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Advanced virologic detection of Coronavirus from infected calves

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Abstract

Bovine Coronavirus (BCoV) is widespread both in dairy and beef cattle throughout the world. The virus is one of the largest RNA virus and has specific tropism for intestinal and pulmonary epithelial cells. In the present study, 50 fecal samples of diarrheic calves up to 3 months age were collected over a one year period from both commercial and household farms around different regions all over Egypt. Fecal samples from all these cases were screened for the presence of bovine corona virus (BCoV) using commercially available indirect antigen capture ELISA kit. Three out of 50 samples were positive to corona virus infection by indirect antigen capture ELISA kit. Furthermore the ELISA positive samples were subjected to conventional PCR for detection and confirmation of BCoV and to amplify the conserved nucleocapsid (N) gene of the virus targeting a 236bp Length of amplified product of BCoV. The screening PCR showed that the same three samples were positive for coronavirus. One of the three samples was submitted to sequencing process and Phylogenetic analysis. The gene sequence in our study have a similar identity to BCV Menofyia, BCV FRA8, BCV FRA10, BCV FRA12 and BCV _3 with 100% percent of identity and FRA03, FRA05, FRA07, FRA11 and FRA14 with 99.6% percent of identity from gene Bank database. The nucleotide sequences generated in this study have been submitted to Gen Bank data and were assigned an accession number of BoCVBeheira,2017MN592991.

Keywords: *Bovine corona virus, ELISA, PCR, phylogentic analysis.*

Introduction

Corona viruses (CoV, family Coronaviridae) are large enveloped viral particles containing a positive sense single stranded RNA genome (26–30 kb), coding for several structural proteins, including polymerase (Pol), nucleocapsid (N), membrane (M), hemagglutinin-esterase (HE), spike (S) proteins and several non-structural proteins (NSPs) **(L.Gunn *et al.* 2015).** Calf diarrhea, one of the most widespread diseases, is a complex syndrome causing important economic losses due to morbidity and mortality, treatment costs, and reduced growth rates in affected calves **(Garaicoechea *et al.* 2006; Reidy *et al.* 2006))**. The etiology of this syndrome involves infectious agents (viruses, bacteria, and protozoa) and also non-infectious causes **(Garaicoechea *et al.* 2006)**. Common etiologic viral agents involved in calf diarrhea include bovine rotavirus (BRoV), and bovine coronavirus (BCoV). BCoV has been identified in both healthy and diarrheic calves, **(Duckmanton *et al,* 1998)**. Coronaviruses (CoVs) are enveloped, positive-sense, single-stranded RNA virus, belong to the Coronaviridae family of the Nidovirales **(Mostafa Hasoksuz *et al.* 2007)**. BCoV is the causative agent of neonatal diarrhea in newborn calves, winter dysentery in adult cows and respiratory tract illness in calves and adults, they are able to replicate both in gastrointestinal enterocytes, as well as in the epithelium of the upper airways **(Lathrop *et al.* 2000).** Transmission from calf-to-calf is mainly by the fecal–oral route but respiratory transmission can be possible **(Boileau *et al,* 2010).** Clinically recovered calves can continue to shed low levels of virus for weeks **(Saif *et al*. 1986).** There are a variety

of diagnostic methods available for the detection of rotavirus and coronavirus including PCR, ELISA, Electron microscope and Immune electron microscope. Rotavirus may also be detected by agglutination and polyacrylamide gel electrophoresis **(Samuel Jakobsson, 2013).** Diagnosis is done through collecting faeces from diseased live calves or intestinal contents of dead animals suffering from diarrhea using laboratory diagnostic tests such as Enzyme-linked immunosorbent assay (ELISA), latex agglutination, polyacrylamide gel electrophoresis, and polymerase chain reaction (PCR) **(Radostitis *et al*. 2007).**

Objectives of this study were to investigate the detection rates of BCoV in feces of calves with diarrhea and or respiratory symptoms and also exploring of the molecular identity of the isolated virus with the gene bankto establish sequence identity to Gen Bank accessions and vaccinal strain used in Egypt.

Materials and Methods

Sample collection and preparation:

Fecal samples from diarrheic live calves and intestinal content from dead calves were collected from both commercial and household farms in different governorates in Egypt. Fecal suspensions (v/v; 50%, watery feces; 20%, loose feces; or 10%, normal feces) were prepared with pure distilled water and were centrifuged at 1400 x g for 10 min at 4°C and supernatants were collected and used for screening of BCoV by ELISA and PCR.

Enzyme Linked Immune Sorbent Assay (ELISA):

All the 50 fecal samples were subjected to an indirect antigen-capture ELISA monoclonal antibodies (mAbs) to detect the presence of BCoV employing commercially available ELISA kit (Bio-X Diagnostics, Belgium kit) following the manufacturer's instructions.

Nucleic acid extractionand amplification.

Bovine corona RNA extraction from 220 mg stool samples was done using QIAampminielute virus spin kit (Qiagen, Germany, GmbH), A preparation step of the stool using the QIAamp DNA stool Mini Kit (QIAGEN, Hilden, Germany).

Target agent	Target gene	Primer sequence (5'-3')	Length of amplified product (bp)	Reference
Bovine corona	N	TGGATCAAGATTAGAGTTGGC	236 bp	**Amer *et al*., 2013**
		CCTTGTCCATTCTTCTGACC		

Analysis of the PCR Products.

The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature using gradients of 5V/cm. For gel analysis, 15 µl of the products was loaded in each gel slot. A gelpilot 100 bp DNA ladder (Qiagen, Germany, GmbH) was used to determine the fragment sizes. The gel was photographed by a gel documentation system (Alpha Innotech, Biometra) and the data was analyzed through computer software.

Phylogenetic analyses:

DNA sequences were obtained by Applied Biosystems 3130 genetic analyzer (HITACHI, Japan), a BLAST® analysis (Basic Local Alignment Search Tool) **(Altschul *et al.,* 1990)** was initially performed to establish sequence identity to GenBank accessions. The phylogenetic tree was created by the MegAlign module of Lasergene DNA Star version 12.1 **(Thompson *et al.,* 1994)** and Phylogenetic analyses was done using maximum likelihood, neighbour joining and maximum parsimony in MEGA6 **(Tamura *et al.,* 2013).**

Results

ELISA: 3 out of 50 samples were positive for corona viral antigen detection using immune capture ELISA kit.

PCR, sequencingand Phylogenetic analysis:

The specific primers set amplified at 236 bp equivalent to the expected amplification product (amplicon) of BCoV N protein gene fragment. The BCoV reference strain and our samples had the same size of 236 bp, without significant differences between them. This result reveled that these specimens contained RNA of BCoV as viewed in Fig (1).

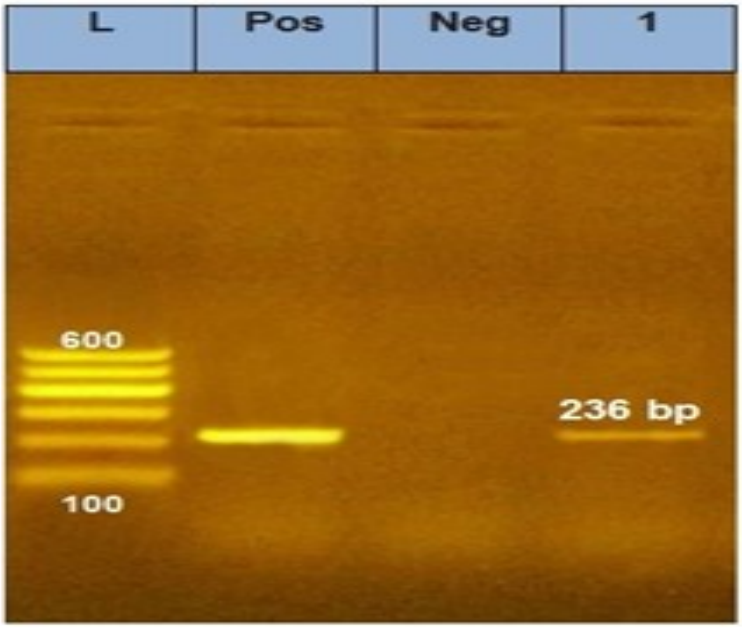

Figure (1). PCR detection of BCoVshowing the amplified product of 236 bp of BCoV N gene in reference strain and our samples had the same size of 236 bp

Our studied sample BoCV Beheira,2017) showed a percentage of nucleotide sequence identity of 100% with the egyptian field isolate BoCVMonfoya and a percentage of nucleotide identity of 99,6% with the egyptian field isolates BoCV Dakhlya1, BoCV Dakhlia2 and Dakhlia3 and phylogenetically clustered with them. .The examined sample did not undergo cell culture passage prior to sequencing. The N gene sequence in our study have a similar identity (100%)to that of BCoV FRA8, BCoV FRA10 and BCoV FRA12, (99,6) with BCoV FRA3, BCoV FRA5, BCoV FRA7, BCoV FRA11, and BCoV FRA14 from gene Bank database as showed in Fig. (2) and (3).

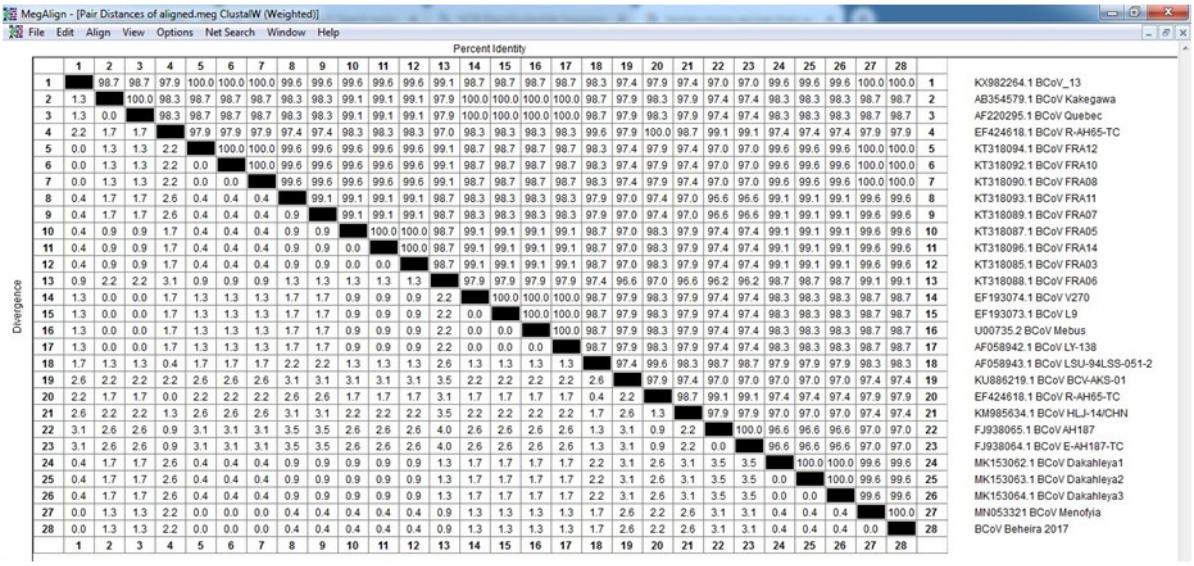

Figure (2). Showing the percent of identity of BCoV_Brheira 2017 isolated from the field samples with other coronavirus in gene bank database.

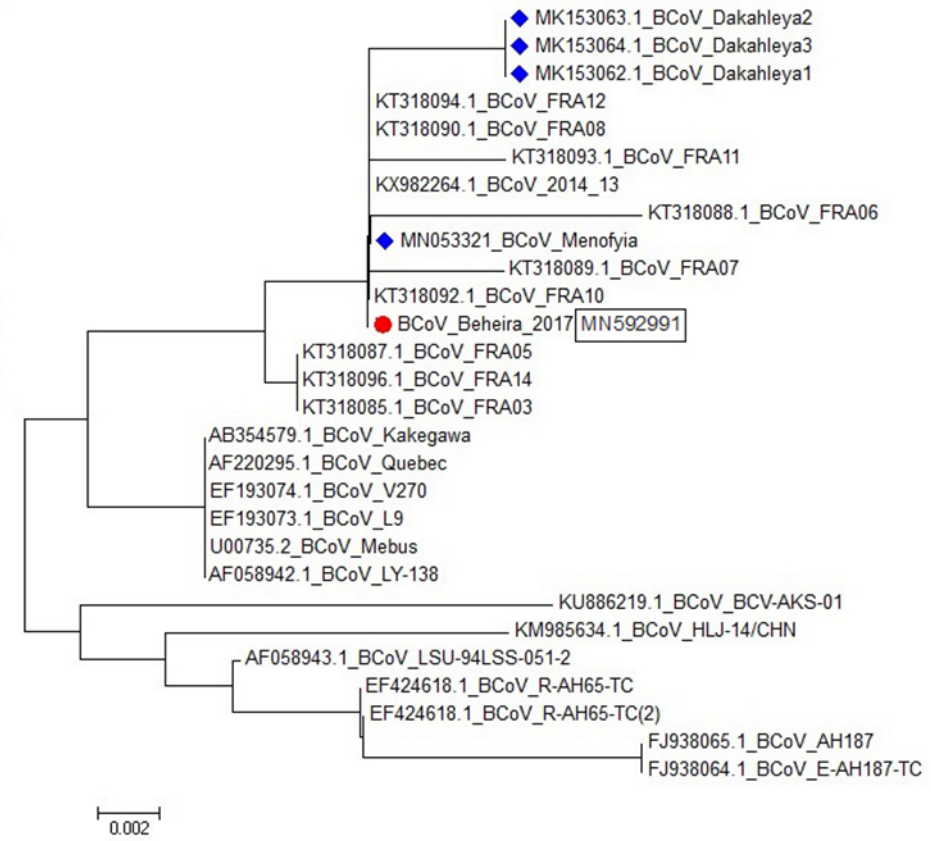

Figure (3). Phylogenetic tree of coronavirus N-gene, showing that the sequence clusters closest to another strain of the Egyptian and other coronavirus present in gene bank database.

Discussion

Corona virus is one of the most prevalent causative agents of diarrhea in farm animals that causes significant economic losses as a result of decreased weight gain, treatment costs, and high mortalities. Therefore, developments of a highly sensitive, rapid and specific test for diagnosis is critically needed by the veterinary producer for rapid control and prevention decisions. Because no "gold standard" test for BCV exists, the greater specificity and sensitivity of ELISA make it more attractive for BCV detection where ELISA test allowed detection of the virus in fecal samples containing as few as104particles, it was easy to test a large number of specimens with BCV ELISA and absence of percentage of nonspecific reactions . According to all these advantages ELISA test considered the test of choice (sensitive, specific, easy and rapid test) for BoCV detection and control. This result was in agreement with **(SchoenthlLer and S. Kpail 1999)**. In the present study three samples out of fifty fecal samples from diarrheic dairy and beef calves up to one month of age were reported coronaviral antigen positive by both immune capture ELISA and PCR. The percentage of occurrence of BCoV at the Egyptian bovine farms at this study was found similar to some other studies as in **(Mohebbi *et al.* 2017)** with a percentage of 7.5%. In contrast, BCoV appeared to be of higher prevalence in some other studies in countries such as **(Hansa *et al.*, 2013)** with a percentage of about 15%. A few studies had lower prevalence, including studies in Argentina with a percentage of 5% **(Bok *et al.*, 2015).**

This study aimed to characterize this Egyptian field sample 0f BCoV (bovine coronavirus) , and to detect its identity with the gene bank database . For this purpose, we determined the partial N gene sequences of BCoV from fecal sample of diseased calves with diarrhea. In our investigation,, nucleotide sequence alignment of 236 bp amplified from the N gene from our field sample BoCV Beheira, 2017 showed a high percentage of nucleotide identity of 100% with the Egyptian field isolates BoCV Monfoya and of 99,6% with BoCV Dakhlya1, BoCV Dakhlia2 and Dakhlia3 and phylogenetically clustered with them. This sample did not undergo cell culture passage prior to sequencing. The N gene sequence in our study have a similar identity (100%) to that of BCoV FRA8, BCoV FRA10 and BCoV FRA12, of (99,6) with BCoVFRA3, BCoV FRA5, BCoV FRA7, BCoV FRA11, and BCoV FRA14from gene Bank database. the N gene is highly conserved among BCoV strains **(Elisabete Takiuchi *et al.* 2006).** The circulating Bcov in Egypt evolved from the same evolutionary pathway in comparison to some studied European ,Asian and American strains. These results show that comparative nucleotide sequence analysis is a useful tool for investigating the molecular epidemiology of BCoV infections and this in agreement with (**Lihong Liu *et al.* 2006).**

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