

# **A survey on detection of coronavirus in neonatal calf diarrhea in dairy farms of Iran**

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## **Abstract:**

**BACKGROUND:** Bovine coronavirus (BCoV) is a primary cause of neonatal calf diarrhea worldwide, and is also associated with acute diarrhea in adult cattle during the winter season, resulting in heavy economic losses to both dairy and beef industry throughout the world. **OBJECTIVES:** The objective of the present study was to screen the fecal samples for BCoV collected from diarrhea from six geographic regions of Iran, with the aim of deepening the knowledge of BCoV prevalence and epidemiology in Iran. **METHODS:** 194 fecal samples from diarrheic calves up to one-month of age, based on the geographic area were collected. Samples from all the cases were screened for the presence of BCoV by commercially available ELISA kit. Furthermore, all samples were subjected to RT-PCR for confirmation. **RESULTS:** ELISA examination revealed that 7.2 % of taken samples were positive. All positive samples in ELISA were also positive in RT-PCR. All samples from northwest, northeast, and west, were negative. The average age of positive calves was nine days. The average stool scores in positive samples and negative samples were 2.5 and 2.1 respectively. 71/4 % of positive samples had fever. **CONCLUSIONS:** The results of the present study showed that the occurrence of BCoV in stool samples of diarrheic calves in dairy farms of Iran is lower than the other reports in the world.

## **Introduction**

Bovine coronavirus (BCoV) is an important livestock pathogen with a high prevalence worldwide. The virus causes diarrhea and respiratory disease in neonatal calves and winter dysentery in adult cattle. These diseases result in substantial economic losses and reduced animal welfare (Boileau and Kapil, 2010; Oma et al., 2016). It has been found that in beef calves, BCoV infection was more frequent among calves up to 30

days of age (Quinn et al., 2002; Afshari et al., 2012).

Serological surveys indicate that approximately 90% of the worldwide cattle population has antibodies (Abs) against BCoV (Boka et al., 2015). A number of researchers showed wild ruminants, dogs, and horses harbor similar strains of BCoV which is transmissible to cattle and vice versa (Barros et al., 2013; Saif et al., 2010).

Inter-herd transmission is possible either directly via import of live animals (Decaro

et al., 2008, Fulton et al., 2011), or indirectly via contaminated personnel or equipment (Mee et al., 2012). Measures to prevent the virus spread between herds must be based upon knowledge of viral shedding, the potential for transmission to susceptible animals and the role of protective immunity (Oma et al., 2016). In two experimental studies, infected calves were not protected against reinfection with a different BCoV strain three weeks after the first challenge, but did not develop clinical signs (Cho KO et al., 2011; El-Kanawati et al., 1996). BCoV is transmitted via the fecal-oral or respiratory route. The virus infects epithelial cells of the respiratory tract (the nasal turbinates, trachea and lungs) and the intestines (the villi and crypts of the small and large intestine) (Park et al., 2007, Saif et al., 1986).

Three antigenic groups of coronaviruses have been established, and all BCoV strains belonged to the subgroup initially designated as 2a (Hasoksuz et al., 2008). The International Committee for Taxonomy Viruses (ICTV) has proposed a revision of the family Coronaviridae to create a new subfamily, Coronavirinae, that includes the Alpha, Beta and Gammacoronavirus genera. Following this new suggested taxonomy, BCoV belongs to the Betacoronavirus genus, cluster within the Coronavirinae subfamily, Coronaviridae family and the order Nidovirales (<http://ictvonline.org/virusTaxonomy.asp>).

The virus genome is comprised of single stranded nonsegmented positive-sense RNA (32 kb) associated to the nucleoprotein (N) and forming a nucleocapsid with helical symmetry (Clark, 1993). Viral particles are large (100-150 nm), pleomorphic and enveloped with four major structural proteins comprising a membrane (M) gly-

coprotein, an envelope (E) protein, a spike (S) glycoprotein and the hemagglutinin-esterase (HE) glycoprotein (Lai, 2001). It is interesting to note that the hemagglutinating activity of the HE from BCoVs strains is lower than the hemagglutinating activity of the S glycoprotein, which forms large spike-like projections in the viral envelope (Schultze et al., 1991).

Moreover, the S glycoprotein harbors domains responsible for receptor binding and induction of neutralizing antibodies, and is the most polymorphic viral protein among CoV species and also among strains of the same species. It is utilized for the molecular characterization of the isolates (Collins et al., 1982). The S glycoprotein consists of two subunits, S1 (N-terminal half) and S2 (C-terminal half). The S1 hypervariable region is useful to study the variability and evolution of this virus (Brandao et al., 2006; Hasoksuz et al., 2002).

BCoV has worldwide distribution and is reported from several countries (Jeong et al 2005; Khalili et al., 2006; Takiuchi et al 2006; Traven et al 2006; Gumusova et al 2007; Park et al 2007, Boileau and Kapil, 2010; Dash et al 2012; Ohlson et al., 2013; Mawatari et al., 2014; Ammar et al., 2014). However epidemiological data on BCoV in Iran is scarce.

Therefore, the aim of the present study was to screen the fecal samples for BCoV collected from clinical cases with the history of diarrhea from six geographic region of Iran covering 27 dairy farms.

## **Materials and Methods**

**Sample collection:** Selected geographical regions for sampling, which were chosen based on density of dairy farms and

climates, were northwest, northeast, southwest, south of Alborz Mountains, west, and central regions of Iran, (Table 1).

Totally 194 samples were collected from diarrheic neonatal calves up to one-month of age from May 2014 to June 2016. Samples were collected directly from the calves' rectum into sterile bottles and transferred to Virology laboratory of the Veterinary Faculty of University of Tehran on ice pack and stored at -18 °C. Sample's specifications such as: name of farm, province, geographic region, age, gender, stool consistency score, and rectal temperature were recorded.

BCoV antigen ELISA. An indirect antigen-capture ELISA kit employing monoclonal antibodies to BCoV was used as described by the manufacturer (IDEXX Rota-Corona-k99, USA).

Then the positive samples in ELISA were conducted on RT-PCR for confirmation.

Preparation of oligonucleotide primers. The oligonucleotide primers used in the RT-PCR were designed from the published sequence of N gene of Mebus strain (GenBank accession No.U00735). The sequences of primers are shown in Table 2, and the predicted RT-PCR product size was 407 bp (Table 2).

RNA extraction and RT-PCR. RNA from faeces was extracted using RNX-Plus solution for total RNA isolation (Sinaclon Bioscience, Iran) as instructed by the manufacturer. Then the complementary DNA (cDNA) was conducted by Maxime RT premix kit (Intron Biotechnology, Korea) as instructed by the manufacturer. The RT-PCR was conducted using the following procedure: in a tube 3 µL of cDNA sample was added to 2 µL of the Reverse and forward primers and 5.5 µL of nuclease-free water, and finally 12.5 µL master mix was

Table 1. Selected geographical regions for sampling, dairy farms and climates.

Region	Province	Sample numbers	Farms numb
northwest	Zanjan	10	1
northeast	Mashhad	30	4
northeast	Gorgan	10	2
south of Alborz	Tehran	40	6
south of Alborz	Varamin	10	1
south of Alborz	Qazvin-Alborz	20	5
Central	Qom	10	2
Central	Isfahan	30	1
Central	Saveh	12	1
west	Kermanshah	10	1
southwest	Ahvaz	12	3
Total	11	194	27

added to the solution. Then the solution was preheated for 5 min at 94 °C, 40 cycles, including 45 seconds at 94 °C, 45 seconds at 52 °C, 1 min at 72 °C and, finally, 10 min incubation at 72 °C was applied. The PCR products were visualized on 1.5% agarose gel stained with ethidium bromide. PCR products of 407 bp were detected.

## Results

ELISA examination of stool samples revealed that 14 samples (7.2%) out of 194 taken samples, which belonged to 10 dairy farms (37.03%) out of 27 farms were positive (7.2%). The positive samples were found in Tehran (three samples), Qom (two samples), Isfahan (three samples), Qazvin (two samples), Alborz (two samples), Saveh (one sample) and Ahvaz. The Zanjan, Mashhad, Gorgan, Varamin, and Kermanshah samples were negative. On the other hand, all samples from northwest, northeast, and west, were negative while samples from three other geographic regions were pos-

Table 2. The sequences of primers and the predicted RT-PCR product size.

virus	target gene	primer	sequence (5'-'3')	position	product length	reference no.
BCV	Nucleocapsid	BCV-N-f	GCCGATCAGTCCGACCAATC	29475-29494	407	Tsunemitsu et al, 1999
		BCV-N-R	AGAATGTCAGCCGGGGTAT			Masaharu et al, 2012

itive. All positive samples in ELISA were positive in RT-PCR.

The average age of positive calves was nine days, and 38% were male and 62 % were female.

The average stool score in the positive samples and negative samples was 2.5 and 2.1 respectively (based on 0-3 scoring). Fecal consistency data were recorded on a scale of 0-3, with score increasing severity (0 = normal feces, 3 = severe diarrhea) (Operario et al, 2015). Degree of fecal consistency: 0 = normal, manure is normal and well formed; 1 = abnormal feces but not diarrhea, manure is pasty (softer than normal); 2 = mild diarrhea (feces are semi liquid, but still have a solid component); and 3 = liquid feces only (Mathur et al, 2001).

10 samples from the positive samples (71/4 %) had fever (rectal temperature  $\geq 40$  °C).

**Discussion**

In the present study, Coronavirus was detected in 7.3% of diarrheic calves. Almost the same results about occurrence of BCoV were found in some other studies in Sweden (De Verdier, 2006), Japan (Kirisawa et al., 2007), Switzerland (Uhde et al 2008) and Holland (Bartels, 2010). In contrast, BCoV appeared to be of more importance with higher prevalence in some other studies in countries such as Spain (De la Fuente et al 1998; Perez et al 1998), France (Bendali et al., 1999), Brazil (Stipp et al., 2009), India (Hansa et al., 2012), Australia (Izzo et al., 2011), Korea (park et al., 2007) and Turkey

(Hasoksuz et al., 2005). A few studies also had lower prevalence, including studies in Pakistan (Alikhan, 2009, 2 %) and Argentina (Bok et al., 2015, 5 %).

In one regional study in Iran, 12.03% of diarrhetic samples were positive with both capture ELISA and RT-PCR (Khalili et al., 2006). Mayameei et al., 2010 in Tehran dairy farms showed that the prevalence of Coronavirus infection in diarrhetic calves is 3.17%. In another study in Mashhad province, Coronavirus antigen was detected in 2.7% and 1.8% of diarrhetic and non-diarrhetic calves, respectively (Afshari et al., 2012). In the present study 28.57 % and 71.43 % of positive samples were in the age group 0-7 days and >7 days respectively. In another study, calves were divided to different age groups 1-15, 16-30, 31-45, and 46-60 days. Age group 16-30 days had most positive samples (29 %), but at least one or more positive samples were seen in other groups (Stipp et al., 2009).

In the present study, there was not any significant association between the presence of Coronavirus in feces and stool score and fever (rectal temperature  $>40$ ) of calves ( $p > 0.05$ ). More than 85% of positive samples had a stool score  $\geq 2$  with an average of 2.5. Also, about 71% of positive samples had a rectal temperature  $>40$ . In some studies (Busato et al 1998; Bjorkman et al 2003; Erdogan et al 2003) no significant association was found between the presence of Coronavirus in feces and clinical diarrhea but in some other studies, there was a significant association between shedding of Coronavirus in feces and diarrhea (Reynolds et al

1986, Perez et al 1998, Bendali et al 1999a, Stipp et al., 2009).

The results of the present study showed that the occurrence of coronavirus in stool samples of diarrheic calves in dairy farms of Iran is lower than the other reports in the world. The occurrence and also diagnosis of enteropathogens in the feces of diarrheic and non-diarrheic calves varies depending on the geographic location, the farm, the age and type of calves being examined and the extent to which the diagnostic laboratory is capable of isolating or demonstrating the causative pathogens. On the other hand, the role of other infectious agents in diarrhea in different countries and regions and the sensitivity and specificity of used tests in each study can explain the observed differences in prevalence of Coronavirus among a variety of studies. In addition, some of the possible reasons for low prevalence of Coronavirus in the present study are that most samples were not collected during winter, the season in which the prevalence of calf diarrhea caused by Coronavirus is higher than other seasons. Also, the sensitivity of ELISA test, used as screening test in this study, is lower than PCR, moreover, Iran has a hot, dry climate, opposed to the Coronavirus desirable stability condition.

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## مطالعه بر روی شناسایی کروناویروس در گوساله‌های نوزاد در گاو اسهال در فارم‌های شیری ایران

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### چکیده

**زمینه مطالعه:** کروناویروس گاوی یکی از علل اصلی اسهال نوزاد گوساله‌ها در جهان می‌باشد، همچنین با اسهال حاد در گاو بالغ در طی فصل زمستان در ارتباط می‌باشد که منجر به ضررهای اقتصادی سنگین به هردو گروه گاو شیری و پروراری صنعتی در جهان می‌شود. **هدف:** هدف از انجام مطالعه حاضر، غربال نمونه‌های مدفوع برای کروناویروس گاوی از نمونه‌های اسهالی جمع‌آوری شده از شش منطقه جغرافیایی ایران، با هدف افزایش دانش در زمینه شیوع و همه گیر شناسی کروناویروس گاوی در ایران، بوده است. **روش کار:** ۱۹۴ نمونه مدفوع از گوساله‌های در گاو اسهال تا سن یک ماهگی، بر اساس منطقه جغرافیایی جمع‌آوری گردید. تمام نمونه‌ها برای بررسی حضور کروناویروس گاوی توسط کیت‌های تجاری الیزا مورد بررسی قرار گرفت. همچنین تمام نمونه‌های مثبت برای تایید نهایی توسط آزمون RT-PCR بررسی شدند. آزمون RT-PCR ۴۰۷ جفت باز از ژن نوکلئو کپسید ویروس کروناویروس را با پرایمر منتشر شده که می‌تواند تمام سویه‌های کروناویروس گاوی را شناسایی کند، هدف قرار می‌دهد. **نتایج:** آزمون الیزا نشان داد که ۷/۲٪ از نمونه‌های گرفته شده مثبت بودند. تمام نمونه‌های مثبت در الیزا همچنین در آزمون RT-PCR مثبت بودند. تمام نمونه‌های شمال غرب و غرب منفی بودند. سن میانگین گوساله‌های مثبت ۹ روز بود. میانگین نمره ی مدفوع در نمونه‌های مثبت و منفی به ترتیب ۲/۵ و ۲/۱ بود. ۷۱/۴٪ از نمونه‌های مثبت تب داشتند. **نتیجه‌گیری نهایی:** نتایج مطالعه حاضر نشان داد که وقوع کروناویروس در گوساله‌های اسهالی فارم‌های صنعتی ایران نسبت به سایر گزارشات در دنیا پایین تر می‌باشد.

**واژه‌های کلیدی:** کروناویروس گاوی، اسهال گوساله، مزارع شیری، تب، RT-PCR

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