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RESEARCH ARTICLE

Detection of bovine coronavirus (BCoV) infection in cattle with clinical respiratory signs by PCR and investigation of the serological status of these animals

Meltem Menekse[1], Atilla Simsek[*2]

[1]Ceren Animal Health Laboratory, Konya, Türkiye
[2]Selcuk University, Veterinary Faculty, Department of Virology, Konya, Türkiye

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*asimsek@selcuk.edu.tr

Solunum yolu klinik belirtileri gösteren sığırlarda bovine coronavirus (BCoV) enfeksiyonlarının PCR ile tespiti ve hayvanların serolojik durumlarının araştırılması

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Öz

Amaç: Bu araştırmada klinik olarak solunum yolu enfeksiyonu gözlenen sığırlarda bovine coronavirus (BCoV) prevalansının antijenik ve serolojik olarak ortaya konulması ve olası virüs saçılımı ile hayvanların serolojik durumları arasındaki ilişkinin değerlendirilmesi amaçlanmıştır.

Gereç ve Yöntem: Araştırmada solunum kanalı klinik belirtileri gözlenen farklı cinsiyetteki 0-2 yaş arasında bulunan toplam 92 sığırdan burun svabı örnekleri alındı. Bu örnekler virusa özgü primerlerin kullanılması ile RT-PCR testine tabi tutuldu. Ayrıca bu hayvanlardan serum numuneleri toplandı, BCoV'ye özgü antikorlar açısından indirekt-ELISA kullanılarak incelendi.

Bulgular: Svap örneklerinden 10 adedinin (% 10.87) BCoV yönünden RT-PCR metodu ile pozitif olduğu belirlendi. İndirekt ELISA kullanılarak yapılan serolojik araştırmada ise tüm hayvanlar arasında seropozitif hayvan oranı % 93.48 bulunurken burun akıntısı ile BCoV saçılımının gözlendiği tespit edilen hayvanlarda % 90 (9/10) olarak belirlendi.

Öneri: Bu araştırmada coronavirusa karşı aşı uygulaması yapılmamış hayvanlarda % 93.47 oranında seropozitiflik saptanmış olması yaşa bağlı olarak bazı hayvanların kolostrum ile bazı hayvanların ise hayatlarının belli bir döneminde BCoV'ye maruz kalmaları sonucu antikora sahip olduklarını göstermektedir. Fakat svap numunelerinde BCoV saptanan 10 adet hayvanın 9 tanesinde aynı zamanda BCoV'ye spesifik antikorların tespit edilmesi bu antikorların solunum yolu BCoV enfeksiyonlarına karşı korunmada oynadıkları rollerini şüpheye düşürmektedir. Sonuç olarak solunum yolu coronavirus enfeksiyonlarında antikorların rolünü ortaya koyacak daha ayrıntılı çalışmalara ihtiyaç olduğu kanısına varıldı.

Anahtar kelimeler: Respiratorik BCoV, RT-PCR, seroloji

Abstract

Aim: It was aimed to reveal the antigenic and serological prevalence of bovine coronavirus (BCoV) in cattle with clinically respiratory infection and to evaluate the relationship between virus shedding and serological status of animals in this study.

Materials and Methods: In the study, nasal swab samples were collected from a total of 92 cattle of different gender between 0 and 2 years of age with clinical respiratory symptoms. These samples were subjected to RT-PCR using specific primer pairs. Furthermore, serum samples were also collected from the same animals and evaluated for BCoV-specific antibodies through an indirect ELISA method.

Results: Ten (10.87%) swap samples were defined as positive for BCoV using RT-PCR. The seropositivity rate was 93.48% (86/92) in cattle by ELISA and 90% (9/10) in virus-shedding animals with nasal swab samples.

Conclusion: The fact that 93.47% seropositivity was detected in unvaccinated animals against coronavirus in this study indicates that the animals were exposed to BCoV through colostrum or at a certain period of their lives. However the fact that 9 out of 10 animals with BCoV detected in swab samples also have BCoV-specific antibodies casts doubt on the efficacy of these antibodies in protecting against respiratory BCoV infections. We concluded that more detailed studies are needed to reveal the role of antibodies in respiratory coronavirus infections.

Keywords: Respiratory BCoV, RT-PCR, serology

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Introduction

BCoV is an enteric and respiratory virus that affects the lower and upper respiratory tract and intestines in cattle. The results of BCoV infection, three different clinical symptoms occur in cattle as etiology: These are called winter dysentery with hemorrhagic diarrhea in adult cattle (WD), calf diarrhea (CD), shipping fever or bovine respiratory disease complex (BRDC) in cattle (Saif 2010).

Coronaviruses infect a wide variety of birds and mammals and cause a wide range of disease syndromes. The ability to easily cross species barriers and variable tissue tropism are well-known characteristics of certain CoVs. In the 21st century, outbreaks of Middle East respiratory virus (MERS-CoV), severe acute respiratory virus (SARS-CoV), and pandemic SARS-CoV-2 have demonstrated these characteristics of CoVs and increased global public health risks. BCoV and bovine-like CoVs have been detected not only in cattle but also in wild ruminants and various livestock species, dogs, cats and humans. There is also a historical case for zoonotic transmission of BCoV (Vlasova and Saif 2021, Soules et al., 2022).

Bovine CoV (BCoV) belongs to the species *Betacoronavirus* 1 of *Betacoronavirus* genus. . BCoV has an enveloped, single-stranded, positive polarity, non-segmented RNA (Bidokhti 2013).

BCoV is transmitted by mucosal secretion of the upper respiratory tract and gastrointestinal secretion (Bidokhti 2013). Some researchers have also detected low titers ocular shedding of BCoV in the herd (Saif et al 2010). Thomas et al (1982) were the first to identify BCoV as one of the contributing agents of calf pneumonia.

There are many methods used in the antigenic detection of BCoV. This virus is very difficult to isolate in cell culture. RT-PCR test and RT-RPA (reverse transcription recombinase polymerase amplification) are used for BCoV detection (Amer et al 2013). BCoV can be diagnosed using Immunohistochemical (IHC), immunofluorescence test (Dar et al 1998), and ELISA (Boileau et al 2010). For the detection of antibodies against BCoV in serum samples, VNT and HI were used to compare the amount of antibodies and antigenic variance between strains (Decaro et al 2008, Fulton et al 2013). Similarly, ELISA is used for collective monitoring of cattle populations in serological studies.

Material and Methods

Animals

Nasal swab and blood serum samples used in this study were obtained from 92 cattle with respiratory infection symptoms in the farms in Konya and Isparta region . A total of 92 animals sampled consisted of cattle of different gender between the ages of 0-2. Sampling was carried out between January 2017 and February 2018; and the age ranges of the sampled animals were divided into age groups as 0-30 days, 1-3 months, 3-6 months, 6-9 months and older than 9 months. The animals used in the research are grouped according to their gender which of 47% are male and 53% are female. In order to obtain statistically correct results, each swap sample was taken from different herds with respiratory system infections.

Swap samples

Sterile PBS (1 cc) was added to each of the nasal swab tubes. It was vortexed for one minute, kept in the refrigerator at +4 °C for 24 hours, and vortexed again for 1 minute. Viral RNA extraction processes were performed with the 'QIAmp cador pathogen mini kit' (QIAGEN, 54104, Germany). The cDNA Synthesis Kit' (BIO-RAD IScript, 170-8891, California) was used to obtain cDNA. The primer sequence used in RT-PCR is NOR 5' CTT AGT GGC ATC CTT GCC AA 3'; NOF 5' GCA ATC CAG TAG TAG AGC GT 3'. In PCR method; 740.5 µl MQ water, 150 µl 10 x Taq Buffer+KCl, 90 µl MgCl 2, 12 µl dNTP mix (3x4), 7.5 µl taq polymerase are prepared and thermal cycler (Bio-Rad T100, Singapore) was used. PCR steps (°C/min) were performed 30 cycles as 94 °C/3, 94 °C/1, 58 °C/2, 72 °C/1, 72 °C/7. For electrophoresis, 1% agarose (Sigma, Germany) was mixed in 0.5xTAE buffer. The agarose gel was placed in the electrophoresis tank (Serva Electrophoresis GmBH, Germany).

Serum samples

At least 5 ml blood samples were taken from the vena jugularis of each animal and centrifuged at 2000 *g* for 10 minutes. The presence of BCoV antibodies was investigated using a commercial indirect ELISA kit (SVANOVIR BCoV-Ab -Uppsala, Sweden).

Results

While 10 (10,87%) of 92 swab samples were positive for the presence of BCoV antigen, 82 (89.13%) were negative by conventional PCR (Table 1-2) . The most common clinical sign was fever in PCR positive animals (Table 2).

In terms of the presence of BCoV antibodies, 86 (93.48%) of the 92 blood serum samples were found to be positive for virus-specific antibodies by the commercial indirect ELISA kit (BCoV Ab-ELISA). It was determined that 6 of the samples (6.52%) were negative for BCoV antibodies (Table3).

Accordingly, a total of 10 samples were determined to be positive for BCoV by RT-PCR method. Six of 92 animals were

Table 1. PCR results by gender of animals

PCR	Gender		Total
	Males	Females	
BoCV +	2	8	10
BoCV-	41	41	82
Total	43	49	92

The result is not significant at p <0 .05.

Table 2. Age distribution and clinical signs of PCR positive animals

Age	Clinical signs				
	Fever+/Enterit+	Fever+/Enterit-	Fever-/Enterit+	Fever-/Enterit-	Total
0-30 days	2	1	-	-	3
30< - 90 days	-	1	1	1	3
90< -180 days	-	3	-	-	3
180 days<	-	1	-	-	1
Total	2	6	1	1	10

found to be seronegative for BCoV by BCoV-Ab ELISA kit. Swap samples of only one of these 6 animals were found to be positive for BCoV. It was determined that the serum samples of the other 9 animals, whose swap samples were positive for BCoV, were positive for the antibodies. Out of total 92 animals used in the study, 5 animals were both antigen and antibody negative, 77 animals were antigen negative-antibody positive, 1 animal was antigen positive-antibody negative and 9 animals were both antibody and antigen positive.

Discussion

In this present study, it was aimed to investigate the virological and serological BCoV infections in cattle, mostly 0-6 months old calves (82/92), showing signs of respiratory tract infection and to reveal the situation among the factors that cause respiratory infections in our country. N gene primers were used with RT-PCR in the study because they have a more stable structure compared to other genes of the virus. The American Center for Disease Control and Prevention (CDC) has reported that the N gene can be used in PCR studies for both monitoring and verification purposes in terms of the diagnosis of the virus.

Lin et al (2001) confirmed the field studies documenting subclinically infected animals and subclinical BCoV infections as a potential source of infection for BCoV, fecal and nasal shedding of virus in calves exposed to heterologous BCoV strains (detected only by RT-PCR). Calves with respiratory BCoV shedding at the initially of epizootic pneumonia had low levels of neutralizing and hemagglutination inhibition (HI) antibodies, while cattle with high levels of antibodies to S and HE viral glycoproteins were negative for respiratory BCoV. Only IgM antibody responses have been detected in animal with fatal respiratory BCoV infections. Intra-nasal (IN) route of administration of the BCoV calf diarrhea strain has been reported to induce cross protection against exposure to respiratory BCoV.

In the study, 9 of 10 animals with BCoV detection consisted of animals up to 6 months old. Only one of them was over 1 year old. The susceptibility to BCoV was not statistically significant (P<0.05) according to the gender of the PCR positive animals (Table 1). It is an interesting finding that the presence of specific BCoV antibodies in eight of the antigen-positive animals up to six months of age is detected. In a study conducted by Hasoksuz et al (2005), which had similar findings to our study, it was reported that serum antibody positivity was detected in a total of 7 animals with virus shedding in the stool, including 4 animals 0-30 days old, 2 animals 4-12 months old, and 1 animal over 2 years old. In the study conducted by Alkan et al (2011) in our country, the presence of neutralizing antibodies was also determined in 4 of 7 calves aged 1 to 2 months and a 3-year-old cattle showing enteric BCoV shedding. Both enteric-nasal BCoV shedding and 1/80 titer of neutralizing antibodies were detected in 2 of these animals (1-2 months old). It is generally thought that the presence of serum antibodies does not play an effective role in protection from enteric

Table 3. Results of ELISA for BCoV antibodies in serum samples obtained from animals

Test	Age				Total
	0-30 days	30< - 90 days	90< 180 days	180 days<	
ELISA +	10	30	36	10	86
ELISA -	-	3	3	-	6
Total	10	33	39	10	92

CoV infections. As a parallel finding, it was mentioned that low-titer maternal antibodies against BCoV in calves do not prevent viral replication in the respiratory tract (Saif, 1993). It has been suggested that low titers in immunoglobulin G (IgG) levels in animals are responsible for the immunological risks in terms of respiratory BCoV infection in cattle (Jee et al 2013). Another approach to the inadequacy of antibodies to protect against BoCV infections is the level of antigenic similarity between previously encountered local strains or strains used in vaccinations. It has been expressed that a single amino acid change (528A→V) in BCoV may cause resistance to neutralization (Temizkan and Alkan 2021, Yoo and Deregt 2001). As a matter of fact, the detection of specific antibodies in 9 out of 10 animals with BCoV antigen shedding in this study recalled the view that either the insufficiency of the protective antibody level or the antibodies developed against different strains as a result of previous infections could not provide protection against infection. The fact that 93.47% seropositivity was detected in animals that were not vaccinated against coronavirus in the study shows that animals are exposed to BCoV through colostrum or at a certain period of their lives. However, the presence of BCoV-specific antibodies in 9 out of 10 animals with BCoV detected in swab samples casts doubt on the efficacy of these antibodies in protecting against respiratory BCoV infections. In recent years, although there are researchers who have the opinion that BCoV contributes to the constitution of respiratory tract infections, especially in beef cattle, and that high serum anti-BCoV antibody titers can reduce the risk of infection (Workman et al 2019), comprehensive experimental studies have not yet been conducted to fully reveal the roles of serum antibodies in protection.

Appropriately with the serological findings in the study, Alkan et al (2002) reported that they detected the presence of BCoV-specific antibodies in the blood serum of 150 (16.3%) blood samples, in which they controlled blood samples from 919 adult cattle belonging to nine different farms for BCoV-specific antibodies with a microneutralization test. The presence of BCoV infection in adult cattle was determined serologically in all the controlled farms, and the seroprevalence values were determined between 4.4-100% with respect to the farms. Yavru et al (2016) examined blood serum samples collected from 184 calves with symptoms of diarrhea and their mothers in Burdur region by indirect ELISA for BCoV-specific antibodies, they determined that they found a high seropositivity rate of 93.99%. In addition, serological studies show that approximately 90% of cattle populations worldwide have antibodies against BCoV (Bok et al 2015).

Conclusion

In conclusion, the data obtained from this study suggested that BCoV may have an important place among the factors causing respiratory tract infections, which are frequently observed in cattle herds in our country. Studies can be aimed to reveal how BCoVs induce a wide spectrum of respiratory tract infections, ranging from mild clinical course to fatal cases, and to reveal the factors and interactions that increase the severity of the disease, virus shedding and transmission. However, more detailed studies are needed to clarify the role of antibodies in respiratory coronavirus infections and to better understand the role of BCoV in respiratory complex diseases (BRCD).

The lack of understanding of the basic features required for effective respiratory BCoV vaccines and their related links to protection are seen as obstacles to vaccine development. It is important to carry out more comprehensive research on the respiratory system throughout the country and to increase sequence analysis studies for BCoV. Especially there is a need for locally developed combine vaccine studies for both respiratory and enteric BCoV, together with nasal vaccine studies that can stimulate cellular immunity for BCoV.

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Conflict of Interest

The authors did not report any conflict of interest or financial support.

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Author Contributions

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Ethical Approval

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