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MOLECULAR CHARACTERIZATION AND PHYLOGENIC ANALYSIS OF CORONAVIRUS IN DIARRHEIC CALVES USING PARTIAL SEQUENCE N GENE FROM SULAYMANIYAH, IRAQ

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ABSTRACT

Diarrhea is one of the common causes of death in calf; it has significant economic impacts in cattle industry. Calf diarrhea is most complicated and has many factors, for example, environmental, management and nutritional. Many viruses like rotavirus, bovine viral diarrhea and corona virus play a very important role in causing diarrhea. Sixty pre-weaning calves were sampled. Samples were taken from four different places at Sulaimaniyah province (Kalar, HalbjaiShahid, Khurmal, and Sharazor) between September 2022 and March 2023.The animals were divided into 3 age groups: first group was (4–30 days), second group was (31–60 days), and third group was (61–90 days). Samples were being collected directly from diarrheic calves by rectal swab for PCR testing. A fragment of the partial N gene was amplified using the Taq DNA master mix (Addbio, Republic of Korea). Phylogenetic trees were constructed based on a partial fragment of the partial N gene. The results showed that the prevalence of bovine coronavirus was (10%) 6 out of 60 collected samples; in addition, the highest prevalence was in group 1 (4-30) day of age, while the lowest prevalence rate was in group 3 (61-90) days of age. This study reported the occurrence of Coronavirus for the first time in four different places in Sulaimaniyah province, northern Iraq, using genetic analysis.

Key word: Corona virus, Calf, Diarrhea, phylogenic analysis.

INTRODUCTION

Bovine corona virus (BCoV) is one of the common causes of calf diarrhea during neonatal period in beef and dairy calves (Ammar *et al.*, 2014; Lotfollahzadeh, Madadgar, Reza Mohebbi, Reza Mokhber Dezfouli, & George Watson, 2020; Singh *et al.*, 2020). BCoV is a worldwide disease in cattle industry and can cause severe infection in neonates. Winter dysentery and respiratory disease can be mentioned as part of bovine respiratory disease and characterize by high morbidity, mortality and growth impedance, drop of milk production and treatment costs with fate such as delayed first calving (Brunauer, Roch, & Conrady, 2021; Kim, Cho, Shin, Park, & Choi, 2022). The mortality rate due to calf diarrhea that reported in Korea was (53.4%) in dairy calves (Amer, 2018; Y.I. Cho & Yoon, 2014). Based on several studies, the incidence risks of diarrhea varies between 15% and 20% (Ammar *et al.*, 2014). Bovine corona virus invades epithelial tissue of the small, large intestines and respiratory epithelium leading to atrophy of villa and crypts of intestine in infected animals with necrosis of lamina

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propria (Aydin *et al.*, 2022). BCoV is belonging to order Nidovirales, Coronaviridae family, subfamily Orthocoronavirinae, genus Betacorona virus, and subgenus Embeco virus which consist of a single-stranded, non-segmented RNA positive-sense with lipid envelope, BCoV replicate in the cytoplasm (Symes *et al.*, 2018; Takiuchi, Stipp, Alfieri, & Alfieri, 2006; Zhu, Li, & Sun, 2022). As well as recent report has reclassified BCoV as Betacorona virus one species, together with human, dog, horse and procine Coronavirus sharing same genetic descriptions. BCoV has three antigenic group; group A has no hemagglutinin-esterase (HE) common member of this group are feline infectious peritonitis and transmissible gastroenteritis virus in swine; group 2 has HE and include BCoV while Group 3 comprise avian virus like infectious bronchitis(D. M. Foster & Smith, 2009; Mansour, Hasso, & Al Rodhan, 2013; Mohamed, Mansour, El-Araby, Mor, & Goyal, 2016). BCoV infection suggesting possible cross-species transmissions between human, alpacas, wild ruminant and dog (Kim *et al.*, 2022). The rate of BCoV depending on many risk factors such as different geographical regions, neonatal calve immunity, seasonal effects and farm management (Mansour *et al.*, 2013). The common methods for BCoV infection diagnosis are viral culture, ELISA, hemagglutination, and PCR and the more recent one pancorona virus reverse transcription RT- PCR assay (Pan CoV-RT-PCR), which was used for identify human CoV from samples of people with respiratory diseases (Gomez, Arroyo, Poljak, Viel, & Weese, 2017). No study has been conducted in our region about neonatal calf diarrhea by Corona virus. Hence, the purpose of this study was to detect and identify the causes of calf diarrhea in Sulaimaniyah Iraq. Sulaymaniyah is one of the biggest cities in Iraq and is located in the North-East. The city includes several districts and many villages with big population of cattle. Different outbreaks of diarrhea in calves have recently occurred. However, a definitive molecular characterization of the causative agent has not been followed by molecular techniques. In this study, we detected and characterized the gene and conducted a phylogenetic analysis of the sequences with different isolates that were published in GenBank databases.

MATERIALS AND METHODS

Sample collection

Fecal samples were collected from 60 pre-weaning calves with diarrhea in four different locations around the Sulaimaniyah province (Kalar, Halbjaishahid, Khurmal and Sharazor) between September, 2022 to March 2023. Animals were indore had a good nutrition with sufficient colostrum and their dam were not vaccinated. Animals were divided into 3 age groups, first group (4-30 days), second group (31-60 days) and third group (61-90 days). All samples were being collected directly from diarrheic calves by rectal swab for RT-PCR. Label was being put to record complete collection data. An index card was then filled for each animal indicating the following data: sampling date, address, breed of animal, clinical signs of diarrhea and dehydration (age, weight, body temperature, respiratory and heart rates) and the number of animal identification. Then the samples were transported to the Sulaimaniyah Veterinary Laboratory in cold packs within hours from collection and stored at -20°C freezer until analyzed.

Sample preparation

Viral nucleic acid extraction

Viral nucleic acid extraction Kit is used to extract the Genomic viral RNA (Genaid, republic korea) according to the manufacturer's protocol. Basically, 200µl of fecal samples were suspended in 500µl PBS. 200µl of fecal suspension were added to 400µlRB buffer. After 10 min, the process followed by adding 700µl of loading buffer in room temperature. The mixture was then applied to a RB spin column followed by centrifugation at 15,000 RPM for 1 min. Loaded RNA was then washed two times using solution W1 and washing buffer, before elution. The extracted RNA was either used

immediately for one-step RT-PCR or was kept at -70 °C.

RT-PCR and sequencing

For the current study we used one sets of primers, that used for amplifying partial N gene, BCoV-F(509-531) AGGCTATTCCGACTAGGTTTCCG and BCoV-R (1185-1207) GTCCRTTCTTCTGRCCRCGMTGA was generated 700 bp(Y.-I. Cho, Han, J.-I., Wang, C., Cooper, V., Schwartz, K., Engelken, T., & Yoon, K.-J., (2013)). Suprime Script RT-PCR Premix has been used to amplify target of the N gene's sequence. A complete solution for quick, efficient, and dependable single tube one-step RT-PCR was provided by this kit. (Genet Bio. Republic Korea). For polymerase chain reaction, PCR was mixed to a final volume of 20 µl. The thermal cycler was used for amplification (Hercuvan Lab Systems, Cambridge, UK). The following processes were used in the PCR: 30 min of cDNA synthesis at 50 °C, followed by 10 min of initial denaturation at 95 °C, 40 cycles of 95 °C for 30 sec, 30 sec of annealing at 56 °C, 40 sec of extension at 72 °C, and 10 min of final extension at 72 °C(Y.-I. Cho, Han, J.-I., Wang, C., Cooper, V., Schwartz, K., Engelken, T., & Yoon, K.-J., (2013)).

Gel electrophoresis

After electrophoresis on 1% agarose gels stained with a safe dye (Urex. Poland) under 100 volt for 60 minutes, the obtained PCR products were visualized under UV light transilluminator. The expected PCR product for bovine coronavirus target gene was 700 bp.

Sequencing and phylogenetic tree

Two PCR positive samples were used for sequencing (Macrogen, Republic of Korea). The samples were then submitted to the national center for bioinformatics and information NCBI/GenBank. The Sequences were then assembled by the MEGA7 software. The Clusta W Multiple sequence alignment tool used to trim and align the N gene (M. D. Foster, & Smith, G., Veterinary Clinics of North America: Food and Animal Practice) The Neighbor joining method was used to infer the phylogenetic and evolutionary tree(Gulliksen, 2009). With The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates)(Lee, (2019, octobar 25)). The evolutionary distances were computed using the Kimura3-Parameter method(Schroeder, 2012).

Sequence Registrations:

In this study, two partial N gene sequences from field viruses were submitted to GenBank employing the accession numbers named (OQ744522 and OQ744523).

RESULT

This study was conducted in four different locations around the Sulaimaniyah province (Kalar, Halbjaishahid and Khurmal and Sharazor), between September 2022 to April 2023, The animals were divided into 3 age groups first group (4-30 days), second group (31-60 days) and third group (61-90 days). The samples were being collected directly from diarrheic calves by rectal swab for RT-PCR. In present study most calves revealed diarrhea with or without blood, with dehydration and loss of weight, the prevalence of BCoV was 10% 6 out of 60collected samples, in addition to as showed in (Table 1), the highest positive percent was in group 1, while the lowest prevalence rate was in group 3.

Table 1: Prevalence rate of BCoV in related with Age.

Age (per day)	Number of samples	PCR Positive infection (%)
Group 1(4 to 30)	20	3 (15%)
Group 2(31 to 60)	20	2 (10%)
Group 3 (61 to 90)	20	1 (5%)
Total	60	6 (10%)

RT-PCR results

RT-PCR is used for detecting viral nucleic acids. A band of 700 base pairs, which is associated with the partial BCV N gene amplification, was found in 6of 60samples tested as positive by RT-PCR (Figure 1).

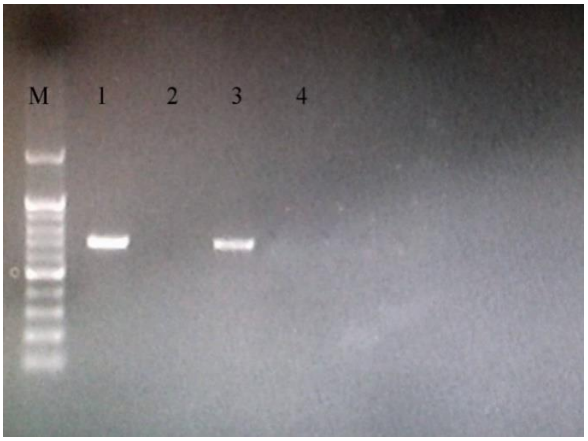

Figure 1: Agarose gel shows amplification of BCV partial sequence N gene amplicon size 700 bp,
Lane M: DNA ladder for 100 bp, Lane 1, and 3 positive sample, Lane 2, 4: Negative sample.

Sequencing result:

Sequencing was done in two samples of PCR products. The strains were named (OQ744522-BCV HF1/23) and (OQ744523-BCV HF2/23) after sequencing. Our strains were connected to the BCV sources in the GenBank to determine their genetic similarity (Table2).

Sequences analysis

The detected BCV two field sequences were highly homologous, with 99–86% identity and 100% amino acid identical, and showed minimal variation.

Genetic analysis of Sulaimani virus with sequence data available in GenBank® in difference countries, showed that highest identity with china, Brazil and Russia strains range (99.42% and 99.32%) respectively (Table 2).

Phylogenetic tree analysis

Phylogenetic algorithms were employed to generate a phylogenetic tree, and the Maximum-Likelihood (ML) tree is illustrated in (Figure 3). All 28 isolates from the N gene were found to be divided into three tree groups (Asian/USA, Classical, and French). The phylogenetic analysis of partial N gene sequence of bovine coronavirus, including two field virus strains (BCV HF1/2023 and BCV HF2/2023) in presented study, showed that the two field virus sequences belonged to the Asia/USA group and clustered with the strains MK095169.1-SWUN/LN4/2018, OL840252.1-KCD_GJ4_2021, and EU401984.1-solate_SUN5.

The partial N gene of both Sulaimani sequences was aligned and compared with referent strains from various countries.

Sequence analysis revealed a single amino acid substitution (Isoleucine) instead of (Methionine) at position 218. (Figure 3). This result serves as an indicator of resistance and genetic evolution in this region.

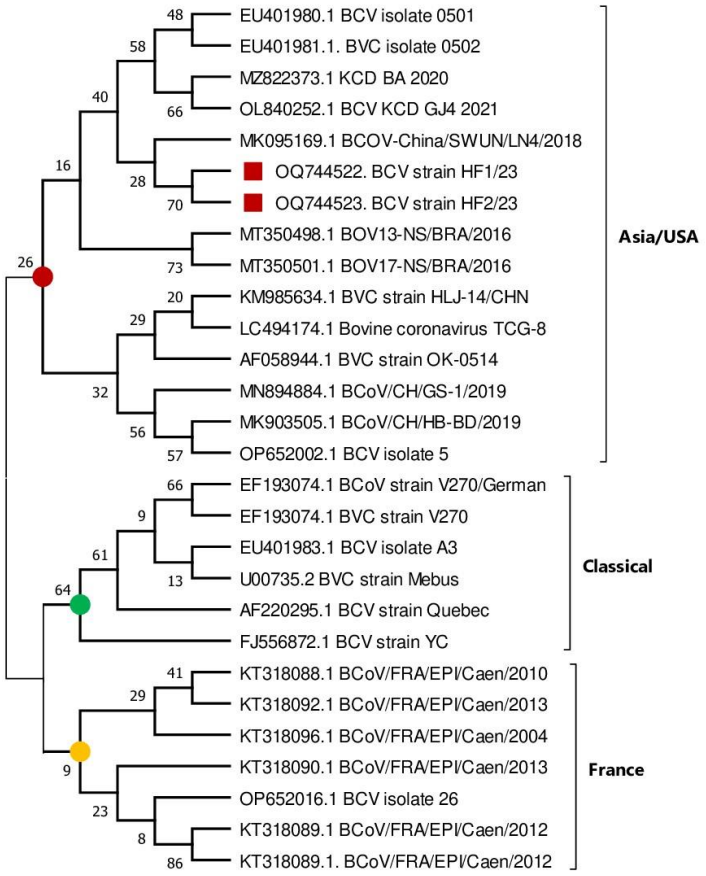

Figure 2: Phylogenetic tree constructed by using Maximum Likelihood (ML),and the nucleotide sequences for the Partial N coding regions of BCV. Iraq/Sulaimani field strains are represented by the red square.

Table 2: Amino acid and DNA sequence identities of nucleocapsid gene of field BCV strains (BCV.HF/2023) with difference strains available Gen-Bank.

Accession No.	Strain/isolate	countries	DNA identity	Group
OL840252.1	KCD_GJ4_2021	Republic Korea	97.84-98%	Asia/USA
LC494174	TCG-8	Japan	98.56%	Asia/USA
MK095169.1	SWUN/LN4/2018	China	99.28 - 99.42%	Asia/USA
U00735.2	BVC_strain_Mebus	Tennessee/USA	98.40-98.55%	Classical
EF193074.1	BVC_strain_V270	Germany	98.27-98.42%	Classical
MT350498.1	NS/BRA/2016	Brazil	99.32%	Asia/USA
OP652002	BCV_isolate_5	Russia	99.32	Asia/USA
OP652016.1	Isolate 26	Russia	98,80%	France
KT318089.1	FRA/EPI/Caen/201	France	98.56-98.70%	France

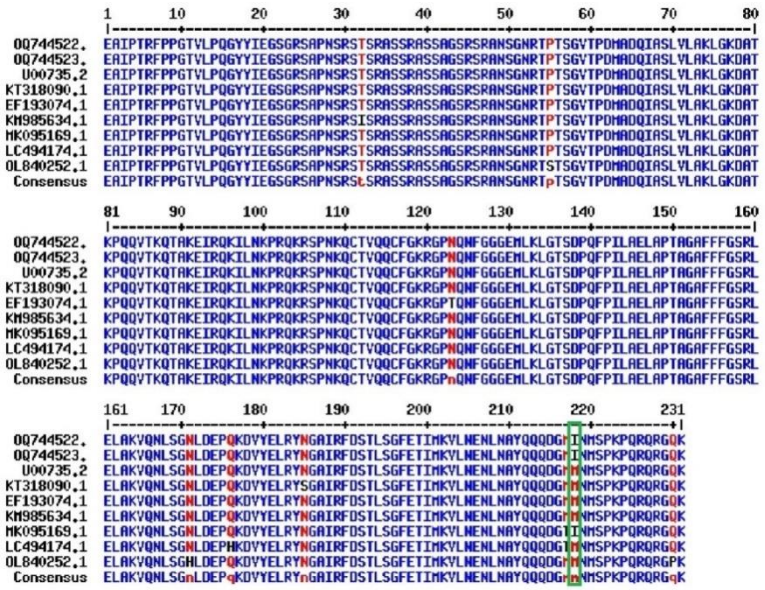

Figure 3: Amino acid alignment was deduced between field virus strains of Bovine Corona virus (BCV) in the partial N gene sequence and reference strains from various countries, spanning positions 170 to 400.

DISCUSSION

Calves with young ages are thought be the main source of livestock production globally for meat or breeding. Diarrhea in adult cattle and neonatal calves is usually associated with bovine coronavirus infection. However, respiratory distress can also be occurred. This can be implicated as etiological agent of bovine respiratory disease (BRD). BCoV infection is one of the main viral infections in beef and dairy calves in young ages. Diarrhea is globally distributed in the early months of age, usually from the first to the third month of age but mostly at the very beginning weeks of ages between first and second week (Amer, 2018). The infection is age susceptible and the risk decreases with increasing ages. This disease has a significant economic impact both in beef and dairy industry(Bok *et al.*, 2015).

In our study, the positive percent of BCoV was 10%, nearly same results have reported in other studies. For instance, in India using RT-PCR found that coronavirus was present in four (8.88%) cases. By contrast with our results, the positive percent of coronavirus infection in other studies were higher, which includes studies in the country of China (18.95% (Keha, Xue, Yan, Yue, & Tang, 2019), in Brazil (15.6% by using semi-nested (SN) PCR) (Stipp *et al.*, 2009), India (14% with ELISA and about 20% by RT-PCR), Australia (21.6% in dairy calves with diarrhea by using RT-PCR, South Korea (15.6% of diarrhea samples using RT-PCR and nested PCR) (Park *et al.*, 2007), and Turkey (37.1% in 0–30 days old calves by indirect antigen-capture ELISA) (Hasoksuz, Kayar, Dodurka, & Ilgaz, 2005). Bok *et al.* (2015) in Argentina conducted a study and used an ELISA test, stated that the viral infection was about 12.13% (29/239) of dairy calves with diarrhea. Furthermore, a possible factor for the low prevalence of Corona virus infection is that most samples are collected in winter, the season in which the prevalence of calf diarrhea caused by Corona virus is higher than other seasons.

Also, our region has a hot and dry climate, which is not suitable for the Coronavirus stability condition.

Some other studies also conducted and reported the lower positive percent than in the present study, as reported by (Singh *et al.*,

20209) in Pakistan, and (23) they tested 424 diarrheic calves with young ages from108 dairy herds and wanted to detect and identified Coronavirus infection and they used a tetra kit assay which had about 88.9% sensitivity and 98.7% specificity relative to an ELISA test. The positive percent of Coronavirus infection was 2.8%. A study conducted in Japan and detected 2 positive samples out of 99 from diarrheic calves (Kirisawa, Takeyama, Koiwa, & Iwai, 2007)by using nested RT-PCR. Singh *et al.*, (Burimuah *et al.*, 2020) tested a total of 816 infected dairy calves having clinical signs with diarrhea and their ages are under 3 months in Central and North India and they used sandwich ELISA. A total of 7 (0.85%) cases tested positive for BCoV. Lotfollahzadeh *et al.* (2020) reported corona virus was (7.2 %.) by using ELISA. In addition, the highest prevalence was in group 1 (4-30) days of age, while the lowest prevalence rate was in group 3 (61-90) days of age because of special characteristics of BCoV in calves with young ages. The protection against diseases is dependent on the presence of specific colostral antibodies in the lumen of the intestine. Colostral antibody does not directly protect but contributes to mucosal immunity by re-secretion into the gut lumen. Protection against clinical disease depends on the amount of immunoglobulin in the lumen of the intestine. Protection from corona virus infection occurs as long as there is a colostrum antibody in the lumen of the intestine, and this explains why corona virus diarrhea commonly occurs between (4-30) days of age. Survival from corona viral diarrhea in calves depends on high levels of serum antibody (Lotfollahzadeh *et al.*, 2020)

Genetic analysis of Sulaimaniyah virus with sequence data available in GenBank® in different countries, showed that the highest identity with China, Brazil and Russia strains range (99.42% and 99.32%) respectively This can be the result of geographical, environmental and natural selection patterns (Keha *et al.*, 2019). A phylogenetic analysis has been constructed using the nucleotide sequences of the N gene from field isolates and additional BCV presence sequences in the GenBank® database. The findings showed that the field virus isolates, BCV HF1/2023 and BCV HF2/2023, belonged to Asia/USA group and clustered with the strains MK095169.1-SWUN/LN4/2018, OL840252.1-KCD_GJ4_2021 and EU401984.1 -solate_SUN5.

The partial N gene in the current study comprises 690 nucleotides, encoding a predicted protein with 230 amino acid residues. However, global genetic information for the N gene is limited. This study holds significance as the first genetic analysis of BCoVs in Iraq, making it challenging to draw precise comparisons.

CONCLUSIONS

In spite of several outbreaks of diarrhea in Sulaymaniyah province, no reports have been conducted to confirm the causative agents. This may cause a failure in the diagnosis of diarrheal calves. Economic losses will then come due to improper treatment. The results confirmed that Coronavirus is one of the common causes of diarrhea in newborn calves in Sulaymniyah province.

Conflict of Interest

Author has no conflicts of interest.

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The funding for this study came from the author.

Ethical approval

The performed procedure of this study was approved by the College of Veterinary Medicine Scientific Research Committee, the University of Sulaimani under the number (AVP-2023-5), on 28/5/2023.

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