Eurasian Journal
of Veterinary Sciences

www.eurasianjvetsci.org

RESEARCH ARTICLE

Comparison of reverse-transcriptase polymerase chain reaction (RT-PCR) and rapid test for the detection of bovine rotavirus and bovine coronavirus in anatolian water buffaloes

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Received:24.03.2020, Accepted: 15.06.2020
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Anadolu mandalarında bovine rotavirus ve bovine coronavirus tespitinde revers-transkriptaz polimeraz zincir reaksiyonu (RT-PCR) ve hızlı testin karşılaştırılması

Eurasian J Vet Sci, 2020, 36, 3, 159-165
DOI: 10.15312/EurasianJVetSci.2020.273

Öz

Amaç: Coronaviruslar ve Rotaviruslar, Türkiye'de ve Dünya'da hayvan ve insan sağlığını tehdit eden önemli virolojik etkenlerdir. Sığırlarda görülen Bovine Rotavirus (BRV) ve Bovine Coronavirus (BCoV) önemli ekonomik kayıplara yol açmaktadır. Bu çalışmada sığırlar ile aynı çiftlikte bulunan Anadolu mandalarında BRV ve BCoV varlığının teşhis edilmesi amaçlanmıştır. Bu amaçla iki virusun varlığı Revers Transkriptaz Polimeraz Zincir Reaksiyonu (RT-PCR) ve BRV-BCoV hızlı testi ile tespit edildi. Bu iki testin sensitivite ve spesifite oranları karşılaştırıldı.

Gereç ve Yöntem: Bu çalışmada Afyonkarahisar bölgesindeki sığır çiftliklerinde bulunan 230 adet Anadolu mandası klinik olarak değerlendirildi. Yirmi yedi adet klinik belirti (zayıflık, dehidrasyon, kusma, sulu kıvam ve sarı renkli dışkı) gösteren mandadan gaita numuneleri toplandı. Gaita numuneleri BRV ve BCoV yönünden Hızlı test ve RT-PCR ile değerlendirildi. Belirtilen analizler, kullanılan ticari kitlerin prosedürüne uygun olarak gerçekleştirildi.

Bulgular: RT-PCR sonucunda %22.2 (27/6) BRV ve % 3.7 (27/1) oranında BCoV pozitiflik tespit edilirken, Rota-Corona Hızlı testle tüm numuneler negatif belirlendi. Her iki etken için rapid testin RT-PCR ile karşılaştırıldığında sensitivite ve spesifitesi sırayla %0 ve %100 olarak belirlendi. Ayrıca analiz edilen örneklerde istatistiki olarak BRV pozitiflik oranı, BCoV'a göre daha önemli bulundu ($p<0,05$).

Öneri: Sonuç olarak rapid test sensitivitesinin düşük çıkması, enterik enfeksiyonların seyri boyunca saçılan virus miktarındaki değişikliğe bağlı olabilir.

Anahtar kelimeler: Anadolu mandası, bovine coronavirus, bovine rotavirus, BRV, BCoV hızlı test, RT-PCR

Abstract

Aim: Coronaviruses and Rotaviruses are important virologic factors for both animal and human health in Turkey and the world. Bovine Rotavirus (BRV) and Bovine Coronavirus (BCoV) in cattle cause significant economic losses. The aim of this study was to determine the presence of BRV and BCoV in Anatolian buffaloes which were on the same farms with cattle. For this purpose, presence of these two viruses were investigated by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) and BRV-BCoV Rapid tests and sensitivity and specificity ratios of these two tests were compared.

Materials and Methods: In this study, 230 Anatolian buffaloes were clinically evaluated in cattle farms in Afyonkarahisar region. Fecal samples were collected from 27 buffaloes which had clinical signs (weakness, dehydration, vomiting, watery consistency and yellow stool). The fecal samples were evaluated by Rapid Test and RT-PCR for Bovine Rotavirus and Bovine Coronavirus. The analyzes were performed according to the procedure of the commercial RT-PCR and rapid kits.

Results: The RT-PCR results were positive as 22.2% (6/27) for BRV and 3.7% (1/27 27/1) for BcoV while Rota-Corona Rapid test results were negative in all samples. When compared with RT-PCR results for both viruses, the rapid test sensitivity and specificity was determined as 0% and 100%, respectively. In addition, positive rates of BRV was statistically important as BCoV rate in analyzed samples ($p<0,05$).

Conclusion: In conclusion, low sensitivity of rapid test may be due to the change in the amount of virus scattered throughout the course of enteric infections.

Keywords: Anatolian water buffaloes, bovine coronavirus, bovine rotavirus, BRV, BCoV rapid test, RT-PCR

Introduction

Rotaviruses comprise a genus within the family *Reoviridae*. A rotavirus is a non-enveloped icosahedral virus with three protein layers that encapsidate 11 segments of the double-stranded (ds) RNA genome (Raming 2004). Different polymerase chain reaction (PCR) techniques (real-time PCR [qPCR], conventional PCR, nested PCR, and multiplex PCR) are used for diagnosis of Bovine Rotavirus (BRV) (Gentsch et al 1992, Min et al 2006).In animal models, rotaviruses have also been documented to spread beyond the intestine after oral infection (Raming 2004). Through the feces of the infected animals, a high level of viral particles (approximately 10^{11} particle/g) is shed into the surrounding area (Murphy et al 1999). Rotavirus diarrhea has been attributed to several different mechanisms, including malabsorption secondary to enterocyte destruction, virus-encoded toxin, stimulation of the enteric nervous system (ENS), and villus ischemia (Raming 2004). Reverse transcriptase (RT)-PCR is a method frequently used in recent years to provide the necessary products (amplicon) for molecular diagnostic and direct analytical studies using type-specific primers against different types of virus.

Bovine Coronavirus is a member of the family *Coronaviridae,* within the order Nidovirales(De Vries et al 1997). It is enveloped and possesses a single-stranded, non-segmented RNA genome of positive polarity (Belouzard et al 2012). Cattle usually become infected orally from feed and water contaminated with infected feces (Hasoksuz et al 2005, Gomez and Weese 2017). Severe infections can lead to diarrhea, dehydration, acidosis, hypoglycemia, and death due resulting from acute shock and cardiac insuffiency (Clark 1993).

The purposes of this study were to determine the presence of BRV and BCoV in Anatolian buffaloes in the Afyonkarahisar region by using RT-PCR and rapid test, and also to evaluate and compare the sensitivities and specificities of these two methods.

Material and Methods

Two-hundred thirty Anatolian buffaloes were clinically checked on cattle farms and the surrounding enviorment in the Afyonkarahisar province. A total of 27 stool specimens were collected from Anatolian buffaloes that showed clinical signs of disease (weakness, dehydration, vomiting, watery consistency and yellow stool). The samples were collected with sterile cotton swabs from the rectums of the animals. The fecal samples were suspended at a ratio of 1/10 in phosphate-buffered saline (PBS), which included 25,000 IU/ml penicillin and 20 mg/ml streptomycin. All samples were centrifuged at +4ºC, 3000 rpm for 15 min. All supernatants were transferred into a sterile tube and stored at –80ºC until RT-PCR assays were done.

RNA extraction and RT-PCR assays

The High Pure Viral RNA isolation kit (Roche. Cat. No: 11858874001, Mannheim, Germany) was used for RNA extraction.

BRV

Dimethyl sulfoxide (0.8 µl), BRV forward and reverse primers (Table 1) (0.6 µl End-9 and 0.6 µl S-Beg) , were added onto each of the 5 µl RNA extracts, and they were mixed with a straw in order to homogenize. The mixture was incubated at 94 ºC for 5 min and then kept on ice. Following incubation, 10 µl 5X Flexi Green Buffer, 5 µl MgCl2 (25 mM), 1 µl dNTP, 1 µl Primer F (20 pmol), 1 µl Primer R (20pmol) (Table 1), 0.5 µl AMV, 0.5 µl RNasin, 0.5 µl Taq Polymerase ve 23.5 µl ddH2O was treated with7 µl mixture (RNA and DMSO). The mixture was then amplified at 42 ºC for 60 min, 94ºC for 3 min, 35 cycles of 1 min at 95ºC, 2 min at 55ºC, 1 min at 72ºC and 10 min at 72ºC.

BCoV

For detection of BCoV RNA, cDNA samples were synthesized from the isolated viral RNA by using Reverse Transcription System synthesis kit (Promega A3500, USA). The cDNAs of the samples (3 µl) were treated with the Master Mix mixture, which included 5 µl 5X buffer green flexi color, 5 µl MgCl2 (25mM), 1 µl dNTP, 0.5 µl Primer F (50 pmol), 0.5 µl Primer R (50 pmol) (Table 2), 0.5 µl Taq polymerase and 34.5 µl ddH2O. It was amplified at 94 ºC for 3 min, at 94 ºC for 1 min, at 52 ºC for 2 min, and at 72ºC for 1 min for 35 cycles, and at 72ºC for 7 min.

Table 1. The primer sets for Rotavirus (Chang et al 1997, Hasoksuz et al 2008)

Primers	Primer Sequence	Gene	Product (bp)
S-Beg	5-GGC TTT AAA AGA GAG AAT TTC-3		
End-9	5-GGT CAC ATC ATA CAA TTC TAA TCT AAG-3	VP7	1062

Table 2. The primer sets for Coronavirus (Cho et al 2001)

Primers	Primer Sequence	Gene	Product (bp)
NOF	5-GCA ATC CAG TAG TAG AGC GT-3		
NOR	5-CTT AGT GGC ATC CTT GCC AA-3	N	730

Table 3. The RT-PCR and rapid tests results of BRV and BCoV in Anatolian water buffaloes

Analysis Method	BRV	BCoV
RT-PCR	6[a,*]	1[b]
Rapid Test	0	0

[a,b] : Different letters in the same line are statistically significant with chi-square test ($p<0.05$). *: RT-PCR test results of BRV is statistically different to rapid test with McNemar test ($p<0.05$). There is no statistically differences between RT-PCR and rapid tests for BCov.

Agarose gel electrophoresis

In order to display the amplification products, 1.5% agarose gel containing ethidium bromide was prepared. The PCR products were electrophoresed at 100 V for 30 to 45 min, and the amplified DNA bands were visualized under UV light.

Rapid diagnostic test

In this study, we used the BoviD-5 Ag Rapid Diagnosis kit (Bionote. Cat. No: RG13-02, Republic of Korea). We followed the manufacturer's protocol. In line with the procedure, first the swab contaminated with the feces was placed in the solution included in the kit during sampling and then homogenized. One drop of the solution was then added into the arrays according to the change of color. The presence of either BRV or BCoV was interpreted as positive or negative.

Ethical approval

All procedures and animal care were in compliance with the guidelines of the Selcuk University Veterinary Faculty Ethics Committee (Ethical approval number 2020/07 on 16/01/2020).

Statistical analyses

The values were statistically tested using SPSS 22.0 (SPSS, Inc., Chicago, IL, USA). Statistical significance between results of RT-PCR for BRV and BCoV were analyzed using the chi-square test. RT-PCR and rapid test results for BRV and BCoV were analyzed with the McNemar test . In all cases were found significant ($p<0.05$).

Results

As a result of the study, 6 (22.2%) samples from 27 fecal samples were observed to be as positive for BRV, whereas 1 (3.7%) fecal sample was positive for BCoV based on RT-PCR assays. The results of the study are presented in Table 3.

On the other hand, all samples were also examined with the BoviD-5 Ag Rapid Diagnosis kit, but no positive results were detected.

The cumulative results of the fecal samples found to be BRV and BCoV positive are presented in Table 3. According to the RT-PCR results, the rapid test sensitivity and specificity were determined as 0% and 100% respectively. In addition, positive rates of BRV was statistically higher than BCoV rate in analyzed samples ($p<0,05$). Also, there was a statistically significant difference between RT-PCR and rapid test results for BRV ($p<0,05$) but there was no differences between rapid test and RT-PCR results for BCoV ($p>0,05$, Table 3).

Discussion

In ruminants, the etiology of diarrhea involves many factors, such as bacteria (Escherichia coli, Salmonella spp., Clostridium perfringens, Campylobacter jejuni, Chlamydia spp.), viruses (rotavirus, coronavirus, adenovirus, parvovirus, astrovirus, calicivirus, bovine viral diarrhea [BVDV]), parasites (Cryptosporidium, Giardia), management, nutrition, and lack of enzymes (Baljer et al 1989, De La Fuente et al 1998, Gulliksen et al 2009). BRV and BcoV are frequently detected in ruminants with diarrhea (Abraham et al 1992, Athanassious et al 1994, Gulliksen et al 2009, Coura et al 2015). In newborn calves, diarrhea is usually caused by BRV and/or BcoV

that remain in the feces for a long time and the addition of new, contaminated animals to the herd (Garcia-Sanchez et al 1993, Gomez and Weese 2017).

In Turkey, Alkan (1998) reported 18% positivity for BCoV and 53% positivity of BRV in the fecal samples from 81 calves with diarrhea. Erdoğan et al (2003) investigated the frequency of BRV and BcoV in the Kars region and found 19% BRV and 1% BCoV antigen positivity with Enzyme-Linked Immunosorbent Assay (ELISA) test in a group of 104 calves with diarrhea. Hasoksuz et al (2005) detected BCoV in 13 (37.1%) calves from a total of 35 calves aged 1–30 days. Gumusova et al (2007) investigated antigen presence in the feces of 100 calves, regardless of diarrhea status, and detected 23% BRV and 1% BCoV antigen positivity in their study. Çabalar (2004) reported 17.97% BRV and 1.12% BCoV presence in 89 calves (aged 1–30 days) with diarrhea in the Van region. Duman and Aycan (2010) studied BRV frequency in the Konya regionby using an ELISA test. They tested fecal samples from diarrheal calves and detected 8.5% (9/106) as BRV positive. Yavru et al. (2016) 3500 cattle and their calves from 25 number of dairy farms 184 calves with diarrhoea and their dams (183) (≥2 - ≤6 age) were sampled for BCoV presence by ELISA. 172 (93.99%) cows and 172 (93.99%) their calves were found antibodies positive. The high levels of antibody for BCoV were detected as 36.05 % in dams 6 years and older ages. In the calves, antibody to BCoV were found at the highest level (25.26%) in the female calves ≥5 - ≤6 months ages. BCoV antigen was detected in only faecal sample of a (0.54%) calf. Uyunmaz Saklı et al (2019) detected 18 cases of BRV positive (18.75%) and 13 cases of BCoV positive (13.54%) in 96 cattle samples by the RT-PCR method in Turkey. BRV can be more widespread than BCoV in the clinically ill animals and it is compatible the results of current study.

For protection against BRV and BCoV infections, proper management and nutrition, hygiene, colostrum and milk supplementation of newborns, and vaccination programs on infected farms are important. Coura et al (2015) were carried out a study in Brazilian , which aimed to determine enteropathogenic agents in a dairy herd with both healthy and diarrheal calves.They reported that there was 68.6% positivity for BCoV based on the SN-PCR method and 49.2% positivity for Group A BRV based on the SS-PAGE method. Coura et al (2015) indicated that enteropathogenic agents causing diarrhea are more common in the first three weeks of a calf's life; thus, farmers and veterinarians must consider biosecurity, immunity, better management, and animal welfare in order to minimize the number of diarrheal calves. Al Mawly et al (2015) investigated the enteropathogenic risk factors in New Zealand dairy herds (n=97) using liquid, half liquid, and hard fecal samples from 1283 cows and reported that the most common agents in calves aged 9–21 days were C. parvum, BRV, BCoV, or mixed infections. They also pointed out that animals in open pens tended to have a higher ratio of liquid defecation compared to those housed in the barns. They also concluded that lack of vaccinations, mastitis, antibiotic use, straw bedding, and gender (females) are important factors contributing to decrease in the ratio of liquid defecation.

The dams of the calves around the Afyonkarahisar region of Turkey are usually vaccinated with commercial vaccines against BRV/BCoV. However, the diarrheal calves were located in the same areas with healthy calves, and as a result, farmers usually complain about the spread of diarrhea to the healthy calves and the death of those calves. The farmers often rely on the symptomatic treatments of diarrhea in order to save the herb but tend to avoid the vaccination of pregnant cows with the prejudice that the vaccine would be harmful to the fetus during pregnancy. Holstein breed cows and calves were also present, and they were in close contact with the water buffaloes on the farms which samples were collected. Holstein cattle also had symptoms of diarrhea and other gastrointestinal problems in sampled farms. However, in this study presence of BRV and BCoV were not investigated in Holstein cattle.

In order to protect the newborns from the BRV and BCoV infections, vaccinations of cow is crucially important. Previous studies reported that following the vaccination of a cow, there is an increase in the serum antibody titer and a decrease in the risk of contracting the disease for the calves born from these cows (Snodgrass 1982, Castrucci et al 1984, Kohara et al 1997, Kohara and Tsunemitsu 2000). Moreover, the severity, duration, and outcome of the disease in calves are improved with the use of vaccinations. Kohara et al (1997) determined that cows vaccinated in Europe with a commonly used commercial vaccine containing BRV, BCoV, BPV, and K99 E. Coli had significantly increased serum titer levels compared to nonvaccinated cows, and also had much higher serum antibody titers even after 3 to 4 weeks after birth than calves born to the nonvaccinated cows. In the present study, the animals sampled were still young, and the water buffaloes had already been vaccinated against BRV and BCoV. Despite this, it is remarkable that six animals were found to have BRV, and one animal had BCoV. Alkan et al (2004), however, reported that the ratios of the calves to be exposed to the disease were 30% and 54.5% regarding the vaccinated and nonvaccinated cows, respectively. The 30% ratio is important considering the fact that the cows were vaccinated, and it could be suggested that these animals shared areas with the Holstein cattle, which could have led to infections caused by different types of the virus. However, the researcher was not determined the viral types. Lu et al (1994) reported that some differences in the P-serotype of BRV caused cross-reactions, and when the cows were vaccinated with different P-serotypes, their calves could not have enough protection from the maternal sources. Lu et al (1994) explained that even though the BRV Lincoln strain

(JG6: P1)-containing vaccines had been used for 20 years in the United States, BRV infection was still present in the newborns possibly due to vaccine and field strain differences. Alkan et al (2010) collected samples from the different parts of Turkey for genotyping with RT-PCR, and it was determined that the ratios of G6 and P11 genotypes were 75% and 98%, respectively. Therefore, the efficiency of commercial vaccines used in our country must be determined with respect to vaccine and field strain differences.

In this study, specific primers for VP7 gene of Group A BRV were used for the molecular diagnosis of BRV and BCoV in the diarrheal fecal samples. In the BRV positive samples, group A cattle rotavirus was detected. The frequencies of G and P-type rotavirus is expected to be high in the diarrheal calves around the Afyonkarahisar region; however,in this study sequence analysis was not carried out to determine the type of BRV. In future studies it is important to include the genotyping of G and P genes in the rotavirus-positive samples in order to prevent the antigenic differences in the vaccination programs in Turkey

Currently, molecular techniques, such as RT-PCR and qRT-PCR, have almost replaced the other diagnostic methods in terms of sensitivity and specificity (Slovis et al 2014). PCR is used to detect viral nucleic acids by increasing their amounts to quantifiable levels (Matson et al 1990). RT-PCR is a method that employs specific primers for different types of the virus for the molecular diagnosis and provides amplicons for sequence analysis. (Gentsch et al 1992, Min et al 2006). Alkan et al (2010) collected samples from different parts of Turkey to be used for genotyping with RT-PCR and determined that the ratios of G6 and P11 genotypes were 75% and 98%, respectively. Izzo et al (2012) compared RT-PCR, ELISA, and immunochromatographic tests for their efficiencies in BRV detection and found 79%, 38%, and 35% positivity, respectively. They explained the discrepancy among these tests with the timing of sampling as the samples were collected at the advanced stage of the disease when viral spread and particle levels were low. The working idea behind the fast immunochromatographic test is to detect the virus by dropping the sample on a strip that is conjugated with a specific antibody. Therefore, it is appropriate to use this test when the virus spread is at the highest level. Since the virus discharge decreases by time, it is recommended to collect samples within the 72 h after the beginning of the disease. However, the PCR technique can detect the virus at very low levels. When the virus level is low, rapid detection diagnostic kits have limited efficiency compared to RT-PCR. In this study, 230 water buffalo were clinically examined for BRV and BCoV infections based on signs characterized by weakness, dehydration, vomiting, watery and yellow-colored feces. As the amount of the virus decreases at the later stages of the disease, the diagnosis may carry some risks and the disease can not be appropriately identified. Another important issue with the disease is the presence of subclinical cases that appear to be clinically healthy. This is a real risk that no veterinarian would like to take, especially with respect to large size farms. Therefore, molecular techniques, such as RT-PCR, may offer a permanent solution for diagnosis such cases (Cho et al 2012) It should be emphasized that accurately selected primers and probes would allow for the detection of BRV and BCoV by RT-PCR with high sensitivity and specificity.

Compared with RT-PCR, Klein et al 2009 found the sensitivity of the rapid test kit for BRV as 71.9% and the specificity for the same as 95.3%. In addition, comparing the commercial rapid test kits with the PCR method, the sensitivity of the rapid test kits has determined as 60% for BCoV, and 42.3% for BRV (Cho et al 2012). In this study, all samples were also examined by rapid test kits, but no positive results were detected. In recent years, thanks to rapid immunochromatographic tests, which are more advantageous under filed conditions, it has become possible to diagnose different enteropathogens in the feces of calves in short time periods, such as 10 to 15 min (Izzo et al 2012). These rapid test kits are important in terms of determining the treatment process and avoiding wrong antibiotic use, but the fact is that the use of rapid detection test kits is unfortunately behind the desired levels.

Conclusion

The sampled Anatolian water buffaloes were together on the same farm with Holstein cows. They were fed together with cows from the same feed sources and were interested by the same animal careers and veterinarians. In conclusion, detection of BRV and BCoV in the water buffaloes that were observed in the dairy cows with diarrhea, it can be speculated that the viral transmission occurred between water buffaloes and diseased cattle by housing and feeding.

Conflict of Interest

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

Funding

During this study, any pharmaceutical company which has a direct connection with the research subject, a company that provides and / or manufactures medical instruments, equipment and materials or any commercial company may have a negative impact on the decision to be made during the evaluation process of the study. or no moral support.

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Ethical Approval

Ethics committee approval (No: 2020/07) was obtained by Selcuk University Veterinary Faculty Experimental Animals Production and Research Center Ethics Committee (SÜVDAMEK)

CITE THIS ARTICLE: *Bulut O, Uyanmaz Saklı G, Hasoksuz M, Dik I, et al., 2020. Comparison of rapid test of bovine rotavirus and bovine coronavirus in anatolian water buffaloes with polymerase chain reaction. Eurasian J Vet Sci, 36, 3, 159-165.*