What is the evidence that bovine coronavirus is a biologically significant respiratory pathogen in cattle?

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Abstract — Coronaviruses, including bovine coronavirus (BCoV), are etiologically associated with enteric and respiratory disease across a wide range of mammalian and avian species. The role of BCoV in calfhood diarrhea is well-established, but its role in the bovine respiratory disease complex (BRDC) has been controversial. This review re-examines the evidence that BCoV is a significant pathogen in the BRDC.

Résumé — Quelle est la preuve que le coronavirus bovin est un agent pathogène biologiquement important chez le bétail? Les coronavirus, y compris les coronavirus bovins (BCoV), sont étiologiquement associés à des maladies entériques et respiratoires chez un vaste éventail d'espèces mammifères et aviaires. Le rôle du BCoV dans la diarrhée des veaux est bien établi, mais son rôle dans le complexe de la maladie respiratoire bovine est controversé. Cet examen se penche de nouveau sur les preuves indiquant que le BCoV est un agent pathogène important pour le complexe de la maladie respiratoire bovine.

(Traduit par Isabelle Vallières)

Can Vet J 2019;60:147–152

Introduction

Bovine coronavirus (BCoV) belongs to a family of microbes that is etiologically associated with enteric and respiratory disease across a wide range of mammalian and avian species (1). The role of BCoV in calfhood diarrhea is well-established, and it continues to be a problem in calf-rearing operations (1). The role of BCoV in the bovine respiratory disease complex (BRDC) has been controversial, and, if anything, the recent increased application of molecular diagnostics to BRDC cases has further muddied the waters. Over the years since its discovery there have been several reviews on BCoV, including some focusing on "respiratory" BCoV (2–5). Beyond the biological precedents linking coronaviruses to respiratory diseases, recent information concerning BCoV is reviewed herein, and the evidence that implicates BCoV in the BRDC is re-addressed.

A brief history of bovine coronavirus

Exemplifying Pasteur's aphorism, "Chance only favors the prepared mind," BCoV was accidently discovered by Mebus et al (6) at the University of Nebraska in 1972. These authors were conducting efficacy studies on a vaccine for the then newly discovered bovine reovirus-like virus (rotavirus) and astutely observed that while the vaccine was apparently effective in reducing diarrhea due to the rotavirus, there were several herds in which vaccinated calves developed diarrhea later than expected with rotavirus, and their feces were free of that microbe. Mebus et al (6) observed a corona-like virus in diarrheic feces and conducted transmission experiments in gnotobiotic calves. They then cultured the virus, determined which cell types would support growth, attenuated the virus, and performed initial protection experiments (7). In the next decade BCoV was recognized as a common cause of calfhood diarrhea (8). In 1982 Thomas et al (9) working in England in a search for new microorganisms in calf pneumonia first implicated BCoV as a respiratory pathogen by inoculating material from nasopharyngeal swabs and lung washes from calves with naturally occurring respiratory disease into gnotobiotic calves. Coronaviruses were then observed using electron microscopy in respiratory samples and supernatants from organ cultures that were inoculated with respiratory samples from the experimentally infected calves (9). The studies by Thomas et al (9) also provided the first indication that the 2 BCoVs associated with enteric and respiratory disease were the same, or at least belonged to the same serotype, by noting that serum raised against enteric isolates of BCoV immunoagglutinated the "respiratory" BCoV. Shortly thereafter, workers in the same laboratory extended investigations of the relatedness of BCoVs in 1985, and demonstrated immunity to heterologous infection and cross-neutralization of BCoVs by porcine antisera to enteric and respiratory isolates (10). Subsequently, numerous investigators have confirmed, using various techniques, that enteric and

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respiratory BCoVs are members of the same quasispecies (11), notwithstanding predictable genotypic and phenotypic differences amongst isolates (1–5).

A primer on coronavirology

The family *Coronaviridae* was originally named in the 1960s in the heyday of electron microscopy based on morphologic features (12). Coronaviruses are spherical to pleomorphic enveloped RNA viruses (1–5). They have distinctive club-shaped 20-nm peplomers or "spikes" protruding uniformly, circumferentially from the envelope. Some coronaviruses, including BCoV, have a secondary fringe of smaller 5-nm spikes (1–5). In electron micrographs the overall appearance of the viral particles was reminiscent of the solar corona to virologists, hence the name "corona" (12). The lipid-containing envelope makes these viruses susceptible to conventional disinfectants and the extra-corporal environment (1). The larger spike is a heterodimeric glycoprotein comprising 2 subunits, S1 and S2 (1,2,5). This spike protein has several important biological features: it interacts with sialic acid-containing receptors on the target cell membrane, probably largely determining tissue tropism and species specificity of different coronaviruses; it is involved in fusing infected cells; it contains, mostly conformational, epitopes that are the major targets for antibody responses and less well-characterized cell-mediated immune responses; and it is the major site of immunologically important antigenic variation amongst BCoV isolates, which likely contributes to vaccines working, or not (1,2,5). The smaller spike is a second envelope glycoprotein, which is also a heterodimer comprising a host membrane (class I) protein and a hemagglutinin-esterase (HE). This spike also interacts with cellular receptors and contains epitopes that are targets for neutralizing antibodies (1,2,5). There are 3 other structural proteins; 2 transmembrane proteins: M and E, that assist in viral assembly, and an internal N or nuclear protein that is associated with the genome to form a helical nucleocapsid. The amino acid structure of N protein is relatively conserved amongst BCoV isolates and is therefore targeted in diagnostic testing such as polymerase chain reactions (PCRs) (1,2,5). Less glamorous, but no less biologically significant, is a group of 4 to 8 (depending on the particular coronavirus) nonstructural "accessory" proteins. These proteins have been best characterized in the emergent bat coronaviruses, notably SARS virus, but have not been thoroughly examined in BCoV. Nevertheless, a major function of these proteins is to inhibit innate (interferon) responses (1,13). Inter-isolate differences in this immunosuppressive function could contribute to differences in virulence amongst BCoV isolates (13); time will tell.

Coronaviruses are in the Order Nidovirales (from the Latin *nidus,* nest), which refers to their complex replicative scheme, involving "nests" of subgenomic RNAs (1). Essentially, this involves 3 major steps. First, part of the +stranded RNA genome acts as messenger RNA for the synthesis of an RNA-dependent RNA polymerase. Then, this enzyme guides cellular machinery in the cytoplasm of the infected cell to transcribe a full length negative (complementary) RNA from which a new copy of the +strand genome is transcribed. The RNA polymerase also directs the transcription of a set, or "nests" of subgenomic messenger RNAs from the complementary RNA strand, which the cell uses to translate individual viral proteins (1). Molecular details aside, this highly error-prone replicative scheme, together with the possibility of recombination between isolates, results in high mutability, making BCoV and other coronaviruses rapidly moving targets.

Historically, coronaviruses were divided into 3 groups based on genetic and serologic properties: Group 1 included feline infectious peritonitis virus and transmissible gastroenteritis virus, and lacked the hemaglutinin-esterase (HE); Group 2 included BCoV and the human "cold" virus, HCoV-043, and had the HE; and Group 3 included avian viruses, notably infectious bronchitis virus (2,5). More recently, the relative ease and low cost of sequencing, together with the discovery of more coronaviruses, have complicated the taxonomy of coronaviruses (1). These viruses are currently divided into 4 genera based on the partial nucleotide sequences of the RNA-dependent RNA polymerase: alpha, beta (contains 4 subgroups A to D), gamma, and delta, with BCoV in the beta A grouping, with its close relationship to the human (and canine) "respiratory" coronaviruses preserved. Genotyping of BCoV isolates is in its infancy (14). However, despite the confusion more cladistics analyses may bring, it is likely that the current bottom line will not change; BCoV exists as a quasispecies with 1 serotype, but with significant variation, including antigenic spectrum, tropism, and virulence, amongst isolates that does not necessarily relate to their clinical origin (enteric *versus* respiratory) (1,5).

Circumstantial evidence that BCoV is a respiratory pathogen

Associations with ex vivo *and postmortem sampling*

As indicated in the first report implicating BCoV as a respiratory pathogen in the early 1980s (9), the presence of a microbe in nasal secretions and other respiratory samples has long been taken as evidence of pathogenicity/causality in BRD cases. Historically, detection was accomplished using electron microscopy and/or virus isolation. Isolation of BCoV in cell culture can be problematic because, as first reported in the seminal studies (7), it is fastidious, especially in the case of field isolates (15,16). The latter usually preferentially grow in primary or very low passage cell cultures, and, for some undetermined reason, in the human rectal adenocarcinoma cell line, HRT-18 (17), which is the cell of choice in most diagnostic settings when culture of BCoV is attempted (1). Currently, as throughout veterinary diagnostic medicine, reverse transcriptase (RT)-PCR-based methods (18–25), and most recently metagenomic analysis (26) have largely supplanted culture in identifying BCoV in clinical samples. The increased sensitivity, however, could lead to false positives with regard to biological significance in the likely case that a certain threshold of viral growth is necessary to cause clinical disease. Attesting to this *caveat emptor,* as is the case with many (all?) endemic pathogens in host populations, BCoV has been identified in respiratory samples from healthy (16,21,26–28) as well as sick cattle (16,18–25). In the latter case, BCoV shedding has been documented in the absence of other recognized, or tested for, respiratory pathogens (27,28), and, more frequently as an apparent co-conspirator with other

respiratory pathogens in clinical specimens (18,22,27–31). Even if it is not consistent with Evan's postulates (29) the presence of BCoV in the nasal secretions of healthy cattle is not exculpatory of pathogenicity; however, it does make the indictment of BCoV as a significant respiratory pathogen, based on presence alone, more tenuous. Simply because a microbe is present in a sick animal does not necessarily mean it is a pathogen. Quantitative PCR (qPCR) has been used experimentally in a small number of calves to correlate amount of nasal shedding with transmissibility (32). No direct link was found, but this study suffered the confounding variable of developing immunity (32). Beyond Evans postulates (29), more data correlating BCoV load with disease would be an obvious theoretical and practical advancement to more definitively establish the conditions of causality with regard to the detection of BCoV in clinical samples.

Associations with immune responses to BCoV

Like a fingerprint at the scene of a crime, traditionally and currently, post-disease seroconversion is often used as evidence of causality. From its initial discovery and indictment in respiratory disease to the present day, numerous serological studies have implicated BCoV; but not without reasonable doubt (28,31,33–37). Conversely, an association between an immune response to BCoV, *a priori* to exposure/challenge, and disease-sparing has also been taken as circumstantial evidence of causality. Several epidemiologic studies have reported that high antibody titers to BCoV, most likely resulting from exposure prior to weaning, can have a respiratory disease-sparing effect. Cattle with high antibody titers on entry to a feedlot or other situations of exposure are less likely than those with low antibody titers to develop the BRDC and/or require treatment (28,31,34,38–40). In addition, in the 1 and only prospective study that examined the effect of (intranasal) vaccination for BCoV it was reported that vaccination before entry to the feedlot and/or antibody titers $>$ 20 were associated with decreased risk of treatment for BRDC; whereas vaccination on arrival was associated with increased risk (41). However, in the absence of specific etiologic diagnosis, preferably visualization of BCoV in lesions, these data may indicate an association and not a causal relationship. The data do not rule out that calves with a broad range of immunological experience may have less BRD simply because they also have antibody and other immune responses to the pathogen(s) which is the true the cause of the observed disease; bovine respiratory syncytial virus (BRSV), for instance. Moreover, at the individual calf level, data are conflicting as to the predictive value of BCoV antibody titers regarding which calves require treatment for BRDC after entry to a feedlot (25,28,34).

Physical evidence that BCoV is a respiratory pathogen

In contrast to the relative plethora of circumstantial evidence in the form of epidemiologic data and routine diagnostic testing of clinical specimens that are often used to indict BCoV as a respiratory pathogen, there is a dearth of physical evidence of its criminality in that organ system. Quite simply, there are very few images demonstrating BCoV in lesions in the respiratory tract in naturally or experimentally infected cattle in the peer-reviewed literature or textbooks, which would arguably constitute a "smoking gun" of direct evidence, at least for pathologists. From the standpoint of naturally occurring disease, this could be due, at least in part, to the timing of sampling. Cattle that die of respiratory disease often present little physical (immunohistochemical) evidence of viral infection in affected organs simply because the acutely infecting respiratory viruses have come and gone by the time an animal succumbs to secondary, more readily demonstrable, bacterial infections (5,42). In the last decade, 1 investigation of outbreaks of acute respiratory disease in intensively reared beef calves showed a series of good quality gross and histological lesions. These included tracheal petechiation together with mucopurulent discharge and bronchointerstitial pneumonia with intra-bronchiolar syncytial cells in an affected airway that could have been compatible with BCoV infection (43). Bovine coronaviral RNA was detected by qRT-PCR in 2 of 15 lesional lungs tested; however, unfortunately, there was no apparent attempt to directly associate BCoV with the histological lesions.

From the perspective of attempting to demonstrate Koch's postulates by experimentally reproducing disease, convincing evidence convicting BCoV in a causal relationship with respiratory disease is scant. Four studies reported a failure to produce clinical respiratory disease using various BCoV-containing inocula (10,44–46). The first was a complicated cross-protection study and used feces from diarrheic calves administered orally or material from nasopharyngeal swabs administered intranasally and intratracheally as inoculum. Bovine coronavirus-positive epithelial cells were demonstrable in nasal turbinates and/or tracheas of 11 of 12 infected calves; however, there was no associated inflammation reported or evident in pictures of immunohistochemically stained tissues (10). No BCoV was similarly identified in lungs of any calf. In a second study, 18 (3- to 50-day-old) gnotobiotic calves and 7 (25- to 63-day-old) colostrum-deprived calves were inoculated intranasally, orally, or by both routes with a suspension of BCoV-containing intestinal contents that had been derived from the 5th passage of similar material in gnotobiotic calves and was "bacteriologically sterile" (44). All the calves developed diarrhea by 2 to 4 d after infection, but none had clinical signs of respiratory disease. Bovine coronavirus-infected nasal and/or tracheal epithelial cells were detected in 16 of the 18 calves, and in the "lung tissue" (impression smears) of 4/18 calves by immunofluorescent staining. Two of the latter calves had "focal interstitial emphysema"; however, assessment of histological changes was not performed, or at least not presented. In the third study, 5 (1- to 10-day-old) colostrum-deprived calves and 2 (5- to 27-day-old) gnotobiotic calves were administered either 40 mL of a HRT-18 cultured (passage level and dose not given) "respiratory" strain (BC930), or "a winter dysentery strain" or "a calf diarrhea strain," "oronasally" (passage levels and doses not given) (45). All calves developed diarrhea of variable severity and duration. No calves developed signs of respiratory disease, and aside from monitoring nasal (and fecal) shedding of BCoV, there was no examination of any tissues from the respiratory tract to determine sites of infection. Arguably the most convincing experimental physical evidence of

the pathogenicity of BCoV in the respiratory tract was obtained with a winter dysentery isolate of the virus, the Korean strain "KWD3" that had been passaged 6 times in HRT-18 cells (46). Six 2- to 4-day-old colostrum-deprived (CD) Holstein calves were inoculated orally with 40 mL of HRT-18 cell culture supernatant containing 1.5×10^8 focus (plaque) forming units/mL; 1 CD calf was mock infected with 40 mL of uninfected HRT-18 cell culture supernatant, and 1 CD calf received 40 mL of inactivated infected HRT-18 supernatant. All of the infected calves developed diarrhea and elevated body temperatures; the mock infected controls did not. Despite the report of no clinical signs of respiratory disease in the infected calves, there were significant lesions described throughout the respiratory tract, including infection and necrosis of epithelia in the nasal turbinates, trachea, bronchioles, and pulmonary parenchyma. There was associated interstitial pneumonia and hyperplasia of type II pneumocytes, and BCoV antigens were demonstrated immunohistochemically in the cytoplasm of degenerate epithelial cells. Unfortunately, these changes were illustrated in figures of only moderate quality, somewhat limiting interpretation. Although the authors did not exclude the possibility of some inhalation of the inocula during administration, they made the point of discussing a probable role for viremia, including cell-free and infection of cells of monocyte macrophage lineage, resulting from transenteric transmission in the pathogenesis of respiratory infection.

Two studies reported reproduction of some level of respiratory disease (47–49). In the first published attempt to produce respiratory disease with BCoV, 7 (< 7-day-old) calves, 5 CD, 2 colostrum fed, were infected with a tracheal-organ culture supernatant containing BCoV (derived from a field case) as inoculum (47). Mild signs of respiratory disease, including cough and nasal discharge, as well as diarrhea, were produced. A few scattered areas of atelectasis were observed in lungs of 3 calves; changes in tracheas and nasal turbinates were not reported. Bovine coronavirus positive cells were visualized by immunofluorescence in the lungs of 2 calves, but no histological examination of respiratory organs was reported. However, this was the result of a draconian, unnatural challenge method involving both intranasal and transtracheal inoculation of 10 mL of supernatant given twice daily for 4 consecutive days. In another study published as 2 brief communications (48,49), 5-day-old CD Holstein calves were infected orally with different doses of an attenuated BCoV (2 calves; Mebus strain; 41 passages *in vitro*) or a "virulent pneumoenteric strain," the "Minnesota strain" in the form of suspended filtered fecal material containing BCoV from a field case (3 calves). All 3 calves that received the field isolate developed diarrhea; 2 had "pneumonia," including respiratory distress, and 1 of these died. The investigators reported fluorescence using BCoV conjugates [but not with conjugates for bovine respiratory syncytial virus (BRSV), bovine parainfluenza virus-3 (BPIV-3), or bovine herpes virus-1 (BHV-1)] of variable intensity in lung sections and "disrupted nasal cells." There were no images of lesions or immunological staining.

Some degree of diarrhea resulted during the attempts to reproduce respiratory disease with inocula containing BCoV, even when "respiratory" isolates were used and some respiratory disease was observed. Ironically, this unintended outcome provides some of the best evidence of the still-debated idea that the BCoVs that cause enteric and respiratory disease in cattle are essentially the same (5). Excluding the possibility that BCoV is not a significant respiratory pathogen, it is not known why respiratory disease caused by BCoV has been so difficult to reproduce. It could involve strain differences, host factors, or environmental co-factors to name a few, but it may be as conceptually and practically simple as how the BCoV inoculum is propagated. As demonstrated in the first paper reporting the *in vitro* culturing of BCoV nearly 50 y ago (7), and confirmed subsequently (2,5), upon repeated passage in cell culture, BCoV isolates become more promiscuous in their ability to grow in a broader range of cell types, and in higher passage cell cultures. This could simply reflect the *de facto* selection of variants within the quasispecies that are more flexible in their growth requirements. Again, the seminal studies indicated that this "adaptation to culture" is associated with attenuation (7); good for vaccine development, not so good for producing inocula to study pathogenesis. Still, the details as to how, and to what extent this promiscuity concerning target cell permissivity to infection *in vitro* is associated with changes in pathogenicity and/or virulence *in vivo* remains to be more fully examined. It has been more than a decade since there has been a publication related to the experimental reproduction of respiratory disease with BCoV in calves.

In conclusion, the answer to the rhetorical question that is the title of this review ultimately turns on one's definition of "*biologically* significant." Certainly, from the standpoint of pulmonary infection and pathology, in contrast to, for example, BRSV (50), available evidence indicates that BCoV has been associated with neither substantial infection of the lower airways or pulmonary parenchyma, nor substantial pulmonary pathology. In this it is different from the avian infectious bronchitis virus (IBV), the prototype virus of the *Coronaviridae* (12); severe respiratory disease and pulmonary (airway) pathology can be sequel to infection with virulent strains of that virus (1,12). With regard to the trachea, BCoV infection has been associated with mild clinical signs consistent with tracheal disease, and BCoV infection has been documented immunohistochemically *in situ* in that organ. However, this infection has apparently not been associated with the severe clinical tracheitis and extensive necrotizing, so-called "stove-pipe" lesions, typical of many cases of bovine herpesvirus-1 infection (50) or IBV (1). Examination of the nasal turbinates and sinuses is generally not a common practice in cases of bovine respiratory disease outside academia. BCoV has been identified, *in situ,* in those parts of the upper respiratory tract, which is consistent with the "cold-like" signs that are associated with BCoV infection in cattle, as well as in humans infected with another prototypical coronavirus responsible for the "common cold" (12).

Although it is certainly cliché to discuss the synergy between various pathogens in the development of the BRDC, it is probably in the case of mixed infections, generally the rule, that BCoV achieves significance as a respiratory pathogen. Certainly, infection and some level of damage to epithelium and resultant

inflammation, relatively innocuous in itself (witness the common cold), could and often probably does serve as a preamble to "secondary infections." Indeed, the first study implicating BCoV as a respiratory pathogen (10) provided evidence of this probable necessity and alluded to the problem of reproducing disease with a monoculture of BCoV (or other recognized respiratory pathogens), proving that Koch's postulates are a limited way of thinking about a syndromic, etiologically complicated, disease. This difficulty in reproducing dramatic respiratory disease, or at least something that can be measured beyond shedding of virus, with BCoV (alone) is also an impediment to having a BCoV vaccine with a label claim for respiratory disease from a regulatory perspective. In the context of this apparent constraint of disease production, it is both interesting and inconsistent that vaccines for the 2 recognized paramyxoviral respiratory pathogens of cattle, BRSV and bovine parainfluenza virus-3 (BPIV-3), were in the case of BRSV (51), and are in the case of BPIV-3 (52), licensed on the basis, essentially, of reduced nasal shedding (of the viruses) alone, with no requirement for producing typical clinical respiratory disease in the experimental models traditionally employed. Perhaps the best way forward is to place more emphasis on studying BCoV in the context of mixed infections, and relatedly, evaluate the efficacy of BCoV-containing vaccines in the context of disease reduction overall, rather than in the traditional regulatory approach to licensure: 1 agent, 1 disease.

Acknowledgments/Disclosures

The author has conducted and conducts vaccine efficacy studies for Pfizer Animal Health, Zoetis, Merial, Merck Animal Health, Fort Dodge Animal Health, Boehringer Ingelheim Vetmedica, Elanco. CVJ

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