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Detection of bovine coronavirus by RT-PCR in a field study

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ABSTRACT

In the present study we used RT-PCR assay for detecting of BCoV, targeting a 730 bp fragment of the nucleocapsid (N) gene of BCoV with published primers that could amplify all BCoV strains. We evaluated presence of BCoV in diarrheic and nondiarrheic samples. 108 faecal samples from diarrheic calves and 80 faecal samples from nondiarrheic calves collected. In 13 of 108 diarrheic samples both ELISA and RT-PCR detected BCoV. In 4 of 80 samples second group (non diarrheic) BCoV was detected by RT-PCR only not capture ELISA. This report is the first detection of BCoV in Iran. The results suggest that RT-PCR is more sensitive than ELISA method to detect BCoV, especially in subclinical cases. Because these animals shed a low amount of virus in faeces we need to apply sensitive techniques, such as RT-PCR, nested PCR and real time RT-PCR.

Key words: bovine coronavirus (BCoV), RT-PCR, ELISA, calf diarrhoea

Introduction

Bovine Coronavirus (BCoV), a member of the family *Coronaviridae*, order *Nidovirales*, possesses a single-stranded, enveloped, non-segmented RNA genome with positive polarity (VRIES et al., 1997). The virion contains five structural proteins: the nucleocapsid (N), the transmembrane (M), the haemagglutinin/esterase (HE), the spike (S) and the small membrane (E) proteins (SAIF, 1993). The BCoV N protein is a 50-60 KD phosphoprotein that is bound to viral genomic RNA to form the helical nucleocapsid. N protein may play a role in replication of viral RNA since the antibody directed against the N protein inhibits the *in vitro* RNA polymerase chain reaction (LAI and CAVANAGH, 1997).

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BCoV is associated with severe diarrhoea in newborn calves, neonatal calf disease (NCD), winter dysentery (WD) in adult cattle, and respiratory tract infections in calves and feedlot cattle (QUINN et al., 2002). BCoV was first recognized as a cause of potentially fatal diarrhoea of neonatal calves in 1972. Economically important NCD and WD outbreaks were reported (LATHROP et al., 2000). There are different methods to detect BCoV, but a high degree of sensitivity is required, especially in subclinically infected calves and chronic shedders of BCoV in faeces. These calves are important sources for the infection of other calves (HASUKSUZ et al., 2002). The RT-PCR assay is useful to detect small quantities of nucleic acid and is widely used for the diagnosis of infectious disease. This study was designed with two aims. The first was to compare a capture EIA to the RT-PCR methods for detection of bovine coronavirus. The second was to determine the prevalence of bovine coronavirus in instance of calves' diarrhoea in north-west Iran.

Materials and methods

Collection of samples. A total of 188 faecal samples collected from diarrheic and nondiarrheic calves were divided into two groups. Samples were collected directly from rectum in sterile bottles. Samples were processed within 24h of reception and were stored in a freezer at -80 °C.

BCoV antigen ELISA. An indirect antigen-capture ELISA kit employing monoclonal antibodies (mabs) to BCoV was used as described by the manufacturer (Bio-x Diagnostic s.p.r.l, Belgium).

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.M16620). The sequence of primers were as follows: upstream primer: 5′-GCA ATC CAG TAG TAG AGC GT-3′ (21-40), downstream primers: 5′-CTT AGT GGC ATC CTT GCC AA-3′ (731-750). The predicted RT-PCR product size was 730 bp.

RNA extraction and RT-PCR. RNA from faeces was extracted using QIAamp virus RNA mini kit (Qiagen, UK) as instructed by the manufacturer.

The optimal annealing temperature was tested in a gradient thermocycler (Corbett, Australia) and used in the following protocols. We then optimized Mg^{++} concentration and other conditions. For each sample we used neat and 1/10 diluted. The reverse transcriptase reaction was conducted using the following procedure: in the tube 10 μL of RNA sample was added to 2 μL of the Reverse primer (100 pMol) and 4 μL of water. The tube was incubated at 70 °C for 5 min and then quenched on ice for 10 min. Subsequently, 4 μL of 5× RT buffer, 2 μL of 0.1 M dithiothereitol, 2 μL of 10 mM dNTPs, 20 units of Rnase Inhibitor (Fermentas, Germany) were added and with nuclease free water it was reached to 19 μL. The mixture was incubated at 37 °C for 5 min. Subsequently, 200 units of Moloney Murine Leukemia Virus (M-MULV) reverse transcriptase (Fermentas, Germany) were

added and incubated at 42 °C for 60 min. For end of reaction, mix was incubated at 70 °C for 10 min, and then quenched on ice for 5 min. Five µL of the RT reaction samples were then added to 45 µL of the PCR mixture. The PCR mixture consisted of 5 µL of 10× PCR buffer, 2 µL of 10mM dNTPs, 1 µL of the upstream primer (100 mM), 1µL of the downstream primer, 35.5 µL of water and 0.5 µL of Taq polymerase (Fermentas, Germany) (5 unit per µL). Then, 5 min of preheating at 94 °C, 35 cycles, including 1 min at 94 °C, 1 min at 58 °C, 2 min at 72 °C and, finally, 7 min incubation at 72 °C were applied. The PCR products were visualized on 1.5% agarose gel stained with ethidium bromide. PCR products of 730 bp were detected (Fig. 1).

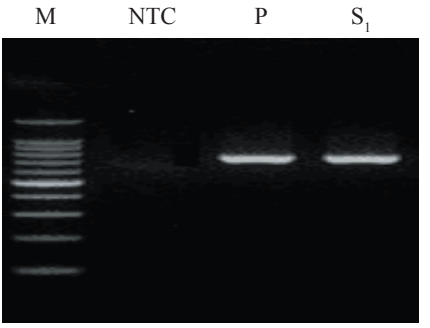

Fig. 1. RT-PCR detection of BCoV in faeces samples. M = molecular marker (100 bp); NTC = non template control; P = positive sample from Mebus strain; S = sample.

Results

Thirteen samples were positive by both ELISA and RT-PCR, while 4 samples from the second group were positive by RT-PCR only. One sample was positive by ELISA and negative by RT-PCR. One hundred and seventy one samples were negative by both methods (Table 1). The percentage of agreement of the two assays (ELISA and RT-PCR) was 97 %.

Agreement beyond chance was calculated with a kappa statistic: $\kappa = 0.82$.

Table 1. Results of the two methods

		CAPTURE ELISA		
		Nº of positive	Nº of negative	Total
RT- PCR	Nº of positive	13	4	17
	Nº of negative	1	170	171
	Total	14	174	188

We reasoned that the poor sensitivity of BCoV capture ELISA detection in nondiarrheic calves could be enhanced by RT-PCR.

Discussion

The BCoV and other pathogens, including BVDv, rotavirus, bovine Torovirus (Breda virus), *E. coli*, *Salmonella* spp., *Cryptosporidium parvum* and *Coccidia*, were associated with diarrhoea in calves (HOET et al., 2003; QUINN et al., 2002). Coronaviruses from wild ruminants can experimentally infect young calves (TSUNEMITSU et al., 1995). All BCoV isolates belong to 1 serotype (2 subtypes) and are pneumotropic. Only point mutations occur in the S gene of EBCoV (Enteric Bovine Coronavirus) vs RBCoV (Respiratory Bovine Coronavirus) strains (SAIF, 1993). Therefore, for detection of BCoV in diarrhoea we need to use a universal primer to that conserved all of strains. A high degree of sensitivity is desirable in BCoV assay, especially for specimens from those calves early or late in the course of illness or after reinfection which may have a low level of BCoV shedding (CHO et al., 2000). In another study, CHO et al. (2001b) showed that prevalence of EBCoV was 22%, and that 45% of cattle shed both EBCoV and RBCoV.

We used published primers for the nucleocapsid gene that was conserved among BCoV strains. Our results showed that RT-PCR is applicable in detecting BCoV in clinically normal calves, an essential approach concerning epidemiological surveillance in calves as it allows for the application of preventative measures prior to the emergence of diarrhoea on a farm. This is because the more sensitive RT-PCR assay may detect BCoV-positive animals than might otherwise be classified as BCoV-negative by ELISA or other methods. When we used the RT-PCR method we observed that sensitivity increased. RT-PCR could detect 4/80 of subclinically infected calves, but with using capture ELISA we were unable to detect any positive samples. This was expected because subclinical calves shed a low amount of virus to the environment, which is what we suggested. Using a novel detection method of real time quantitative RT-PCR technology can increase sensitivity of detection. MUNIER and KIBENGE (2004) reported that the sensitivity of Real Time RT-PCR is 100 times greater than one step RT-PCR. CHO et al. (2001a) reported that the sensitivity of RT-PCR and nested PCR compared with ELISA was very high, but that specificity was low. BRANDAO et al. (2003) proved that nested PCR based on detection of S gene is a specific and sensitive tool for bovine Coronavirus diagnosis. The assay protocol to detect BCoV RNA should be as simple as possible so that it could be simultaneously applied to a large number of clinical samples. However, ELISA is more suitable than RT-PCR for testing large numbers of samples. But we suggest that ELISA is not a reliable method for detection of subclinically calves infected with BCoV. This report is the first detection of BCoV in Iran.

We suggest that in order to to reduce mortality rates and economic losses caused by BCoV, that in addition to vaccination and good management it is necessary to ensure that subclinical individuals are detected by very highly sensitive methods, such as RT-PCR, nested PCR, quantitative real time RT-PCR, and then to isolation them from other animals.

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SAŽETAK

U istraživanju je rabljena lančana reakcija polimerazom uz prethodnu reverznu transkripciju (RT-PCR) za dokaz goveđega koronavirusa (GKV), odnosno ciljnoga fragmenta od 730 pb njegova nukleokapsidnoga (N) gena uz upotrebu objavljenih početnica koje se mogu upotrijebiti za amplifikaciju svih sojeva GKV. Pretraženi su uzorci proljeva kao i normalno formirane balege. Sakupljeno je 108 fekalnih uzoraka uzetih od teladi s proljevom i 80 uzoraka od teladi bez proljeva. Imunoenzimnim testom i RT-PCR-om ustanovljen je GKV u 13 od ukupno 108 uzoraka proljeva. U četiri od ukupno 80 uzoraka druge skupine (bez proljeva) GKV je ustanovljen samo RT-PCR-om, ali ne i ELISA-om. U ovom izvješću prvi je put dokazan GKV u Iranu. Polučeni rezultati pokazuju da je RT-PCR osjetljivija metoda od ELISA-e za dokaz GKV, osobito u supkliničkim slučajevima. Budući da životinje izlučuju malu količinu virusa izmetinama, potrebno je primjenjivati osjetljivije tehnike kao što je RT-PCR, ugniježđeni PCR i PCR u stvarnom vremenu («real-time» PCR).

Ključne riječi: goveđi koronavirus, RT-PCR, ELISA, proljev teladi