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Investigation of bovine coronavirus and bovine rotavirus by rapid diagnosis kit and RT-PCR in diarrheic calf feces*

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ABSTRACT

This study has investigated bovine coronavirus (BCoV) and bovine rotavirus (BRV), which are among the most important causes of diarrhea in calves leading to financial losses in Turkey and all over the world BCoV and BRV were detected by Reverse Transcriptase Polymerase Chain Reaction (RT-PCR), which is one of the most reliable method of diagnosis, The results obtained by RT-PCR were compared to the sensitivity of the commercial Rota-Corona Rapid Test Kits used by clinical veterinarians in fields. In this study, 96 fecal samples were examined from diarrheic calves in cattle farms in the cities of Konya and Afyon for BRV and BCoV firstly by BoviD-5 Ag rapid test kit, and then we applied the RT-PCR test. A comparison of the rapid test kit with the RT-PCR in terms of sensitivity and specificity revealed the 83% sensitivity and 100% specificity of the BRV and 7.6% sensitivity and 100% specificity of BCoV. In conclusion the practical and rapid diagnosis of the disease using of Rapid Diagnosis kit used by the clinician veterinarians may be useful, but the results must be interpreted with caution since the sensitivity of the test decreases due to the reduction in the number of viruses in the later stages of the infection.

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Introduction

Neonatal calf diarrhea caused by viral, bacterial and protozoon agents is one of the infections characterized by enteritis leading to weight loss and deaths in calves under one month of age (Murphy et al.,1999). Neonatal calf diarrhea is among the most important reasons for financial losses in the meat and milk industry all over the world (Boileau et al.,2010). Although its causes show variations depending on the regional and stable conditions, the role of rotavirus and coronavirus in the cases of calf diarrhea have been found to reach up to 50% and 80% respectively. Rotaviruses generally cause infections characterized by diarrhea in dairy calves (Al Mawly et al., 2015) and beef calves (Cho et al., 2013) up to 9-21 days old. Bovine group A rotavirus, bovine coronavirus, enterotoxigenic K99+ Escherichia coli (K99), Cryptosporidium parvum and Salmonella spp. are reported to be the most common enteric infection

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agents (Bartels et al., 2010; Izzo et al., 2012). Among the factors that affect the course of rotavirus and coronavirus infections are whether the newborns received colostrum, time of weaning, climate conditions, their immune conditions, and other present enteropathogenic agents. The main mode of transmission of rotaviruses is the fecal-oral route. Through the feces of the infected animals, a high level of viral particles (approximately 1011 particle/g) is shed around. This shedding reaches the highest level on the third and fourth days, and the virus can survive in the feces for several months (Murphy et al., 1999). Virus isolation (Mebus et al., 1969; Hasoksuz et al.,2002; Gulyaz et al., 2005), immunochromatographic rapid diagnostic assays (Al-Yousif et al., 2001; Uhde et al., 2008; Klein et al., 2009; Bartels et al., 2010; Altug et al., 2013), IEM (Immunelektron microscopy) (Saif et al., 1980), ELISA (Alkan, 1998; Murphy et al., 1999; Gulyaz et al., 2010) and RT-PCR (Cho et al., 2001; Hasoksuz et al., 2002; Aich et al., 2007; Decaro et al., 2008; Asano et al., 2010; Bok et al., 2015) are among the most preferred methods to diagnose rotavirus and coronavirus infections.

Materials and Methods

Specimens: The samples were collected from calves (1 to 30 days old) with acute diarrhea cases in stables in Konya and Afyon. Total of 96 fecal samples were collected for this study. The age distribution of the collected samples is presented in Figure 1. The fecal samples were collected from the rectums of the animals with sterile cotton swabs. One swab taken out of the rectum was put in the solution in the rapid test kit, and another was homogenized by putting it into Phosphate Buffer Saline (PBS), and stored at -20ºC until it was used for RT-PCR. The samples for the rapid test kits were examined under stable conditions, and their results were reported to the owners in 15 minutes. Necessary notes were taken and the positive samples were taken into account for the next examination.

Rapid Diagnostic Test: In this study, we used the Bionote BoviD-5 Ag (Cat. No: RG13-02) rapid diagnosis kit. We followed the test procedure of the producer. In line with the procedure, firstly the swab contaminated with the feces was placed in the solution included in the kit during the sampling and was homogenized. Then one drop of the solution was added onto the arrays and according to the change of color, coronavirus or rotavirus was interpreted as positive or negative.

RT- PCR Materials: Extraction of the Viral RNA: We used High Pure Viral RNA isolation kit of Roche (Roche, Cat. No: 11858874001). The fecal samples were suspended at a rate of 1/10 in PBS including 25000 U/ml Penicillin and 20 mg/ml Streptomycin, centrifuged at +4oC, 3000 rpm for 15 minutes, and then supernatant was transferred into a sterile tube. Following the centrifugation 200 µl of the supernatant was taken and transferred into a 1.5 ml RNase-free sterile tube. Each sample was mixed with working solution containing 4 µl Poly A and 400 µl Binding Buffer. The working solution and the sample mixture were treated with the Removal Buffer, Wash Buffer, Elution Buffer included in the kit by using the silica gel spin column. At the end of the extraction process, 50 µl of viral nucleic acid was isolated, and stored at -80^{o}C.

cDNA Synthesis: We used Reverse Transcription System (Promega A3500) to obtain cDNAs. In order to synthesize cDNA from the isolated viral RNA, we used the Promega Reverse Transcription System synthesis kit (Promega A3500), and followed the recommended protocols. 5 µl of the isolated viral RNA was transferred into the PCR tubes and was incubated at 70ºC for 10 minutes. Following the incubation, it was kept in ice for 2 minutes. Master Mix (15 µl for each sample) was prepared in a different PCR tube and 15 µl of Master Mix was added onto each of the 5 µl RNA samples.

Viral RNA and Master Mix mixture totaling to 20 µl was put into the Thermal Cycler and amplified at 22ºC for 10 minutes, at 42 ^{o}C for 15 minutes, at 95 ^{o}C for 5 minutes at 4^{o}C for 5 minutes, and then the cDNA was synthesized. The resulting products were stored at -20^{o}C.

Polymerase Chain Reaction (PCR): We used Promega Go Taq Flexi DNA Polymerase (Promega M8305) To detect the presence of bovine rotavirus in the fecal samples of the calves with the one-step RT-PCR method. The primers reported by Hasoksuz et al., (2008) and Chang et al., (1997) were used. These primers are specific to the VP7 gene region of the group A rotaviruses. We used the primers reported by Cho et al., (2013) for the detection of bovine coronavirus. These primers are specific to the N protein gene of the virus. We used S-Beg5-GGC TTT AAA AGA GAG AAT TTC-3, End-9, 5-GGT CAC ATC ATA CAA TTC TAA TCT AAG-3 primers of 1062 bp for bovine rotavirus and NOF-5-GCA ATC CAG TAG TAG AGC GT-3, NOR-5-CTT AGT GGC ATC CTT GCC AA-3 primers of 730 bp for bovine coronavirus.

BRV: We used the one-step RT-PCR method for the viral RNAs obtained. 0.8 µl DMSO, 0.6 µl End-9 of the rotavirus primers and 0.6 µl S-Beg were added onto each of the 5 µl RNA products, and they were mixed with a straw to homogenize. The mixture was incubated at 94°C for 5 minutes, and then kept in ice. Following the incubation, 43 µl Master Mix composed of Primer F (20 pmol), Primer R (20 pmol), and solutions of the Promega M8305 kit and the Promega A3500 cDNA kit was treated with 7 µl RNA and DMSO mixture. It was amplified at 42°C for 60 minutes, at 94°C for 3 minutes, (at 95°C for 1 minute, at 55°C for 2 minutes, at 72°C for 1 minute at 35 cycles), and at 72°C for 10 minutes.

BCoV: cDNAs of the samples were treated with the Master Mix mixture of the Promega M8305 kit including Primer F (50 pmol) and Primer R (50 pmol) and, for the PCR reaction, Go Taq Flexi DNA Polymerase, 5X buffer green flexi color, MgCl2 (25 mM) and dNTP. It was amplified at 94°C for 3 minutes (at 94°C for 1 minute, at 52ºC for 2 minutes and at 72°C for 1 minute at 35 cycles), at 72°C for 7 minutes.

To display the amplification products, 1.5% agarose gel containing ethidium bromide was prepared. The PCR products were run at 100 V for 30-45 minutes and the amplified DNA bands were controlled under UV light.

Statistical analysis: We used the chi-square test ($\chi 2$) for the statistical analysis of the diagnostic tests. We recorded $p<0.05$ as statistically significant.

Results

We examined 96 diarrheic fecal samples in total for BCoV and BRV by rapid diagnostic test and RT-PCR method. The collective results of the study are presented in Table 1. According to the results, the

Table 1: Number of positive samples identified by the rapid test kit and RT-PCR

Pathogen	Rapid Test Kit	RT-PCR
BRV (Group A)	15/96 (15.62%)	18/96 (18.75%)
BCoV	1/96 (1.04%)	13/96 (13.54%)
BRV (Group A)- BCoV	-	4/96 (4.16%)

rapid diagnostic test revealed 15 samples as BRV positive (15.62%) and 1 sample as BCoV positive (1.04%). The RT-PCR method detected 18 cases of BRV presence (18.75%), 13 cases of BCoV presence (13.54%), and 4 cases of both BRV and BCoV presence (4.16%). The electrophoresis images are presented in Figure 2 and Figure 3. According to these results, four samples were found to be both BRV and BCoV positive by RT-PCR method. However, only one sample was found to be BRV and BCoV positive with the rapid diagnostic test.

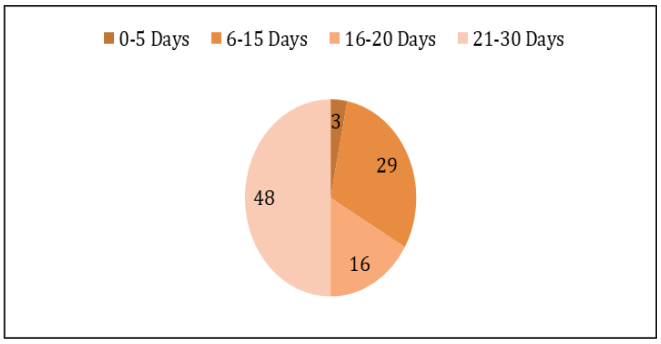

Figure 1: Distribution of the samples of calve feces by age (days)

This sample showed a quite strong DNA band appearance after the agarose gel electrophoresis using RT-PCR. (Figure 3). For the 0-5, 6-15, 16-20 and 21-30 days old calves found to be BRV positive by the rapid test kit, the rates of positivity were 67%, 24%, 0% and 13% respectively. Only 4% of the 6-15 days old calves were found to be BCoV positive. For the 0-5, 6-15, 16-20 and 21-30 days old calves found to be BRV positive by the RT-PCR method, the rates of positivity were 67%, 31%, 6% and 13% respectively. In terms of BCoV, while RT-PCR found no positive samples in the 0 -5 days old group, the other groups found to be 34%, 13% and 2% positive respectively (Figure 4).

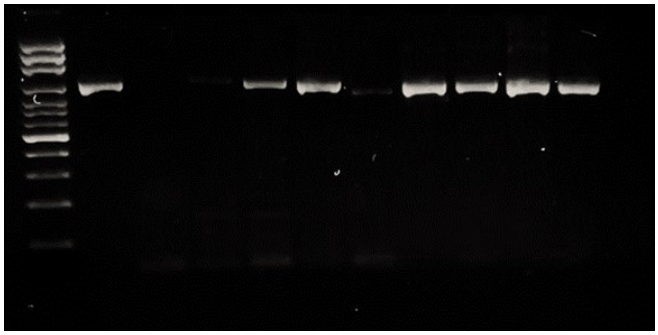

Figure 2: Electrophoresis image of the BRV (1062 bp) positive samples, DNA Ladder (100 bp Fermentas), PC (Positive Control), NC (Negative Control), DNA bands of the samples; 27, 28, 12, 13, 14, 15, 16 and 17

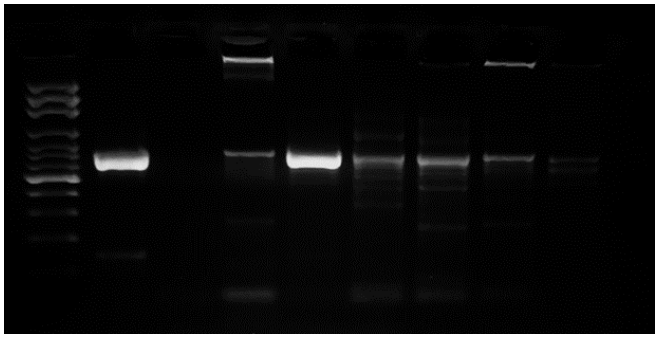

Figure 3: Electrophoresis image of the BCoV (730 bp) positive samples; DNA Ladder (100 bp Fermentas), PC (Positive Control), NC (Negative Control), DNA band of the samples 16, 17, 19, 22, 23 and 25

Table 2. Sensitivity, Specificity, PPV and NPV rates of the Rapid Test Kit versus RT-PCR in terms of BRV/BCoV

Reference Test	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
BRV Rapid Test BRV RT-PCR	83	100	100	96
BCoV Rapid Test BCoV RT-PCR	7.6	100	100	88

PPV: Positive Predictive Value, NPV: Negative Predictive Value

Discussion

Rapid and accurate diagnosis of BRV and BCoV infection is important for the control and eradication of the disease in newborn animals in cattle farms in many developed and developing countries. Therefore, it is important to diagnose BRV and BCoV rapidly in the field, and to detect it through rapid and effective test techniques in veterinary diagnostic laboratories. Among these methods, isolating the RNA of the virus and converting it into DNA (cDNA) and multiplying the cDNAs by using specific primers (RT-PCR) has the highest sensitivity and originality. However, as these techniques can only be applied under laboratory conditions and require time, clinical veterinarians need rapid test kits to diagnose the infection under field conditions. These rapid test kits are important in terms of determining the treatment process and avoiding wrong antibiotic use, but the use of rapid test kits is unfortunately behind the desired levels.

Table 3. Statistical comparison of the positive results by the tests applied

	Rapid Test Kit (n)	RT-PCR (n)
BRV Positive	15/96[a]	18/96[a]
BCoV Positive	1/96[b]	13/96[a]
BRV-BCoV Positive	0/96[b]	4/96[b]

a, b: Different letters within the same column are statistically different. ($p<0.05$) a is statistically higher than b.

The most important reason behind this is the righteous suspicion about the sensitivity and specificity of these rapid test kits. In recent years, the rapid immunochromatographic tests, which are more advantageous under filed conditions, it has become possible to diagnose different enteropathogens in the feces of calves in approximately such short time periods as 10 to 15 minutes, and to plan prophylaxis and treatment (Klein et al., 2009).

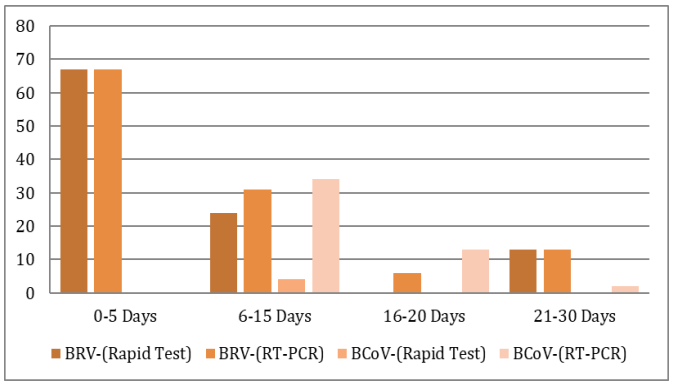

Figure 4. Percentage distribution of the BRV and BCoV positive calves by age (days)

Many investigators have reported that the immunochromatographic rapid test kits are a simple and easy-to-apply method for the diagnosis of enteropathogens in feces, and that they may be preferred by clinical veterinarians and investigators more often as they do not require specialist and fully-equipped laboratory, are cheap and rapid in comparison to other techniques, and can be applied under any laboratory or office conditions which can be found in each private clinic and even under field conditions (Thorns et al.,1992; De la Fuente et al., 2009; Klein et al., 2009). Klein et al. (2009) examined the fecal samples collected from 1 day to 42 days old 180 calves (98 of them had the symptoms of diarrhea) both with immunochromatographic rapid test kit and RT-PCR method. Compared to RT-PCR, the investigators (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%, and its sensitivity as 60% for BCoV and its specificity as 96.4% for the same. In their study comparing the commercial rapid test kits with the multiplex PCR method, Cho et al. (2012) found the sensitivity of the rapid test kits as 60% for BCoV, and 42.3% for BRV, and they, therefore, stated that the rapid test kits had to be interpreted carefully in terms of originality and sensitivity. In a study

In a study comparing real-time RT-PCR, ELISA and immunochromatographic tests, Izzo et al. (2012) found the sensitivity of the rapid test kit as 32.7% for BRV and as 28.2% for BCoV in comparison to RT-PCR technique. The investigators reported that the sensitivity and specificity levels of the immunochromatographic rapid test kits were very low in comparison to real-time PCR, and that it was possible to interpret the course of the disease at the clinic since the viral RNA amount is known due to real-time PCR method. In their study on the rapid etiological diagnosis of neonatal calf diarrhea by immunochromatographic test kits, Altug et al. (2013) reported 14 cases of BRV (27.5%) and 1 case of BCoV (1.96%) among the samples from 51 diarrheic calves. In this study, among the samples examined by rapid test kit, we found 15.6% (15/96) to be BRV-positive and 1.04% (1/96) to be BCoV-positive. The same samples were tested using RT-PCR method and the positivity rates for BRV and BCoV were found 18.75% (18/96) and 13.5% (13/96), respectively. A combination of BRV and BCoV infections was detected in 4% of the diarrheic feces (4/96).The results obtained in this study were found to be compatible to those of Altug et al. (2013). Besides, in comparison to RT-PCR technique, the sensitivity of the immunochromatographic rapid test kits for BRV was 83% and the specificity of the same was 100%, its sensitivity for BCoV was 7.6% and specificity for the same was 100%. The results for bovine rotaviruses were found to be compatible with those of (Klein et al., 2009), who have previously contrasted the immunochromatographic rapid test kits to RT-PCR in terms of sensitivity and specificity, while they were determined to be higher than those of Cho et al. (2012) and Izzo et al. (2012). In terms of bovine coronavirus, our results were significantly lower than those of many other investigators (Klein et al. 2009; Cho et al., 2012; Izzo et al., 2012) who have studied the same subject matter. The possible reason for this might be the fact that the sampling is carried out in the late course of the disease when the level of virus shedding and the amount of viral particles are low. The rapid immunochromatographic diagnostic method is based on the attachment by the agent within the sample dropped on the test stripe to the conjugated specific antibodies. Therefore, it is essential to carry out the sampling during the peak time of virus shedding. It is necessary to collect the samples within 72 hours after the onset of the disease, because virus shedding decreases in time. However, it is possible to detect even very low levels of viruses by the RT-PCR method. The diagnostic ability of the rapid test kit can be inferior to that of RT -PCR in samples containing small amount of virus. “In this study, we identified both BRV and BCoV by RT-PCR in four samples. Only one sample was found positive in terms of BoCV using the rapid test kit. The electrophoresis images from the RT-PCR diagnosis of this positive sample presented/showed a stronger DNA stripe image in comparison to the other positive samples. This indicates that the rapid test kit determines positive results if there is high amount of coronavirus in the fecal samples. Therefore, it is necessary to support the results with a lot of samples. Examining the age ranges of the calves and the infection-positive results by RT-PCR for these age ranges, we see that the highest level of BRV-positivity was found as 67% in calves of 0-5 days of age. This rate was identified as 31% in the 6-15 days age group, 6% in the 16-20 days age group and 13% in the 21-30 days age group. In our investigations by RT-PCR for BCoV, we identified no positivity in the 0-5 days age group, but 34% in the 6-15 days age group, 13% in the 16-20 days age group, and 2% in the 21-30 days age group (Figure 4). While the rates identified for BRV by this study are close to those reported by Al Mawly et al. (2015) (20% in calves of 1-5 days of age, and 19% in calves of 9-21 days of age), but in terms of BCoV, the results of Al Mawly et al. (2015) (5.4% in calves of 1-5 days of age, 6.1% in calves of 9-21 days of age) are lower than those of this study. Alkan (1998) has pointed out that this situation can be associated with the colostrum that calves receive from their mothers. Alkan (1998) has reported that one of the most important factors affecting the average infection age is maternal immunity. In this study, we know that the calves from which we collected the samples had generally received colostrum from their mothers. Ellens et al., (1978) and Wood et al., (1975) have reported that there were no rotavirus specific antibodies in the second week after birth, but antibodies specific to coronavirus reached significantly high levels in the third week. Contemplating on the fact that the coronavirus antibodies are secreted for a long time in milk, Wellemans and Van Opdenbosch (1981) have explained it with the fact that mothers were considerably infected with coronavirus during the diarrheic periods, their immune systems were stimulated as they shed the virus through their feces on the day of giving birth, inducing the mammary gland to secrete Ig antibody. Therefore, the total rate of BRV-positivity in the first two weeks (0-15 days) in this study is 34% while it decreases to 6% and 13% in

the third and fourth weeks, respectively. With regards to BCoV, the positivity rate in the first 3 weeks is 26% while it decreases to as low as 2% in the fourth week. The distribution of positivity by the age groups identified in this study was found to support the ideas of Ellens et al. (1978), Wood et al. (1975) and Wellemans and Van Opdenbosch (1981). This fact shows that in this study colostrum received from the mothers of calves stimulated the maternal immunity, and affected the positivity rate by age specified in the study. Although this study identifies a low level of sensitivity for immunochromatographic rapid test kits, one might think that the most important advantage of these kits for clinical veterinarians with regard to BRV diagnosis is to avoid wrong treatment with antibiotics. Nevertheless, as the amount of virus decreases in the late course of the disease, one must not ignore the fact that it is not a very effective method. This might lead to an inaccurate interpretation of the disease. The presence of subclinical carrier animals is another important issue to bear in mind while evaluating the disease. This is a point a good veterinarian would not like to ignore while assessing the cases of diarrhea in calves posing a problem especially in big farms. As a permanent solution, molecular methods such as RT-PCR play an essential role in identification of these animals. İt is possible to identify even one virus particle in the feces by RT-PCR (Klein et al. 2009). Thus, veterinarians will be able to interpret the disease accurately, and to take such protective measures as vaccination. It is of paramount importance to identify the field strains of infections such as bovine rotavirus and bovine coronavirus present in Turkey. Apart from the group A rotavirus identified in this study, identifying the G and P type rotavirus strains present in Turkey is of significant importance for the effectiveness of vaccinations. The results of this study indicates that the rapid test kits used by veterinarians under field conditions to diagnose the diseases quickly can be beneficial, but a careful interpretation is advisable since the sensitivity of the rapid test kits has been found to be low (especially for BCoV) in comparison to RT-PCR. In order to be able to interpret diseases more effectively and to seek more permanent solutions, it is advisable to support the results through molecular techniques such as RT-PCR.

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