Research Article

# Isolation and Molecular Characterization of Bovine Coronavirus from Calves with Acute Gastroenteritis in Egypt

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**Abstract** | This study was intended for the antigenic and molecular characterization of betacoronavirus 1 (bovine coronavirus) BCoV in newborn gastroenteritis calves in the Delta Region, Menofia Province, Egypt, also for isolation and identification of the local circulating BCoV strain for further diagnosis or vaccination. In cattle, Betacoronavirus 1 bovine coronavirus (BCoV) is primarily involved in enteric infections which leads to serious complication which may be fatal in young calves up to 3 months of age. A total of 20 fecal samples were collected in the winter season of 2018 and 2019, all samples were serologically screening by antigen-capture ELISA, then molecular confirmation by RT-PCR targeted to nucleocapsid protein gene (N-gene) was carried out followed by phylogenetic analysis. Positive samples were isolated on Vero cell culture and identified by TEM and immune-peroxidase technique. Betacoronavirus 1 was detected by ELISA in 6 out of 20 fecal samples (30%), PCR detected 4 out of 6 ELISA positive samples at specific M.W. band of 236 bp by electrophoresis, and one sample was sequenced and submitted on Genbank with acc.no. MW173144, further phylogenetic sequence analysis revealed high percentage of identity with reference strains from different countries. Phylogenetic tree cleared that current research strain was found related to France strains 2013 and 2014, also to local Egypt strain 2019 with acc.no. MN053321, two samples were successfully isolated in Vero cells and positively identified after the 3rd passage by TEM and immune-peroxidase technique. ELISA results are considered an alarmingly high prevalence that requires further statistically designed epidemiological studies, phylogenetic analysis revealed minimum evolution rate and stability of BCoV genome.

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## Information

Diarrhea is one of the most serious problems affecting cattle, particularly younger ones from 1 week to 3 months of age, leading to serious dehydration, which in many cases can be fatal (Blowey and Weaver, 2011). The most common viral enteric pathogens were known include Bovine Rotavirus (BRoV) and Betacoronavirus 1 Bovine Coronavirus (BCoV) (Cavanagh, 1997), can cause enteric and respiratory infections in calves and adult livestock. (Lathrop et al., 2000; Hoet et al., 2003). Betacoronavirus 1 infection is a significant cause of economic losses for the dairy industry worldwide (Saif, 2004). In addition, the maximum decrease in dairy production is about 10 percent and can last for 1-2

weeks, after dairy production levels are recovered in mild BCoV epidemics, but dairy production decreases to 30 percent in extreme epidemics and continues for up to 1 month (Radostits et al., 2007).

Betacoronavirus 1 BCoV is classified in the order Nidovirales, family Coronaviridae, subfamily Coronavirinae; genus Betacoronavirus (Group 2 Coronavirus) subgroup 2A (Graham et al., 2013). Coronaviridae members have enveloped viruses with a single-stranded non-segmented positive-sense RNA genome of 27-32 kb in length (Saif, 2004). Virions are pleomorphic to spherical, about 80–200 nm in diameter.

Betacoronavirus 1 BCoV genomeconsists of 13 open reading frames (ORFs) flanking 5′ and 3′ untranslated regions encodingfivestructural proteins (SP) and non-structural proteins (NSP). Five majorstructural proteins are encoded within the genomic RNA suchas hemagglutinin-esterase (HE) protein (ORF3), spike (S) glycoprotein (ORF4), small membrane (E) protein (ORF8), transmembrane (M) protein (ORF9), and nucleocapsid (N) protein (ORF10) (Chouljenko et al., 2001; Masters, 2006).Coronavirus host spectrum and tissue tropism variations are referred to as the spike (S) glycoprotein, which is split into two functional domains, S1 (N-terminal half) and S2 by intracellular protease (C-terminal half) (Gallagher and Buchmeier, 2001). The peripheral S1 subunit is responsible for host-cell receptor virus attachment (Kubo et al., 1994), neutralizing antibody induction (Yoo and Dereget, 2001), and haemagglutination function (Schultze et al., 1991). The S1 sequence is variable, with mutations correlated with altered antigenicity and pathogenicity of the virus in this area (Ballesteros et al., 1997); which explains why this region was used as a target to research the molecular epidemiology of BCoV infection (Jeong et al., 2005; Liu et al., 2006). The S2 subunit sequence is more conserved, and this subunit is responsible for the operation of cell membrane fusion (Yoo et al., 1991). Most research investigating the importance of BCoV in neonatal calf diarrhea (NCD) have been carried out in the northern hemisphere (Ammar et al., 2014; Bidokhti et al., 2013; Decaro et al., 2008b; Hasoksuz et al., 2002; Jeong et al., 2005; Lu et al., 1991; Mawatari et al., 2014; Ohlson et al., 2013), On the other hand, little epidemiological information is available on the identification, occurrence, and characterization of Betacoronavirus 1 BCoV in cattle from the Middle East in general, especially Egypt. In nasal secretions and in fecal samples, BCoV may be diagnosed. The diagnosis depends on the identification of the virus, viral antigen, or viral RNA; the detection of viruses requires isolation in cell culture, which is not the first option since certain BCoV strains have trouble adapting to cell culture.

While Betacoronavirus 1 BCoV antigens are most detected using ELISA, which has the good qualities of being fast and timesaving. Also, viral RNA detection through PCR (with different formats) was more widely used (Saif, 2010).

The present study aimed to determine the infection rate of Betacoronavirus 1 BCoV BCoV in diarrheic calves from some Egyptian governorates, additionally, to conduct a phylogenetic study with the Egyptian Betacoronavirus 1 BCoV strains in comparison with the BCoV strains; characterized worldwide and finally to prepare a BCoV (cell culture adapted) identified strain for further studies.

## Materials and Methods

### *Ethics statement*

This statement confirms that sampling for this research was performed as non-experimental clinical work with respecting the rules of the veterinary profession. Sampling was performed strictly on the owner's request international, national, and\or institutional guidelines for sampling were followed with the approval of the local ethics committee on animal experimentation at the animal health research institute, agriculture research center (ARC), Egypt.

### *Clinical specimens' collection and preparation*

A total of 20 fecal samples were obtained between 2018 and 2019 from calves with acute gastroenteritis diarrhea, restlessness, loss of appetite, dry muzzle, fever, and dehydration finally deaths and aged from 5-21 days from some dairy herds in Egypt (Menofia governorate). Samples were submitted to the Animal health research institute, Virology department for laboratory diagnosis, considering biosecurity measures according to OIE Manual, 2018 (transport of biological material). Fecal samples were prepared in 0.01 M phosphate-buffered saline (PBS) (pH 7) centrifuged at 1500 x g and the supernatants were stored in sterile vials at -80 ° C (Barry et al., 2009).

*Detection of BCoV antigen in fecal samples by antigen capture ELISA*

BCoV antigen was detected in fecal samples by indirect antigen-capture sandwich ELISA (Monoscreen Ag ELISA, BIO K 344/2 Rochefort, Belgium), test procedures were performed according to manufacturing instructions.

*Molecular characterization of BCoV*

Nucleic acid was extracted from fecal samples using QIAamp mini elute virus spin kit (Qiagen, Germany, GmbH) according to manufacturing instructions. Nucleic acid was finally eluted with 100 µl of elution buffer.

RT- PCR protocol as previously described by Amer et al. (2013), targeted to N gene, by using a commercial kit (My Taq One-Step RT-PCR Kit) according to kit instructions. Primers used were (F: TGGATCAAGATTAGAGTTGGC, R: CCTTGTCCATTCTTCTGACC), expected length of the amplified sequence was 236 bp. Positive control RNA was included ready to use in the kit.

The PCR products were separated by 1.5% agarose gel electrophoresis (AppliChem, Germany, GmbH). A gene ruler 100bp DNA ladder (Fermentas, Thermo, Germany) was used to determine the fragment size. The gel was scanned by a gel documentation system (Alpha Innotech, Biometra) then the data was analyzed through computer software.

One representative sample of PCR products was purified using the QIAquick PCR Product extraction kit. (Qiagen, Valencia). Bigdye Terminator V3.1 cycle sequencing kit (Perkin-Elmer) was used for sequencing using Applied Biosystems genetic analyzer (HITACHI, Japan), to check the sequence identity; a BLAST® analysis (Basic Local Alignment Search Tool) was initially performed (Altschul et al., 1990). Then, the phylogenetic tree was constructed using MegAlign module of Lasergene DNA Star version 12.1 by maximum likelihood (Tamura et al., 2013).

*Isolation of BCoV from ELISA positive, molecular characterized samples in VERO cells*

VERO cell line (Vero-1008, ATCC-CCL-81) was used for isolation of the virus from two representative samples (PCR high positive samples). The procedures were carried out according to Hansa et al. (2014). The VERO cell line was kindly obtained from the tissue culture unit (T.C), VACSERA, Egypt. It was grown with Earl's Minimal Essential Medium (EMEM). After being inoculated with 100 µl of prepared, filtered samples; prescription flasks were incubated for 1 hour in a CO2 incubator at 37 ºC. Then maintenance media was added (10 ml at each flask). Flasks were incubated at 37 ºC with daily inspection for 7 days for observation of any cytopathic changes. Positive control virus was supplied by veterinary serum and vaccine research institute (SERVAC), Egypt. Harvest of tissue culture suspension (row virus isolate) was prepared by three repetitive cycles of freezing and thawing, centrifugation at 3000 rpm\10 min.\4 ºC, collecting the supernatant, allotment in Eppendorf tubes at -80 ºC for further passaging or examination.

*Identification of BCoV isolate by negative staining transmission electron microscopy (TEM)*

As previously described, tissue culture suspensions have been prepared. On a carbon-coated grid, drops of the acquired suspension were placed; next, the grids were drained with filter paper before drying and negatively stained with 2% phosphotungstic acid, pH 5.0, then examined with TEM at EM-Unit, Central research laboratories, Faculty of Agriculture, Cairo University and examined according to Catroxo et al., 2010.

*Identification of BCoV isolate by indirect Immuno-peroxidase test (IP) on VERO cell line*

According to Schacherer et al. (1988), the test was performed using IP kit (Power-StainTM 1.0 Poly HRP DAB Kit) for BCoV detection in VERO cells, IP was performed in 16 microwells lab-Tek glass chamber slides TM on 24h infected cells, then slides were examined for specific reactions.

## Results and Discussion

Implementation of Ag capture ELISA procedures detected BCoV antigen (Positive) in 30% of fecal samples, (6/20).

The used RT-PCR protocol detected BCoV in (4/20) 20% of fecal samples with a particular M.W band at 236 bp (Figure 1).

Partial nucleotide sequence of N-gene of one representative sample named (BCoV_EGY_AHRI_2020) was submitted to Genbank with accession number (MW173144). Analyzing the

sequence data of the aligned strain revealed that our strain is distantly identical (at the nucleotide and amino acid levels) with other strains with accessions: KX982264, KT318095, and KT318094 (France 2014), KT318094 and KT318090 (France 2013), also with the local strain; accession: MN053321 (Egypt, 2019), meanwhile; with another local strain with accession: MK153062 (Egypt 2018) it showed lower identity (99.6% at the nucleotide level and 98.7% at amino-acid level), and less identity was observed (96.2% at both nucleotide and amino acid levels) with a recent strain with accession: LC494174 (Japan 2020), As described in nucleotide, amino-acids sequence identity table (Tables 1 and 2).

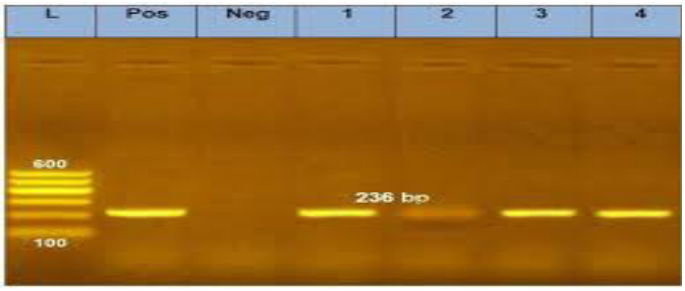


**Figure 1:** *Electrophoresis of the amplified PCR product of BCoV N-gene (M.W band at 236 bp), first Lane (L) is 100bp DNA standard ladder, the second lane is a positive control, the third one is a negative control, and lanes from 1-4 are positive samples.*

A phylogenetic tree was constructed as described (Figure 2), revealed that sequences aligned with our isolates in the present research were grouped, and more related to France isolates 2013, 2014, also with the latest local strain (Egypt 2019, Acc. No. MN053321).

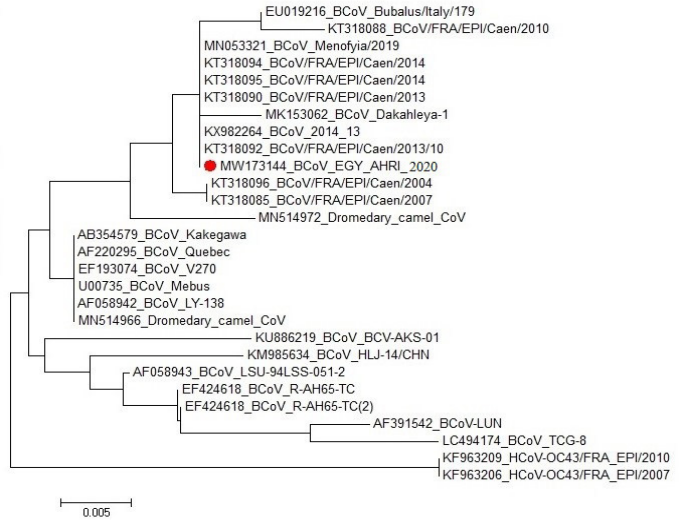


**Figure 2:** *Phylogenetic tree for the partial nucleotide sequence of BCoV N-gene (BCoV_AHRI_EGY_2020- Acc. No. MW173144, Red circled) compared with other reference strains in the Genbank database. The scale bar represents the number of substitutions per nucleotide.*

Two representative samples (Full molecular characterized positive samples) were isolated in the VERO cell line, four successive serial passages were carried out. Inoculated flasks were inspected daily for specific BCoV CPE which was completely developed within 6 days after inoculation in the first passage, decreased gradually till reached 3 days in the fourth passage. Negative control cells did not record any specific CPE in contrast with the positive control cell which observed with clear specific CPE.

The observed BCoV CPE started with rounding 24 H.P.I (hours post-inoculation), then granular, swollen, or enlarged cells within 48 H.P.I., then the membranes of the enlarged cells showed vacuolation and clumping 72 H.P.I, then were fused to form syncytia with intra-cytoplasmic inclusions 96 H.P.I (which is indicative of viral infection),followed by detachment with CPE progression, focal to diffuse cytoplasmic vacuolation was prominent 5-6 days H.P.I., CPE produced came in agreement with (El-Kenawy et al., 2019), also with those of earlier reports (Tektoff et al., 1983). CPE of inoculated cells, positive and negative controls are cleared in Figure 3.

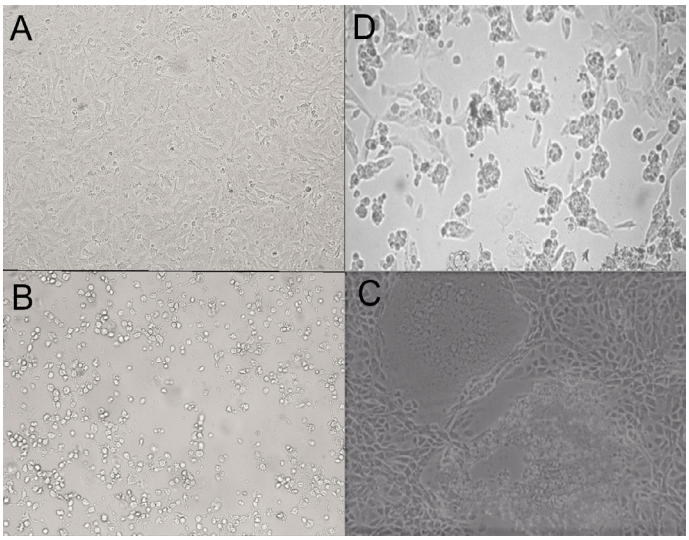


**Figure 3:** *Microscopic pictures for BCoV CPE in Vero cells (3rd passage) at 100X magnification power; image A: control uninfected Vero cells, image B: rounding of cells 24 H.P.I, granulation, image C: swelling and syncytium formation 96 H.P.I., image D: cell lysis and detachment 5 days post-inoculation.*

High loads of virus particles with marked pleomorphism were reported for the identification of two BCoV-suspected isolates (after four active serial passages), which appeared as rounded and elongated particles ranging from 72-206 nm with an average diameter of 140 nm, with characteristic radial projections, as shown in Figure 4.

**Table 1:** *Percentage of nucleotide sequence identity between BCoV_AHRI_EGY_2020 strain and BCoV reference strains published on Genbank based on partial sequence of N-gene.*

Percent Identity

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | 98.7 | 100.0 | 98.3 | 98.7 | 98.7 | 98.7 | 98.7 | 99.1 | 99.1 | 98.3 | 97.9 | 100.0 | 100.0 | 100.0 | 98.7 | 97.9 | 98.3 | 97.9 | 98.7 | 98.3 | 98.3 | 100.0 | 97.0 | 96.6 | 97.0 | 97.0 | 98.7 | 1 | AB354579 BCoV Kakegawa |
| 2 | 1.3 | | 98.7 | 97.9 | 100.0 | 100.0 | 100.0 | 100.0 | 99.6 | 99.6 | 99.6 | 99.1 | 98.7 | 98.7 | 98.7 | 98.3 | 97.4 | 97.9 | 97.4 | 100.0 | 99.6 | 98.7 | 98.7 | 96.6 | 96.2 | 95.7 | 95.7 | 100.0 | 2 | KX982264 BCoV_2014_13 |
| 3 | 0.0 | 1.3 | | 98.3 | 98.7 | 98.7 | 98.7 | 98.7 | 99.1 | 99.1 | 98.3 | 97.9 | 100.0 | 100.0 | 100.0 | 98.7 | 97.9 | 98.3 | 97.9 | 98.7 | 98.3 | 98.3 | 100.0 | 97.0 | 96.6 | 97.0 | 97.0 | 98.7 | 3 | AF220295 BCoV Quebec |
| 4 | 1.7 | 2.2 | 1.7 | | 97.9 | 97.9 | 97.9 | 97.9 | 98.3 | 98.3 | 97.4 | 97.0 | 98.3 | 98.3 | 98.3 | 99.6 | 97.9 | 100.0 | 98.7 | 97.9 | 97.4 | 97.4 | 98.3 | 98.7 | 98.3 | 96.2 | 96.2 | 97.9 | 4 | EF424618 BCoV R-AH65-TC |
| 5 | 1.3 | 0.0 | 1.3 | 2.2 | | 100.0 | 100.0 | 100.0 | 99.6 | 99.6 | 99.6 | 99.1 | 98.7 | 98.7 | 98.7 | 98.3 | 97.4 | 97.9 | 97.4 | 100.0 | 99.6 | 98.7 | 98.7 | 96.6 | 96.2 | 95.7 | 95.7 | 100.0 | 5 | KT318095 BCoV/FRA/EPI/Caen/2014 |
| 6 | 1.3 | 0.0 | 1.3 | 2.2 | 0.0 | | 100.0 | 100.0 | 99.6 | 99.6 | 99.6 | 99.1 | 98.7 | 98.7 | 98.7 | 98.3 | 97.4 | 97.9 | 97.4 | 100.0 | 99.6 | 98.7 | 98.7 | 96.6 | 96.2 | 95.7 | 95.7 | 100.0 | 6 | KT318094 BCoV/FRA/EPI/Caen/2014 |
| 7 | 1.3 | 0.0 | 1.3 | 2.2 | 0.0 | 0.0 | | 100.0 | 99.6 | 99.6 | 99.6 | 99.1 | 98.7 | 98.7 | 98.7 | 98.3 | 97.4 | 97.9 | 97.4 | 100.0 | 99.6 | 98.7 | 98.7 | 96.6 | 96.2 | 95.7 | 95.7 | 100.0 | 7 | KT318092 BCoV/FRA/EPI/Caen/2013 |
| 8 | 1.3 | 0.0 | 1.3 | 2.2 | 0.0 | 0.0 | 0.0 | | 99.6 | 99.6 | 99.6 | 99.1 | 98.7 | 98.7 | 98.7 | 98.3 | 97.4 | 97.9 | 97.4 | 100.0 | 99.6 | 98.7 | 98.7 | 96.6 | 96.2 | 95.7 | 95.7 | 100.0 | 8 | KT318090 BCoV/FRA/EPI/Caen/2013 |
| 9 | 0.9 | 0.4 | 0.9 | 1.7 | 0.4 | 0.4 | 0.4 | 0.4 | | 100.0 | 99.1 | 98.7 | 99.1 | 99.1 | 99.1 | 98.7 | 97.0 | 98.3 | 97.9 | 99.6 | 99.1 | 98.3 | 99.1 | 97.0 | 96.6 | 96.2 | 96.2 | 99.6 | 9 | KT318096 BCoV/FRA/EPI/Caen/2004 |
| 10 | 0.9 | 0.4 | 0.9 | 1.7 | 0.4 | 0.4 | 0.4 | 0.4 | 0.0 | | 99.1 | 98.7 | 99.1 | 99.1 | 99.1 | 98.7 | 97.0 | 98.3 | 97.9 | 99.6 | 99.1 | 98.3 | 99.1 | 97.0 | 96.6 | 96.2 | 96.2 | 99.6 | 10 | KT318085 BCoV/FRA/EPI/Caen/2007 |
| 11 | 1.7 | 0.4 | 1.7 | 2.6 | 0.4 | 0.4 | 0.4 | 0.4 | 0.9 | 0.9 | | 99.6 | 98.3 | 98.3 | 98.3 | 97.9 | 97.0 | 97.4 | 97.0 | 99.6 | 99.1 | 98.3 | 98.3 | 96.2 | 95.7 | 95.3 | 95.3 | 99.6 | 11 | EU019216 BCoV Bubalus/Italy/179 |
| 12 | 2.2 | 0.9 | 2.2 | 3.1 | 0.9 | 0.9 | 0.9 | 0.9 | 1.3 | 1.3 | 0.4 | | 97.9 | 97.9 | 97.9 | 97.4 | 96.6 | 97.0 | 96.6 | 99.1 | 98.7 | 97.9 | 97.9 | 95.7 | 95.3 | 94.9 | 94.9 | 99.1 | 12 | KT318088 BCoV/FRA/EPI/Caen/2010 |
| 13 | 0.0 | 1.3 | 0.0 | 1.7 | 1.3 | 1.3 | 1.3 | 1.3 | 0.9 | 0.9 | 1.7 | 2.2 | | 100.0 | 100.0 | 98.7 | 97.9 | 98.3 | 97.9 | 98.7 | 98.3 | 98.3 | 100.0 | 97.0 | 96.6 | 97.0 | 97.0 | 98.7 | 13 | EF193074 BCoV V270 |
| 14 | 0.0 | 1.3 | 0.0 | 1.7 | 1.3 | 1.3 | 1.3 | 1.3 | 0.9 | 0.9 | 1.7 | 2.2 | 0.0 | | 100.0 | 98.7 | 97.9 | 98.3 | 97.9 | 98.7 | 98.3 | 98.3 | 100.0 | 97.0 | 96.6 | 97.0 | 97.0 | 98.7 | 14 | U00735 BCoV Mebus |
| 15 | 0.0 | 1.3 | 0.0 | 1.7 | 1.3 | 1.3 | 1.3 | 1.3 | 0.9 | 0.9 | 1.7 | 2.2 | 0.0 | 0.0 | | 98.7 | 97.9 | 98.3 | 97.9 | 98.7 | 98.3 | 98.3 | 100.0 | 97.0 | 96.6 | 97.0 | 97.0 | 98.7 | 15 | AF058942 BCoV LY-138 |
| 16 | 1.3 | 1.7 | 1.3 | 0.4 | 1.7 | 1.7 | 1.7 | 1.7 | 1.3 | 1.3 | 2.2 | 2.6 | 1.3 | 1.3 | 1.3 | | 97.4 | 99.6 | 98.3 | 98.3 | 97.9 | 97.9 | 98.7 | 98.3 | 97.9 | 95.7 | 95.7 | 98.3 | 16 | AF058943 BCoV LSU-94LSS-051-2 |
| 17 | 2.2 | 2.6 | 2.2 | 2.2 | 2.6 | 2.6 | 2.6 | 2.6 | 3.1 | 3.1 | 3.1 | 3.5 | 2.2 | 2.2 | 2.2 | 2.6 | | 97.9 | 97.4 | 97.4 | 97.0 | 97.9 | 97.9 | 96.6 | 96.2 | 95.7 | 95.7 | 97.4 | 17 | KU886219 BCoV BCV-AKS-01 |
| 18 | 1.7 | 2.2 | 1.7 | 0.0 | 2.2 | 2.2 | 2.2 | 2.2 | 1.7 | 1.7 | 2.6 | 3.1 | 1.7 | 1.7 | 1.7 | 0.4 | 2.2 | | 98.7 | 97.9 | 97.4 | 97.4 | 98.3 | 98.7 | 98.3 | 96.2 | 96.2 | 97.9 | 18 | EF424618 BCoV R-AH65-TC |
| 19 | 2.2 | 2.6 | 2.2 | 1.3 | 2.6 | 2.6 | 2.6 | 2.6 | 2.2 | 2.2 | 3.1 | 3.5 | 2.2 | 2.2 | 2.2 | 1.7 | 2.6 | 1.3 | | 97.4 | 97.0 | 97.0 | 97.9 | 97.4 | 97.0 | 95.7 | 95.7 | 97.4 | 19 | KM985634 BCoV HLJ-14/CHN |
| 20 | 1.3 | 0.0 | 1.3 | 2.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.4 | 0.4 | 0.4 | 0.9 | 1.3 | 1.3 | 1.3 | 1.7 | 2.6 | 2.2 | 2.6 | | 99.6 | 98.7 | 98.7 | 96.6 | 96.2 | 95.7 | 95.7 | 100.0 | 20 | MN053321 BCoV Menofyia/2019 |
| 21 | 1.7 | 0.4 | 1.7 | 2.6 | 0.4 | 0.4 | 0.4 | 0.4 | 0.9 | 0.9 | 0.9 | 1.3 | 1.7 | 1.7 | 1.7 | 2.2 | 3.1 | 2.6 | 3.1 | 0.4 | | 98.3 | 98.3 | 96.2 | 95.7 | 95.3 | 95.3 | 99.6 | 21 | MK153062 BCoV Dakahleya-1 |
| 22 | 1.7 | 1.3 | 1.7 | 2.6 | 1.3 | 1.3 | 1.3 | 1.3 | 1.7 | 1.7 | 1.7 | 2.2 | 1.7 | 1.7 | 1.7 | 2.2 | 2.2 | 2.6 | 3.1 | 1.3 | 1.7 | | 98.3 | 96.2 | 95.7 | 95.3 | 95.3 | 98.7 | 22 | MN514972 Dromedary camel CoV |
| 23 | 0.0 | 1.3 | 0.0 | 1.7 | 1.3 | 1.3 | 1.3 | 1.3 | 0.9 | 0.9 | 1.7 | 2.2 | 0.0 | 0.0 | 0.0 | 1.3 | 2.2 | 1.7 | 2.2 | 1.3 | 1.7 | 1.7 | | 97.0 | 96.6 | 97.0 | 97.0 | 98.7 | 23 | MN514966 Dromedary camel CoV |
| 24 | 3.1 | 3.5 | 3.1 | 1.3 | 3.5 | 3.5 | 3.5 | 3.5 | 3.1 | 3.1 | 4.0 | 4.4 | 3.1 | 3.1 | 3.1 | 1.7 | 3.5 | 1.3 | 2.6 | 3.5 | 4.0 | 4.0 | 3.1 | | 98.7 | 94.9 | 94.9 | 96.6 | 24 | AF391542 BCoV-LUN |
| 25 | 3.5 | 4.0 | 3.5 | 1.7 | 4.0 | 4.0 | 4.0 | 4.0 | 3.5 | 3.5 | 4.4 | 4.9 | 3.5 | 3.5 | 3.5 | 2.2 | 4.0 | 1.7 | 3.1 | 4.0 | 4.4 | 4.4 | 3.5 | 1.3 | | 94.4 | 94.4 | 96.2 | 25 | LC494174 BCoV TCG-8 |
| 26 | 3.1 | 4.4 | 3.1 | 4.0 | 4.4 | 4.4 | 4.4 | 4.4 | 4.0 | 4.0 | 4.9 | 5.3 | 3.1 | 3.1 | 3.1 | 4.4 | 4.4 | 4.0 | 4.4 | 4.4 | 4.9 | 4.9 | 3.1 | 5.4 | 5.8 | | 100.0 | 95.7 | 26 | KF963209 HCoV-OC43/FRA_EPI/2010 |
| 27 | 3.1 | 4.4 | 3.1 | 4.0 | 4.4 | 4.4 | 4.4 | 4.4 | 4.0 | 4.0 | 4.9 | 5.3 | 3.1 | 3.1 | 3.1 | 4.4 | 4.4 | 4.0 | 4.4 | 4.4 | 4.9 | 4.9 | 3.1 | 5.4 | 5.8 | 0.0 | | 95.7 | 27 | KF963206 HCoV-OC43/FRA_EPI/2007 |
| 28 | 1.3 | 0.0 | 1.3 | 2.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.4 | 0.4 | 0.4 | 0.9 | 1.3 | 1.3 | 1.3 | 1.7 | 2.6 | 2.2 | 2.6 | 0.0 | 0.4 | 1.3 | 1.3 | 3.5 | 4.0 | 4.4 | 4.4 | | 28 | MW173144 BCoV_EGY_AHRI_2018 |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | | |

Divergence

**Table 2:** *Percentage of amino acids sequence identity between BCoV_AHRI_EGY_2020 strain and BCoV reference strains published on Genbank based on partial sequence of N-gene.*

Percent Identity

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | 98.7 | 100.0 | 100.0 | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 98.7 | 97.4 | 100.0 | 100.0 | 98.7 | 97.4 | 97.4 | 97.4 | 98.7 | 1 | AB354579 BCoV Kakegawa |
| 2 | 1.3 | | 98.7 | 98.7 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 100.0 | 98.7 | 98.7 | 98.7 | 97.4 | 96.2 | 96.2 | 96.2 | 100.0 | 2 | KX982264 BCoV_2014_13 |
| 3 | 0.0 | 1.3 | | 100.0 | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 98.7 | 97.4 | 100.0 | 100.0 | 98.7 | 97.4 | 97.4 | 97.4 | 98.7 | 3 | AF220295 BCoV Quebec |
| 4 | 0.0 | 1.3 | 0.0 | | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 98.7 | 97.4 | 100.0 | 100.0 | 98.7 | 97.4 | 97.4 | 97.4 | 98.7 | 4 | EF424618 BCoV R-AH65-TC |
| 5 | 1.3 | 0.0 | 1.3 | 1.3 | | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 100.0 | 98.7 | 98.7 | 98.7 | 97.4 | 96.2 | 96.2 | 96.2 | 100.0 | 5 | KT318095 BCoV/FRA/EPI/Caen/2014 |
| 6 | 1.3 | 0.0 | 1.3 | 1.3 | 0.0 | | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 100.0 | 98.7 | 98.7 | 98.7 | 97.4 | 96.2 | 96.2 | 96.2 | 100.0 | 6 | KT318094 BCoV/FRA/EPI/Caen/2014 |
| 7 | 1.3 | 0.0 | 1.3 | 1.3 | 0.0 | 0.0 | | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 100.0 | 98.7 | 98.7 | 98.7 | 97.4 | 96.2 | 96.2 | 96.2 | 100.0 | 7 | KT318092 BCoV/FRA/EPI/Caen/2013 |
| 8 | 1.3 | 0.0 | 1.3 | 1.3 | 0.0 | 0.0 | 0.0 | | 100.0 | 100.0 | 100.0 | 100.0 | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 100.0 | 98.7 | 98.7 | 98.7 | 97.4 | 96.2 | 96.2 | 96.2 | 100.0 | 8 | KT318090 BCoV/FRA/EPI/Caen/2013 |
| 9 | 1.3 | 0.0 | 1.3 | 1.3 | 0.0 | 0.0 | 0.0 | 0.0 | | 100.0 | 100.0 | 100.0 | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 100.0 | 98.7 | 98.7 | 98.7 | 97.4 | 96.2 | 96.2 | 96.2 | 100.0 | 9 | KT318096 BCoV/FRA/EPI/Caen/2004 |
| 10 | 1.3 | 0.0 | 1.3 | 1.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | | 100.0 | 100.0 | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 100.0 | 98.7 | 98.7 | 98.7 | 97.4 | 96.2 | 96.2 | 96.2 | 100.0 | 10 | KT318085 BCoV/FRA/EPI/Caen/2007 |
| 11 | 1.3 | 0.0 | 1.3 | 1.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | | 100.0 | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 100.0 | 98.7 | 98.7 | 98.7 | 97.4 | 96.2 | 96.2 | 96.2 | 100.0 | 11 | EU019216 BCoV Bubalus/Italy/179 |
| 12 | 1.3 | 0.0 | 1.3 | 1.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 98.7 | 100.0 | 98.7 | 98.7 | 98.7 | 97.4 | 96.2 | 96.2 | 96.2 | 100.0 | 12 | KT318088 BCoV/FRA/EPI/Caen/2010 |
| 13 | 0.0 | 1.3 | 0.0 | 0.0 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 98.7 | 97.4 | 100.0 | 100.0 | 98.7 | 97.4 | 97.4 | 97.4 | 98.7 | 13 | EF193074 BCoV V270 |
| 14 | 0.0 | 1.3 | 0.0 | 0.0 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 0.0 | | 100.0 | 100.0 | 100.0 | 100.0 | 100.0 | 98.7 | 97.4 | 100.0 | 100.0 | 98.7 | 97.4 | 97.4 | 97.4 | 98.7 | 14 | U00735 BCoV Mebus |
| 15 | 0.0 | 1.3 | 0.0 | 0.0 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 0.0 | 0.0 | | 100.0 | 100.0 | 100.0 | 100.0 | 98.7 | 97.4 | 100.0 | 100.0 | 98.7 | 97.4 | 97.4 | 97.4 | 98.7 | 15 | AF058942 BCoV LY-138 |
| 16 | 0.0 | 1.3 | 0.0 | 0.0 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 0.0 | 0.0 | 0.0 | | 100.0 | 100.0 | 100.0 | 98.7 | 97.4 | 100.0 | 100.0 | 98.7 | 97.4 | 97.4 | 97.4 | 98.7 | 16 | AF058943 BCoV LSU-94LSS-051-2 |
| 17 | 0.0 | 1.3 | 0.0 | 0.0 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 0.0 | 0.0 | 0.0 | 0.0 | | 100.0 | 100.0 | 98.7 | 97.4 | 100.0 | 100.0 | 98.7 | 97.4 | 97.4 | 97.4 | 98.7 | 17 | KU886219 BCoV BCV-AKS-01 |
| 18 | 0.0 | 1.3 | 0.0 | 0.0 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | | 100.0 | 98.7 | 97.4 | 100.0 | 100.0 | 98.7 | 97.4 | 97.4 | 97.4 | 98.7 | 18 | EF424618 BCoV R-AH65-TC |
| 19 | 0.0 | 1.3 | 0.0 | 0.0 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | | 98.7 | 97.4 | 100.0 | 100.0 | 98.7 | 97.4 | 97.4 | 97.4 | 98.7 | 19 | KM985634 BCoV HLJ-14/CHN |
| 20 | 1.3 | 0.0 | 1.3 | 1.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | | 98.7 | 98.7 | 98.7 | 97.4 | 96.2 | 96.2 | 96.2 | 100.0 | 20 | MN053321 BCoV Menofyia/2019 |
| 21 | 2.6 | 1.3 | 2.6 | 2.6 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 2.6 | 2.6 | 2.6 | 2.6 | 2.6 | 2.6 | 2.6 | 1.3 | | 97.4 | 97.4 | 97.4 | 96.2 | 94.9 | 94.9 | 98.7 | 21 | MK153062 BCoV Dakahleya-1 |
| 22 | 0.0 | 1.3 | 0.0 | 0.0 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.3 | 2.6 | | 100.0 | 98.7 | 97.4 | 97.4 | 97.4 | 98.7 | 22 | MN514972 Dromedary camel CoV |
| 23 | 0.0 | 1.3 | 0.0 | 0.0 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.3 | 2.6 | 0.0 | | 98.7 | 97.4 | 97.4 | 97.4 | 98.7 | 23 | MN514966 Dromedary camel CoV |
| 24 | 1.3 | 2.6 | 1.3 | 1.3 | 2.6 | 2.6 | 2.6 | 2.6 | 2.6 | 2.6 | 2.6 | 2.6 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 2.6 | 2.6 | 1.3 | 1.3 | | 98.7 | 96.2 | 96.2 | 97.4 | 24 | AF391542 BCoV-LUN |
| 25 | 2.6 | 4.0 | 2.6 | 2.6 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 2.6 | 2.6 | 2.6 | 2.6 | 2.6 | 2.6 | 2.6 | 4.0 | 4.0 | 2.6 | 2.6 | 1.3 | | 94.9 | 94.9 | 96.2 | 25 | LC494174 BCoV TCG-8 |
| 26 | 2.6 | 4.0 | 2.6 | 2.6 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 2.6 | 2.6 | 2.6 | 2.6 | 2.6 | 2.6 | 2.6 | 4.0 | 5.3 | 2.6 | 2.6 | 4.0 | 5.3 | | 100.0 | 96.2 | 26 | KF963209 HCoV-OC43/FRA_EPI/2010 |
| 27 | 2.6 | 4.0 | 2.6 | 2.6 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 2.6 | 2.6 | 2.6 | 2.6 | 2.6 | 2.6 | 2.6 | 4.0 | 5.3 | 2.6 | 2.6 | 4.0 | 5.3 | 0.0 | | 96.2 | 27 | KF963206 HCoV-OC43/FRA_EPI/2007 |
| 28 | 1.3 | 0.0 | 1.3 | 1.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 1.3 | 0.0 | 1.3 | 1.3 | 1.3 | 2.6 | 4.0 | 4.0 | 4.0 | | 28 | MW173144 BCoV_EGY_AHRI_2018 |
| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | | |

Divergence

Identification of the isolates referred to above (TEM identified); followed by the immune peroxidase (IP) test, which is considered positive if stained cells have been detected. The number of cells stained increased with incubation; the maximum recovery was reached in the time period between 1 and 8 h; the negative control cells were not stained, while the samples tested displayed brownish coloration, which

gradually increased with incubation time (Figure 5), an incubation of at least 18 h was chosen.

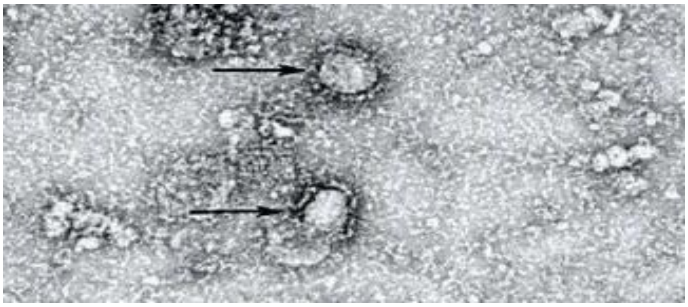

**Figure 4:** *Image of BCoV visualized by TEM (negative staining) showing characteristic radial projections forming a crown shape like pointed by the black arrow, with an average of 140nm diameter confirming identity of BCoV isolate.*

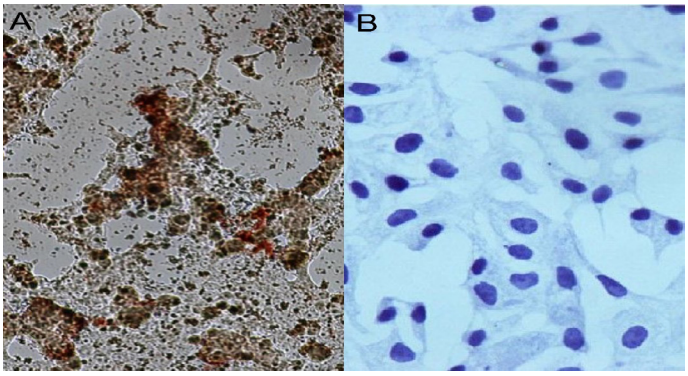


**Figure 5:** *The image of IP test on BCoV isolate in Vero cells 18 H.P.I, image A: tested sample at 200X MP showed a high average of brownish stained cells confirming virus identity, where image B: negative control cells at 400X MP were not stained.*

The goal of this study was to detect Betacoronavirus 1 BCoV antigen in tested samples through Ag capture ELISA and RT-PCR, complete molecular characterization of positive PCR samples, then isolation and identification of circulating strain on cell culture, in order to prepare a new local Betacoronavirus 1 BCoV strain; full molecularly characterized to be further used in diagnostics and vaccine solutions. In Egypt, detection of Betacoronavirus 1coronavirus antigen in fecal samples obtained from newborn buffalo calves, bovine-like CoVs were detected in the faeces of suckling buffalo calves with profuse diarrhoea in Egypt using monoclonal antibody (MAb)-based enzyme-linked immunosorbent assay (ELISA) and immunoperoxidase assays (Abd El-Karim et al., 1990).

In egypt, Detection of coronavirus antigen in fecal samples obtained from newborn buffalo calves, bovine-like CoVs were detected in the faeces of suckling buffalo calves with profuse diarrhoea in Egypt using monoclonal antibody (MAb)-based enzyme-linked immunosorbent assay (ELISA) and immunoperoxidase assays (Abd El-Karim et al., 1990).

The CoV particles were identified in two further studies in Egypt using negative-contrast transmission electron microscopy (EM) in 20 and 55.2% of the faecal samples collected from diarrhoeic buffalo calves, and 8.3 and 19.1% of the faecal samples collected from apparently healthy buffalo calves, respectively (Byomi et al., 1996; Abd El-Rahim, 1997). The epidemiological data presented by the Tri-national Health Research Project, USAID, indicated that CoV is the second most-common pathogen causing diarrhoea in buffalo calves in Egypt with a prevalence rate of 37.7% (Saleh, 1994). The different bovine-like CoVs that have been identified in domesticated ruminants (water buffalo, sheep, goat, dromedary camel, llama and alpaca) and wild ruminants (deer, wild cattle, antelopes, giraffes and wild goats) are discussed in terms of epidemiology, transmission and virus characteristics. The presented data denote the importance of these viruses in the persistence of BCoV in nature, spread to new geographical zones, and continuous emergence of disease epidemics in cattle farms (Amer, 2018).

In the present study, a total of 20 fecal samples were collected from diarrheic calves aged up to 21 days during the calving season of 2018 and 2019, which had clinical findings like Betacoronavirus 1 BCoV as described by Lefèvre et al. (2010).

All samples were screened by BCoV antigen capture ELISA, this test was used due to its high sensitivity, and because of most of immunodiagnostic tests produced for enteric pathogen have either low specificity and/or sensitivity (Selim et al., 1991; Kelkar et al., 2004).

Thirty percent of fecal samples were tested positive by Ag capture ELISA, this overall prevalence is considerably high, suggesting that Betacoronavirus 1 BCoV has a significant position in the epidemiology of neonatal calf diarrhea in Egypt, with a prevalence of only 3.7 percent in the latest local prevalence study (Egypt) on BCoV revealed (El-Kenawy et al., 2019).

The effect can be explained by decreasing passive immunity due to numerous possible causes, lack of natural resistance to infection due to lack of protocols for vaccination, as mentioned by (Uhde et al., 2008;

Ammar et al., 2014).

For further clarification, a molecular tool (RT- PCR) was performed for rapid detection and confirmation of ELISA results. Standardization of RT-PCR was done according to Amer et al. (2013), by selecting viral N-gene-based primers as a conserved region among BCoV strains.

The correlation between the above-mentioned tests results even though the difference in overall prevalence in tested samples; possible explanations could be due to lower specificity of ELISA compared with RT PCR assay (Cho et al., 2001).

Phylogenetic analysis of tested sample suggests stability and minimum evolution rate for the current circulating BCoV strain due to nearly complete identity with the previous local samples of 2018 and 2019 Betacoronavirus 1 BCoV strains.

Both ELISA and PCR data indicate that further statistically designed surveys are needed to systematically evaluate Betacoronavirus 1 BCoV local prevalence as well as other enteropathogens associated with neonatal calf diarrhea in Egypt (Cho and Yoon, 2014).

Betacoronavirus 1 BCoV isolation is little used as a means of diagnosis, as it is difficult, requires well trained skilled analyst, however; BCoV can be readily grown on large no. of cell lines including VERO cell line which is used in our study according to (Clark, 1993; Benfield and Saif, 1990; Tsunemitsu and Saif, 1995). The Vero cell line was explicitly selected for isolation as it is said to produce consistent CPE; agreed with Tektoff et al. (1983). We selected two samples for isolation due to the difficulty of viral adaptation and replication on tissue culture, which linearly increases with no. of samples for isolation, which came in agreement with Hansa et al. (2014).

As Brandt et al. (1981), stated earlier, TEM (negative staining technique) is widely used for the detection and identification of BCoV based on morphological characteristics, this characteristic morphology evinced the identity of BCoV, which agreed with an early previous report by (Stair et al., 1972).

IP test results came in harmony with Katz et al. (1987); who cleared that the indirect IP staining technique was used to identify BCoV cell culture-based isolates.

## Conclusions and Recommendations

Prevalence of Betacoronavirus 1 BCoV is increasing alarmingly, from 3.7% to 30% within 2 years between both recent studies (as mentioned), which requires further broad, statistically designed observational analytical epidemiological studies (cohort studies) to systematically evaluate, follow up BCoV situation and detect any evolution in circulating strain.

Vaccination programs with an efficient vaccine are required to raise passive immunity for calves and initiate an effective immune response in adults.

Detection of Betacoronavirus 1 BCoV by indirect antigen capture ELISA is a rapid sensitive tool for screening of fecal samples, but false-positive results may develop.

The VERO cell line can be used for BCoV isolation, in which coronavirus produces a characteristic CPE, however; not all strains can be readily adapted for growth in VERO cells, which reveals a kind of strain variation, which needs focused studies among antigenic and genomic levels.

## Novelty Statement

This manuscript entitled "Isolation and Molecular Characterization of Bovine Coronavirus from Calves with Acute Gastroenteritis in Egypt" by Moussa S.A et al., intended to antigenic and molecular characterization of betacoronavirus 1
(bovine coronavirus, BCoV) in newborn gastroenteritis calves in the Delta Region, in Egypt , that enabled following up the virus activity and circulation through large sector of the country within two years of sampling process.
also it was performed for isolation and identification of the local circulating strain for further research or to used in vaccination protocols, that has a potential application on clearance of the viral activity and lowering the costs of imported vaccines.

## Author's Contribution

**SAM:** Developed the theory.
**AFA and SS:** carried out the experiment and wrote the manuscript.

**AH:** conceived the presented data and revised the manuscript.

*Conflict of interests*

The authors have declared no conflict of interest.

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