 °C for extension. Also, for each run, dissociation curve analysis from 65 °C to 95 °C was performed to identify amplification curves within the estimated temperature of 77.0 °C  $\pm$  1 °C of the 132 bp PCR product and discriminate between artifacts other than the amplification of the expected RNA template.

### **Interpretation of results**

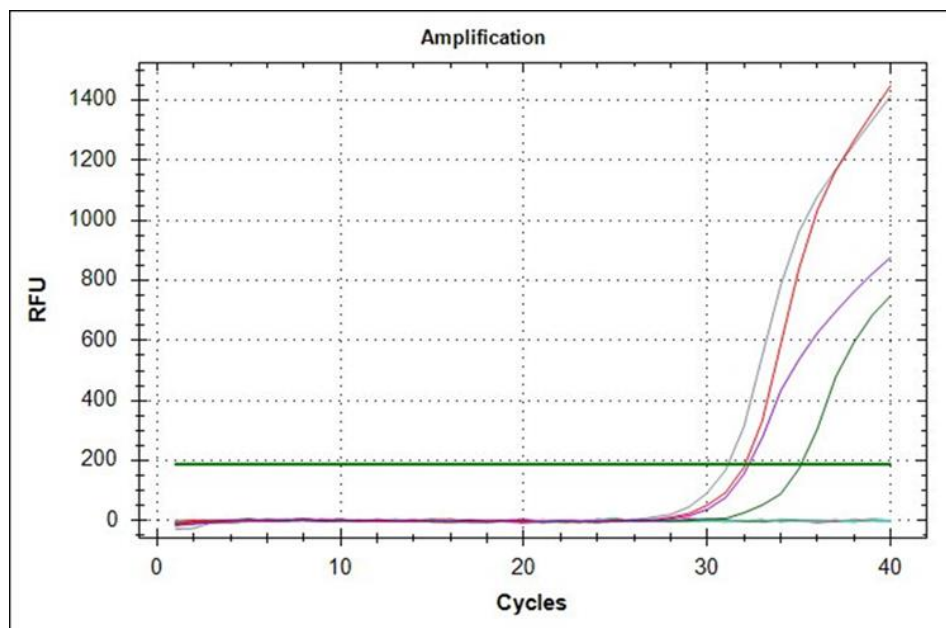
rt-PCR test results for BCV were considered positive when the corresponding sample obtained a fluorescent amplification signal before cycle 40, above the threshold control line automatically established by the CFX96 program, and amounting to 10 times the standard deviation of the average fluorescence index generated by all the samples during the first 10 cycles of each run. Results were considered negative when the corresponding sample failed to develop a fluorescent amplification signal above the threshold line of the negative reference control within a maximum of 40 cycles.

## **Results**

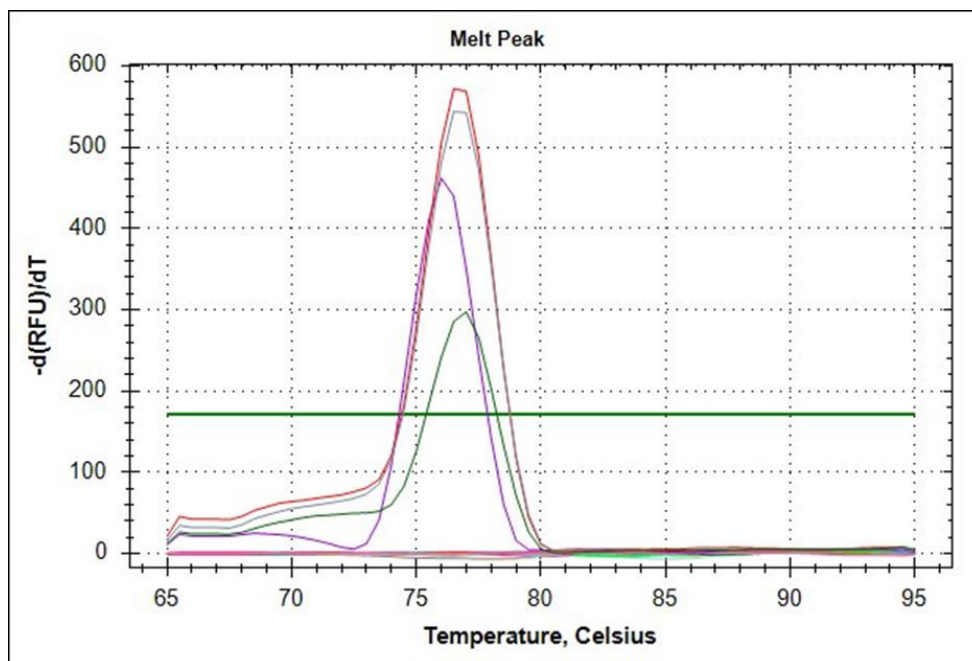
### **RT-PCR standardization for BCV (o)**

The amplification graph and dissociation curve calculated with Bio-Rad's CFX96 package for the BVC rt-PCR system showed that the optimal combination of reagents for maximum amplification of the oligonucleotides BCVf and BCVr is achieved at a concentration of 400 nM with 2  $\mu$ L RNA template. Under these conditions the positive controls extracted from the Scourguard 4 K/C vaccine developed a signal above the threshold line with an average amplification cycle (Cq) of 31.09 in 40 total cycles of each amplification run; the negative controls showed no evidence of amplification (Figure 1). Also, analysis of the dissociation curve (Tm) for the positive control RNA showed a dissociation temperature range between 76.0 and 78.0 °C, with an average temperature of 77.0 °C (Figure 2); these parameters allow considering the rt-PCR test for CVB as valid.

**Figure 1:** Amplification curve of bovine coronavirus controls extracted from Scourguard 4 K/C vaccine using the oligonucleotides BCVf and BCVr at a concentration of 400 nM with a 2  $\mu$ l RNA template



**Figure 2:** Dissociation curve of rt-PCR positive controls for bovine coronavirus showing an average temperature of 77.0  $^{\circ}$ C





## RT-PCR results for nasal exudate samples

Fifty RNA samples of nasal exudate from stabled cattle were tested in duplicate, of which 19 (38.0 %) achieved amplification above the threshold line established by CFX96 and were therefore considered positive. Of the samples that were positive, 5 (10.0 %) belonged to Group 1, which corresponds to animals with signs and symptoms associated with the BRC, and 14 (28.0 %), to Group 2, which is made up of animals without signs and symptoms of respiratory disease. The average C<sub>q</sub> of the samples of both groups was 34.60 cycles with a range of C<sub>q</sub> between 30.87 and 35.95 cycles, and average T<sub>m</sub> of 77.0 °C (Figure 2).

## Discussion

BCV is a globally distributed pathogenic virus that causes enteric diseases in young calves and winter dysentery in adult cattle. BVC is also implicated in BRC-associated infections in beef cattle. Although infections by BCV produce a mortality smaller than 2 %, the morbidity of this virus can reach 100 % of the animals of a farm, causing respiratory or digestive syndromes that negatively affect the rate of gain of weight or milk production and increase the costs due to veterinary services, antibiotics and other medicines that altogether cause economic losses for the cattle sector<sup>(10,15)</sup>.

The development and implementation of the rt-PCR platform for BCV presented here, arises in response to the need for reliable, accurate and rapid diagnostic tools to detect a disease of viral origin that has been reported as part of the BRC; however, due to the large number of signs and symptoms that common pathogens produce, it is difficult to establish precisely the main causal agent of the pathology in an animal or herd<sup>(22,23)</sup>; especially when the infectious process develops with minimal or imperceptible symptoms, causing a delay in the initiation of the corresponding therapy, extending the time required to recover the state of health and, therefore, negatively affecting the levels of productivity of the sick animals<sup>(20,24)</sup>.

In this work, an rt-PCR platform for detecting and amplifying a fragment of the gene that encodes for the BVC S protein was designed, developed and instrumented, which turned out to be a highly sensitive and specific molecular diagnostic platform for the detection of BCV from nasal exudate samples. Although in this work the viral particles were not quantified, the sensitivity of rt-PCR platforms to BCV from nasal exudate samples has been previously reported as having detection ranges of 10<sup>2</sup> cDNA<sup>(25)</sup> to 10<sup>3</sup> cDNA<sup>(26)</sup> copies per reaction, with amplification curves developed after cycle 34 for both studies. This agrees with the average C<sub>q</sub> of 34.60 developed by the samples analyzed in this work; therefore, can be propose that the rt-PCR platform for CVB reported here has an estimated sensitivity between 10<sup>2</sup> and 10<sup>3</sup> cDNA copies per reaction.



Due to the genetic properties of the S protein, characterized by a high homology between the different viral strains and a high immunological reactivity<sup>(22,24)</sup>, different fragments of the BCV S protein gene have been used as a reference methodological basis for the development of molecular and serological diagnostic platforms for the rapid detection and diagnosis of this virus with high levels of sensitivity and specificity, even in samples containing small amounts of virus, where conventional diagnostic tests may be inconclusive<sup>(23)</sup>.

The results obtained indicate that 19 samples (38 %) from both study groups tested positive with the rt-PCR platform for BCV. Notably, while five of the samples from the group of sick animals (n= 30) were positive, 14 of the samples from the group of apparently healthy animals (n= 20) were positive to the tests. Contrary to what was anticipated, 70 % of the samples from the group of apparently healthy animals were positive to the rt-PCR platform for BCV. This may be because BCV can infect up to 45 % of newly arrived cattle without showing obvious signs or symptoms of disease<sup>(27,28)</sup>. However, apparently healthy cattle with nasal shedding of BCV have been shown to be 1.6 times more likely to suffer at least one episode of respiratory disease and 2.2 times more likely to develop lung lesions than animals that do not shed virus by this route<sup>(7,29)</sup>; therefore, these animals may have been incubating the virus while not yet developing the clinical respiratory profile characteristic of BRC.

The prevalence of 38 % is similar to that reported in other regions of the world. In a study carried out in Australia in beef cattle for export, nasal exudate samples were analyzed using an rt-PCR platform similar to the one used here, finding a prevalence of 40.1 % for BVC, followed by 0.4 % for BVDV, 0.3 % for IBR, 0.3 % for RSV and 0.3 % for BPI3, evidencing the magnitude of the influence of BCV on the occurrence of respiratory disease of BRC in that country<sup>(29)</sup>. Likewise, these results show a higher positive rate than the one reported in Ireland, where a study was carried out to establish the prevalence of pathogens associated to BRC from samples of nasal exudate using rt-PCR and found a positive rate of 22.9 % for BCV, 11.6 % for BRSV, 7.0 % for BPI3, 6.1 % for IBR, and 5 % for BVDV, highlighting the fact that BCV is the virus associated to BRC that is most frequently diagnosed in the beef cattle of that country<sup>(30)</sup>. BCV is also the most prevalent virus associated with BRC in beef cattle in the United States of America. Prevalence reports of pathogens associated to BRC in nasal exudate samples from beef cattle analyzed with rt-PCR techniques indicate a prevalence of 62.8 % for BCV, followed by BVDV, with 15.7 %; IBR, with 14.9 %; BRSV, with 9.1 %, and BPI3, with 8.3 %<sup>(23)</sup>. The prevalence rate in the USA exceeds that reported for Australia and Ireland, as well as that reported for Mexico through this work, mainly because beef cattle farms in the USA house hundreds of thousands of cattle in a given region, where close contact between healthy and sick animals can favor the transmission and persistence of BCV among cattle<sup>(31)</sup>.

The results presented here constitute the first report on the presence of the BCV associated to the BRC in the cattle region of northwestern Mexico and place the BCV in the second position of the table of prevalence of virus associated to the BRC previously detected in the area, where the bovine respiratory syncytial virus (BRSV) occupied the first position with 80.6 % of prevalence, followed by the parainfluenza virus 3 (BPI3), with 23.8 %; the bovine herpes virus 1 (BHV)-1, with 20.4 %, and the bovine viral diarrhea virus (BVDV), with 11.3 %<sup>(15)</sup>.

## Conclusions and implications

It concludes that the BCV is present in the cattle stables of the Mexicali Valley, Baja California. The rt-PCR platform for BCV reported here is a fast, sensitive and specific molecular diagnostic tool to detect BCV in nasal exudate samples from cattle in feedlots. The prevalence of 38.0 % for BCV reported in this work should be the starting point for future researches on the role that this virus plays in the presentation of the BRC pathology in the beef cattle exploitation systems in our region and across the country.

The authors of this paper declare that they have no conflict of interests of any kind.

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