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# Molecular Characterization of Bovine Coronavirus by Whole Genome Sequencing from Clinical Samples

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## ABSTRACT

Bovine coronavirus (BCoV) is a significant cattle pathogen that causes three distinct clinical syndromes: calf diarrhea (CD), winter dysentery (WD), and respiratory disease. In this study, 200 nasal swab samples were collected from calves aged one day to six months exhibiting respiratory symptoms. RNA was extracted and analyzed using quantitative reverse transcription polymerase chain reaction (qRT-PCR). From the positive samples, six were selected for further analysis using multiplex PCR, an amplicon-based whole genome sequencing (WGS) approach, and spike gene-targeted sequencing with Oxford Nanopore Technology (MinION MK1B). Phylogenetic analysis of both targeted and whole genome sequences revealed that all six samples belong to the GIb subgroup. Coverage depth at 20× and Q-scores were calculated for both the spike (S) gene and the whole genome. Of the six samples, two had full genome coverage, one exceeded 95%, and the remaining three had coverage ranging between 60% and 70%. For the S gene, three samples were fully covered, while the other three showed coverage ranging from 74% to 86%. Q-scores for both the S gene and WGS ranged from 18.7 to 18.8. The complete genomes were annotated using BLAST, which identified the location and length of each gene. Single-nucleotide polymorphism (SNP) detection was performed manually using SNAPGENE software on both WGS and Sanger sequencing data. A comparison revealed that 22 nucleotide SNPs out of 70 were shared between the two methods, as well as three amino acid SNPs. This study employed an amplicon-based sequencing strategy using Oxford Nanopore Technology to characterize BCoV from clinical samples collected in northern Jordan molecularly. There is a notable lack of data regarding circulating BCoV strains in Jordan and the broader Middle East. Therefore, genomic characterization of local strains is essential to better understand the virus and implement targeted control strategies. This study represents the first whole-genome characterization of a BCoV strain in Jordan using Oxford Nanopore sequencing technology.

**Key words:** Next generation sequencing, Bovine coronavirus, Oxford nanopore technology, Whole genome sequencing, Bovine viruses.

## INTRODUCTION

Bovine coronavirus (BCoV) is classified as an enveloped, single-stranded, positive-sense RNA virus belonging to the family *Coronaviridae*, subfamily *Orthocoronavirinae*, genus *Betacoronavirus*, and subgenus *Embecovirus*. The *Betacoronavirus* genus includes several important human pathogens, such as severe acute respiratory syndrome-related coronavirus (SARS), Middle East respiratory syndrome-related coronavirus (MERS), and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Saif & Jung 2020; Yilmaz et al. 2024).

Among RNA viruses, coronaviruses possess the largest genomes, with BCoV comprising approximately 32 kilobases and containing 10 open reading frames (ORFs) flanked by 5' and 3' untranslated regions. These ORFs—including ORF3, ORF4, ORF8, ORF9, and ORF10—encode essential structural proteins: hemagglutinin-esterase (HE), spike glycoprotein (S), envelope protein (E), membrane protein (M), and nucleocapsid protein (N), which together constitute the virion. In parallel, ORF1a and ORF1b encode a polyprotein that undergoes enzymatic

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cleavage to yield numerous non-structural proteins (Suzuki et al. 2020).

The spike (S) protein of BCoV is characterized by two hydrophobic regions: one at the N terminus, acting as a signal sequence, and the other at the C terminus, facilitating membrane anchoring. The S protein undergoes cleavage at positions 768 and 769, resulting in two subunits—S1 and S2—representing the N- and C-terminal halves of the protein, respectively (Yoo & Deregt 2001; Chineka et al. 2025). The S1 subunit is responsible for binding to specific cellular receptors, thereby determining viral attachment to host cells and influencing both tissue and host tropism, while also inducing an immune response through antibody production. The S2 subunit contains transmembrane domains that anchor the glycoprotein to the viral envelope and plays a critical role in membrane fusion (Saif 2010; Li 2012).

Molecular clock analysis suggests that the ancestral BCoV emerged in the 1940s (Salem et al. 2020), likely via a recombination event similar to that which led to the emergence of SARS (Hu et al. 2017). Phylogenetic analysis based on the S gene reveals two global groups of BCoV strains: GI (European) and GII (Asian-American) (Kanno et al. 2007). Further subdivisions include the GIa subgroup, which comprises early classical strains from various regions, including the original Mebus strain, and the GIb subgroup, which includes European BCoV strains (Kanno et al. 2007; Nathalie et al. 2016). The GII group consists primarily of strains from Asia and the Americas, with the GIIa subgroup arising independently from Korean BCoV strains and the GIIb subgroup comprising strains from China, Japan, Vietnam, and the United States (Zhu et al. 2022).

Viral genome sequencing plays a critical role in understanding disease outbreaks and monitoring viral spread (Gardy et al. 2015). Genomic surveillance enables insights into viral dissemination, evolution, and transmission, thereby facilitating more effective outbreak management (Gardy et al. 2015). Achieving this requires direct sequencing of clinical samples without the need for virus isolation in cell culture, providing a less labor-intensive, faster, and more adaptable alternative to conventional methods (Quick et al. 2017).

Third-generation sequencing technologies, such as Oxford Nanopore Technologies (ONT) and Pacific Biosciences (PacBio), have advanced genomic surveillance by enabling long-read, high-throughput sequencing directly from native DNA, without amplification. This eliminates nucleotide bias and reduces the variability in template abundance often observed in short-read sequencing datasets (Acinas et al. 2005; Pareek et al. 2011; Kebschull & Zador 2015).

In this study, we employed amplicon-based sequencing using ONT to molecularly characterize BCoV from clinical samples collected in northern Jordan.

## MATERIALS AND METHODS

### Sample collection, RNA extraction and real-time reverse transcription polymerase chain reaction (RT-qPCR)

A total of 200 nasal swab samples were collected from calves aged 1 day to 6 months showing clinical symptoms of respiratory disease. These samples were obtained from 16 cattle farms, including both feedlot and dairy operations.

RNA extraction was performed using the Quick-RNA Viral Kit (Zymo Research, USA). Quantitative reverse transcription polymerase chain reaction (RT-qPCR) was conducted on all RNA extracts using the Rotor-Gene Q Real-Time PCR Cycler (QIAGEN, Germany) with the FTD qRT-PCR Master Mix (Fast Track Diagnostics, Luxembourg). Of the 200 samples tested, 76 were positive for bovine coronavirus (BCoV), targeting the nucleocapsid (N) gene. From these, 51 RNA samples were selected based on their cycle threshold (Ct) values and tested using Q5-PCR. Six of these samples tested positive and were selected for sequencing.

### Multiplex PCR and primer pool preparation

Complementary DNA (cDNA) synthesis was carried out using LunaScript RT SuperMix (New England Biolabs, USA) with 8μL RNA template and 2μL of enzyme mix in a GeneMax Thermal Cycler (Bioer, China). The thermal conditions were 25°C for 2 minutes, 55°C for 10 minutes, and 95°C for 1 minute, followed by a 4°C hold.

Initial Q5-PCR primers were designed using the Primal Scheme website provided by the ARTIC Network (Quick 2020). Due to primer dropouts and mismatches, additional primers were designed manually using NEB TM Calculator and Multiple Primer Analyzer. Two pools of primers were created—odd (primers 1, 3, 5, 7) and even. Primers sequences are listed in Table 1 and 2.

Lyophilized primers were reconstituted in 1× TE buffer to a concentration of 100μM. For each pool, 5μL of each primer was combined and mixed thoroughly by vortexing and centrifugation. A 10× working primer pool was made via 10-fold dilution. A PCR reaction (25μL) was prepared using 12.5μL of 2× Q5 High-Fidelity Master Mix (New England Biolabs, USA), 6μL nuclease-free water, 4μL primer pool, and 2.5μL DNA. Thermal cycling: 98°C for 30 seconds, followed by 40 cycles of 98°C for 15 seconds, and 5 minutes of annealing at 65°C (pools 1, 3, 4) or 63°C (pool 2), with a final hold at 4°C. PCR products were analysed using ENDURO GDS Gel Documentation System (Labnet International, USA).

### Library preparation

Library preparation followed the Quick Sequencing Protocol for direct viral RNA sequencing (Quick 2020). Strong amplicon bands were selected for whole genome sequencing (WGS) and spike gene sequencing using Oxford Nanopore Technologies (ONT). PCR products were diluted 1:1. Native barcoding (EXP-NBD104) was used for sample identification. Amplicons were purified using AMPure XP beads, and libraries were prepared with adapter ligation before final elution.

### Sequencing by MinION MK1B

Sequencing was performed on the MinION MK1B device (ONT, UK) using a Flongle R9 flow cell. The flow cell was washed with 117μL Flush Buffer and 3μL Flush Tether. The inlet was primed with a mixture of 13.5μL Sequencing Buffer II, 11μL Loading Beads II, and 5.5μL of prepared library. MinKNOW software (v23.04.3) was used for run control. Guppy (v6.5.7) performed basecalling, trimming and demultiplexing.

Fast5 files were processed by Guppy using a Recurrent Neural Network (RNN). Minimap2 aligned reads to

**Table 1:** Primers designed by Pimalscheme website

| Primer name | Primer sequence |
|---|---|
| b-COscheme_1_LEFT | TCCCTGTAGTCTATGCCTGTGG |
| b-COscheme_1_RIGHT | GCATAAGCCTTCTTAGAAAACTTACCC |
| b-COscheme_2_LEFT | CTTGCGTAAGTGTGGCGAAAAG |
| b-COscheme_2_RIGHT | GTGCTGGACTCCAATACACACA |
| b-COscheme_3_LEFT | GCATACTAAGAGTGCAGCAGGT |
| b-COscheme_3_RIGHT | CAGTACAACTTTAGCAACACTCCG |
| b-COscheme_4_LEFT | GCATGGGTTTTATTTCATGTTTTGCA |
| b-COscheme_4_RIGHT | AGCACAATACAATTTAGAGGCAAGAAC |
| b-COscheme_5_LEFT | TCCATGTAAGGAGCTTGAAGGTG |
| b-COscheme_5_RIGHT | CAAAGTGGGCTGTAAATGGCAC |
| b-COscheme_6_LEFT | AGCTTAAAGGCTTGGATGCTATGT |
| b-COscheme_6_RIGHT | TCTGCCAATTTCTTAGTGCCCTC |
| b-COscheme_7_LEFT | GTGTGCCTTCTGATGTGTCTTTAAC |
| b-COscheme_7_RIGHT | CAGATTCAAACTCTGGAGCATTAAGC |
| b-COscheme_8_LEFT | GCTTGCCTACTACAACATGCTTG |
| b-COscheme_8_RIGHT | GACGGTGTGTCCTATCAATTTAAAATTAGT |
| b-COscheme_9_LEFT | AGAAAATTGAGTATAAACCTGACTTGTCAC |
| b-COscheme_9_RIGHT | GCTTTGATGCAACTTCTGTGGTG |
| b-COscheme_10_LEFT | AATGTGGTTAAAGCTGTGCGAAA |
| bCOscheme_10_RIGHT | CGTAATAGCGTATCATGCCACCA |
| b-COscheme_11_LEFT | GCATGTTGCCTATGGTTGTAGTAAAC |
| bCOscheme_11_RIGHT | CACCCTTCAAATATGTTGGCACC |
| b-COscheme_12_LEFT | GCTGTGCTCGTAAAAGTTGTTCT |
| bCOscheme_12_RIGHT | CCATCGGCCATTGCAAACATAG |
| b-COscheme_13_LEFT | ATGGAGTGCAGTGTTATACGCC |
| bCOscheme_13_RIGHT | AACAGAAGTACCAAGCCGTCTG |
| b-COscheme_14_LEFT | ACAATGGCTAGTTATGTATGGCACT |
| bCOscheme_14_RIGHT | CACCCATTAGCACATAACCAACAG |
| b-COscheme_15_LEFT | GCTGCTTATAACGGCAAACCAC |
| bCOscheme_15_RIGHT | AGCCATGCATAAACATAGCCTCT |
| b-COscheme_16_LEFT | GTTTGGCCATGTTGTTGGTTAAAC |
| bCOscheme_16_RIGHT | CAACTGAGCAAGCTTTTCAAAAGC |
| b-COscheme_17_LEFT | GTTGTCTTGCTTAATTGCTTGCAAC |
| bCOscheme_17_RIGHT | CCTGAGTTTTCAACTTAGCAGGC |
| b-COscheme_18_LEFT | CTGATGATTGTAACTGGCCACTAGT |
| bCOscheme_18_RIGHT | CGTACCCCGAACCCGTTTAAAA |
| b-COscheme_19_LEFT | TGTCAAGTTTGTGGATTTTGGCG |
| bCOscheme_19_RIGHT | CACAATCCAATGCATGACACATGG |
| b-COscheme_20_LEFT | TTTTGGTGACTATGTTATTGCAGCC |
| bCOscheme_20_RIGHT | AACAATGACTTGTGATGCCGGT |
| b-COscheme_21_LEFT | AAGTACAATTTGCCCACCATGG |
| bCOscheme_21_RIGHT | CACTTCTATACACATGTGAGTATAAGCG |
| b-COscheme_22_LEFT | CAGTTTTTAACATATGTCAAGCTGTTTCAG |
| bCOscheme_22_RIGHT | CACTCAAAACATATTTATGATCAGTTGCC |
| b-COscheme_23_LEFT | TATGCAGAGTGTTGGAGCTTGC |
| bCOscheme_23_RIGHT | AGCAAGATGTGACTTTCCTGTACC |
| b-COscheme_24_LEFT | GTGTGCTTGAGACGTTTCAGAAC |
| bCOscheme_24_RIGHT | TGCAAACCCAAAACACGCTTAG |
| b-COscheme_25_LEFT | TGAAAGTTCTAGTGCTGTAAATATGCAG |
| bCOscheme_25_RIGHT | CTAGGTCTAACAACATCCCAGCG |
| b-COscheme_26_LEFT | GAAGCCACTGGTTTGTTTGCTG |
| bCOscheme_26_RIGHT | ACCTCCATTACAGCCAGGAAGA |
| b-COscheme_27_LEFT | GGCACATAAGGACTCTTTTAAAGATGG |
| bCOscheme_27_RIGHT | TGTCGTGGAAATGTAAACGCCA |
| b-COscheme_28_LEFT | GCAGTAATACCTATGGTGTCTGCA |
| bCOscheme_28_RIGHT | GCTTCCAGTCAGATGCAGCTT |
| b-COscheme_29_LEFT | ACTGTGTGAGTAAGGTTGTTAACGT |
| bCOscheme_29_RIGHT | GCCAACTTAAGCGGGAATTTAGC |
| b-COscheme_30_LEFT | AGCCCAAGGTTGAGATAGATGGA |
| bCOscheme_30_RIGHT | CATCCAGTGGCTAGCTATGGTTT |
| b-COscheme_31_LEFT | CGGATGCTGCACTTTCCATTAAG |
| bCOscheme_31_RIGHT | CAAAAGTAAGGCTGCGATACACAG |
| b-COscheme_32_LEFT | GCCTTTAAATGCACCACTTCTGG |
| bCOscheme_32_RIGHT | ACACACAAATAGGTACATCAGGGG |
| b-COscheme_33_LEFT | CTTGTTTTTCGCAGCAAGGTGT |
| bCOscheme_33_RIGHT | ACGCTTATATAAACAGGAAACAACACC |
| b-COscheme_34_LEFT | GCCAGTATACTATGTGCGAGTACC |
| bCOscheme_34_RIGHT | GCAGCAGGTAGATTATAATACAACTGAC |
| b-COscheme_35_LEFT | TGATGCTGCTAAGATATATGGTATGTGT |
| bCOscheme_35_RIGHT | ACCATTAGAATCATATAAAAGGTTCTGCC |
| b-COscheme_36_LEFT | CACAGACATAATTCTTGGTGTTTGTGT |
| bCOscheme_36_RIGHT | CAATCGCTTCCTAAACAACCTAATACAG |
| b-COscheme_37_LEFT | TGCAAGTAGCTAATAGTTTAATGAATGGTG |
| bCOscheme_37_RIGHT | CAAACCATATGGAGCATTCTGCAC |
| b-COscheme_38_LEFT | GCACAAGCTATGGAGAAGGTTAATG |
| bCOscheme_38_RIGHT | CAGCAGGCATTGTGGTAGCTAT |
| b-COscheme_39_LEFT | TGGACACCAGGAGTTAGTAATTAAAAC |
| bCOscheme_39_RIGHT | ACATACCGCAAAGTTGAATACACAATT |
| b-COscheme_40_LEFT | TGCAGACACTGTGTGGTATGTG |
| bCOscheme_40_RIGHT | CAGCAAAACCACTAGTATCGCCT |
| b-COscheme_41_LEFT | GCAAGGTATAAAACTAGGTACTGGCT |
| bCOscheme_41_RIGHT | GCGCGTGAAGTAGATCTGGAAT |
| b-COscheme_42_LEFT | AGCGATGAGGCTATTCCGACTA |
| bCOscheme_42_RIGHT | TTCAGTAAAGGGCTCATCCATCTTC |
| b-COscheme_43_LEFT | AACCCCTACCTCTGGTGTAACA |
| bCOscheme_43_RIGHT | GTCTTTCACTTTCTCCAACACTATACATT |

**Table 2:** Primers designed manually to fill the gaps

| Primer name | Primer sequence: |
|---|---|
| 1F: | GATCATAAACGTGGCGGTTTTG |
| 1R: | CCTGCTGCACTCTTAGTATGC |
| 2F: | GCTAGTGCTGTTGCACAAG |
| 2R: | GATGTCTCCACACATGGACC |
| 3F: | ATGGTCCATGTGTGGAGAC |
| 3R: | TGCATAACTTTGTTTACCCTGTGT |
| 4F: | CTTGCTTAATGCTCCAGAGTTTG |
| 4R: | CCTACAACCTGTAAGCCACATATC |
| 5F: | GATATGTGGCTTACAGGTTGTAGG |
| 5R: | GCCACCATTAGCCATAACATC |
| 6F: | GGTGGCATGATACGCTATTACG |
| 6R: | AACAGAGACATTAGCCATCTGC |
| 7F: | CTGTATATGGTCTGTGGATGCT |
| 7R: | CTGCACTCCATCAGCAGA |
| 8F: | ACGGCTTGGTACTTCTGTTC |
| 8R: | GGTTTGCCGTTATAAGCAGC |
| 9F: | CCTGTGCTTTTTACACTGCT |
| 9R: | AGTGGCAAGTATTTCATTGTGC |
| 10F: | AGTGGTCCAGATCAGACTTG |
| 10R: | CCGCCAAAATCCACAAACTTG |
| 11F: | GACCATGTGTCATGCATTGG |
| 11R: | GCGGGCTCTATTCTTAGCA |
| 12F: | TGCTAAGAATAGAGCCCGC |
| 12R: | CCTTTTGGTATTCTTCATTTTCGT |
| 13F: | CCTCCTGGTACAGGAAAGTC |
| 13R: | CTTTCATGTGTTGTAACGCCTT |
| 14F: | GCTGGGATGTTGTTAGACCT |
| 14R: | GTAAAACCTGCTGTAGTAGCTG |
| 15F: | TGCTGAAGAGTATCGTGAGTAC |
| 15R: | TCGGTACAAGCCTATTAACAAATG |
| 16F: | GCATTTTGGACGGTTTTCTGC |
| 16R: | AATGTCCAATCCACTTCCTGG |
| 17F: | TGAGGGTGTTAATTTTACGCC |
| 17R: | ACAAACCACTGAGTATGCTAGT |
| 18F: | CGCGTAGAACTATGGCATTG |
| 18R: | GTCAATCTTCCTACCATTGGGTAT |
| 19F: | TGAGCAGCCTGATGTCTT |
| 19R: | CCCAAAAATGCTTGTGGTTGG |
| 20F: | TTGGGTTGGTCTGTTGACTC |
| 20R: | GCTACTTGCAACTGTGTAGTGT |
| 21F: | ATCCACTTTAGCTATGTCCCTA |
| 21R: | CTTAAAACAACTAGTCCCACATCC |
| 22F: | TTGCAGACACTGTGTGGTA |
| 22R: | GTAAGGGTATGGTAGTCCTCAA |

reference genomes. Phred scores and quality metrics were assessed with FastQC. SAM and BAM files were generated using SAM tools (Danecek et al. 2001). Bed tools calculated depth and coverage (Quinlan & Hall 2010). Consensus sequences were polished using Medaka (Kim et al. 2021). Alignments and coverage were visualized with IGV (Robinson et al. 2011), and phylogenetic trees were constructed using MEGA11 (Tamura et al. 2021) with the Neighbor-Joining method.

**Spike and HE gene mutation detection**

S and HE gene consensus sequences were aligned to a reference genome (GenBank: MG757141) using SnapGene (Weber et al. 2020) to detect nucleotide and amino acid mutations.

**Sanger sequencing of S and HE genes**

Sanger sequencing was performed to validate ONT results. PCR products (Table 3) were purified using a Gel DNA Recovery Kit (Zymo Research, USA). Sequencing reactions included 4.5µL PCR product, 1.75µL buffer, 0.35µL RR mix (Brilliant Dye Terminator, Nimagen, Netherlands), 0.5µL of each primer, and 5.9µL nuclease-free water (NFW). Primers were used at 3.2 µM rather than 10×. Cycling conditions: 96°C for 1 minute, followed by 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes, ending with a hold at 4°C. Cleanup was done with D-Pure™ DyeTerminator Magnetic Beads (Nimagen, Netherlands), and sequencing was performed using the SeqStudio Genetic Analyzer (ThermoFisher, USA). Chromatograms were analysed and edited using FinchTV, and alignment was completed with SnapGene's MUSCLE algorithm.

**Table 3:** Primers used in sanger sequencing

| Target gene | Primer's | sequences |
|---|---|---|
| S gene | 18f | CGCGTAGAACTATGGCATTG |
| | 18r | GTCAATCTTCCTACCATTGGGTAT |
| | 33f | CTTGTTTTTCGCAGCAAGGTGT |
| | 33r | ACGCTTATATAAACAGGAAACAACACC |
| | 35f | TGATGCTGCTAAGATATATGGTATGTGT |
| | 35r | ACCATTAGAATCATATAAAAGGTTCTGCC |
| | 36f | CACAGACATAATTCTTGGTGTTTGTGT |
| | 36r | CAATCGCTTCCTAAACAACCTAATACAG |
| | 37f | TGCAAGTAGCTAATAGTTTAATGAATGGTG |
| | 37r | CAAACCATATGGAGCATTCTGCAC |
| | 38f | GCACAAGCTATGGAGAAGGTTAATG |
| | 38r | CAGCAGGCATTGTGGTAGCTAT |
| HE gene | 17f | TGAGGGTGTTAATTTTACGCC |
| | 17r | ACAAACCACTGAGTATGCTAGT |
| | 31f | CGGATGCTGCACTTTCCATTAAG |
| | 31r | CAAAAGTAAGGCTGCGATACACAG |
| | 32f | GCCTTTAAATGCACCACTTCTGG |
| | 32r | ACACACAAATAGGTACATCAGGGG |

## RESULTS

**Multiplex PCR amplification results**

Six Q5-PCR–positive samples were selected for sequencing. A negative template control (bovine Nebo virus) was included and yielded no amplification, confirming the specificity of the assay. All six selected samples were successfully amplified using both odd and even primer pools. Strong and distinct ~800bp bands were observed for pools A and B on agarose gel electrophoresis (data not shown). For pools C and D, two clear bands were observed at approximately 650bp and 1000bp, respectively (data not shown).

**MinION sequencing results for Whole Genome Sequencing (WGS)**

The sequencing run was completed after 20 hours and 28 minutes, having generated sufficient data. All six samples were successfully sequenced. Two samples achieved greater than 97% coverage at 20× depth, one sample achieved 92%, and the remaining three samples showed more than 62% coverage. The mean Q-scores ranged between 18.7 and 18.9.

A line plot representing coverage depth per base for the BCoV whole genome is shown in Fig. 1. The six samples were analysed using BLAST (Boratyn et al. 2012) against the NCBI database. Phylogenetic analysis conducted with MEGA11 software revealed that all six samples clustered within the GIb subgroup.

**Targeted spike gene sequencing**

The spike gene sequencing run, conducted alongside WGS, also lasted 20 hours and 28 minutes and included the same six samples. All six samples were successfully sequenced. Complete (100%) spike gene coverage was achieved in samples 5 and 6. Sample 1 had 99.90% coverage, followed by sample 2 with 86.20%, sample 3 with 74.50%, and sample 4 with 77.00%. The Q-scores ranged from 18.7 to 18.8.

A line plot illustrating depth per base for the spike gene is presented in Fig. 3, clearly showing variations in depth across the six samples. These variations likely reflect differences in clinical viral load and possible primer-binding mismatches.

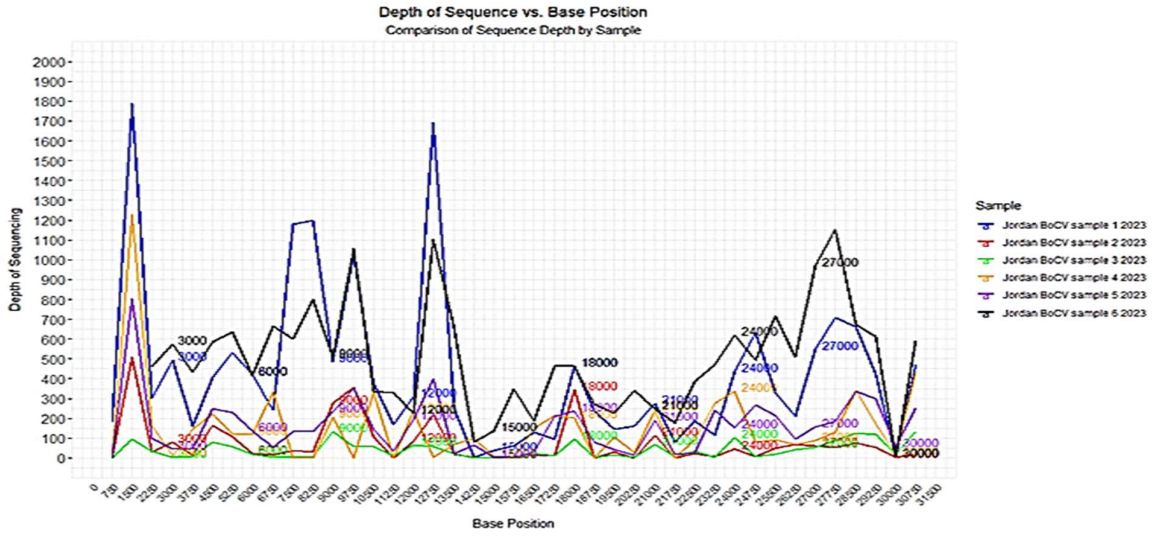


**Fig. 1:** Line plot represents the coverage per base position for WGS six samples. The x-axis shows the base positions for the whole genome for BCoV from 0 to 31kb with a 700 step. The y-axis represents depths from 0 to 2200 with 100 steps.

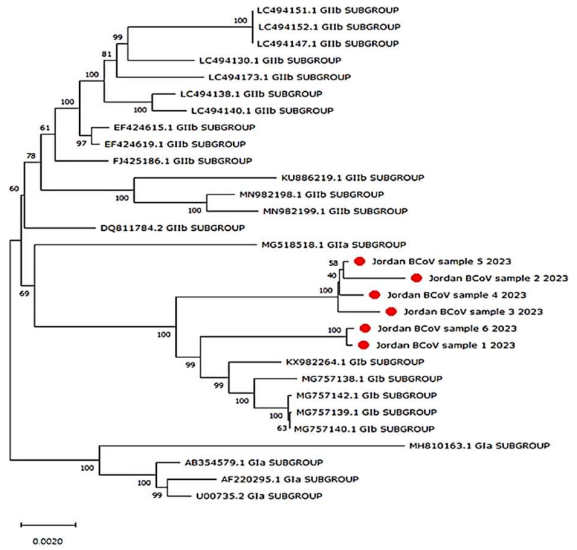


**Fig. 2:** Phylogenetic tree of the resulting six WGS samples (Red dots). The six samples were clustered with the GIb subgroup. The Neighbor-Joining Method algorithm was used for this phylogenetic tree with bootstrapping replications of 1000. The substitute model for this tree was the Tajima-Nei model, and distance bar scale is shown at the lowermost left in this figure.

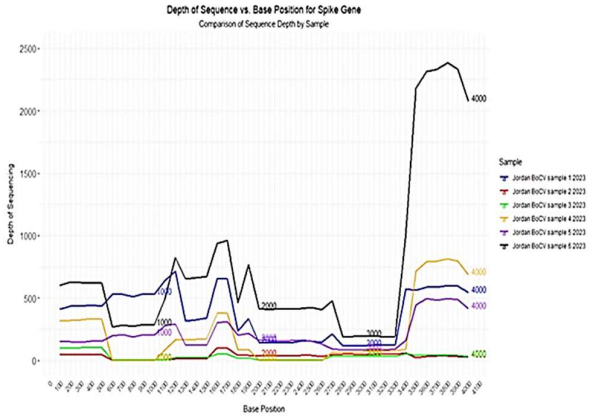


**Fig. 3:** The Line plot represents the coverage per base position for S gene for six samples. The x-axis shows the base positions for the BCoV spike gene from 0 to 4100bp with a 1000 step. The y-axis represents depths from 0 to 2500 with 500 steps.

Subsequent BLAST analysis confirmed that the sequences were similar to the reference strain (GenBank accession: MG757141). Phylogenetic analysis using MEGA11 revealed that all six samples clustered within the GIb subgroup based on S-gene sequences (Fig. 2). This classification was further confirmed by WGS (Fig. 4).

**SNP detection in spike and HE genes from NGS and sanger sequencing**

Single nucleotide polymorphisms (SNPs) were identified in both the spike (S) and hemagglutinin-esterase (HE) genes at the nucleotide and amino acid levels. These SNPs, detected through both Oxford Nanopore (NGS) and Sanger sequencing, are summarized in Table 4, which presents all variations observed at both the nucleotide and protein levels.

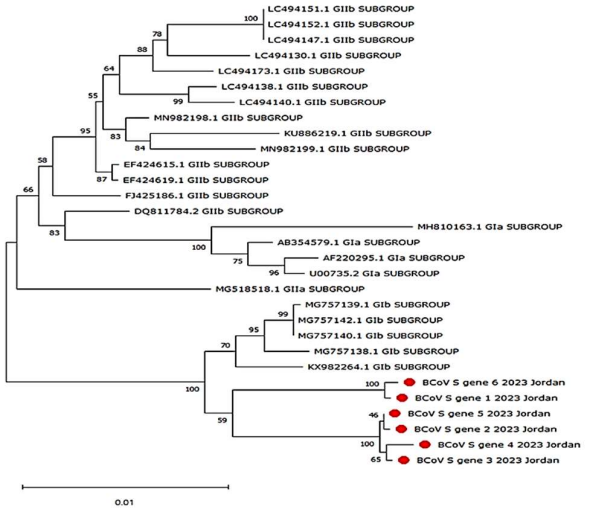


**Fig. 4:** Phylogenetic tree of the resulting six S gene samples (Red dots). The six samples were clustered with the GIb subgroup. The Neighbor-Joining Method algorithm was used for this phylogenetic tree with bootstrapping replications of 1000. The substitute model for this tree was the Tajima-Nei model, and the distance bar scale is shown at the lowermost left in this fig.

## DISCUSSION

In cattle, bovine coronavirus (BCoV) has been linked to both respiratory and enteric diseases, including respiratory tract infections, winter dysentery (Saif et al. 1991; Snodgrass et al. 1986) and neonatal calf diarrhea (NCD). Regardless of clinical presentation, all BCoV isolates identified to date—whether excreted in feces or nasal secretions—belong to the same serotype/genotype, based on genotyping and virus cross-neutralization studies (Vlasova & Saif 2021).

BCoV is divided into two major groups: GI (European) and GII (Asian-American) (Salem et al. 2020). Each group is further subdivided into GIa, GIb, GIIa, and GIIb. The GIa subgroup includes the original Mebus strain and other early classical strains from North America, Europe, and Asia, while the GIb subgroup comprises more recent European strains (Zhu et al. 2022). In our study, phylogenetic analysis based on both whole genome sequencing (WGS) and targeted S gene sequencing revealed that the Jordanian samples are clustered with GIb, which may be attributed to the frequent calves trade between Jordan and European countries.

A study conducted in South Korea (2004–2005) demonstrated that although sampled calves exhibited different clinical syndromes (diarrhea, respiratory illness, and winter dysentery), the isolated coronaviruses were genetically closely related and grouped distinctly from reference strains from the United States and Canada. This led the authors to conclude that circulating BCoV strains in South Korea had evolved along a unique evolutionary path (Park et al. 2007).

Similarly, an Argentine study using seven representative samples found that BCoV strains circulating on farms clustered distinctly from strains isolated in other regions. At both the nucleotide and amino acid levels, the Argentine strains differed

significantly from historical reference strains (Bok et al. 2015). In a separate phylogenetic analysis of Irish isolates based on the spike gene, the strains grouped with recently identified European BCoV strains, suggesting a shared evolutionary lineage.

Consistent with these findings, we observed multiple SNPs in both the spike (S) and hemagglutinin-esterase (HE) genes at the nucleotide and amino acid levels. These genes are critical for viral functions such as receptor binding, cell entry, and elicitation of host immune responses (Tsunemitsu & Saif 1995). Mutations in the S and HE genes have been linked to changes in host range and tissue tropism (Gallagher & Buchmeier 2002; Langereis et al. 2012; Bakkers et al. 2017) and enhanced virulence in Group 2.

To validate the SNPs identified by Oxford Nanopore sequencing, we also performed Sanger sequencing on the same genes. A total of 22 out of 70 nucleotide-level SNPs were confirmed by both methods, along with three amino acid substitutions observed in both datasets.

**Table 4:** SNP locations at both levels, nucleotide, and amino acid for both genes HE and S gene from NGS and sanger

| Gene name | Gene location | SNP location at nucleotide level | The SNP | SNP location at amino acid level | A.A mutation | Notes |
|---|---|---|---|---|---|---|
| HE | 22335-23609 | 294 | C>T * | 108 | H>Y | Sanger resulted: Y>H |
| | | 322 | T>C * | 158 | P>S | |
| | | 351 | C>T * | 177 | K>R | |
| | | 472 | T>C | 189 | Q>E | |
| | | 504 | T>C | 235 | F>L | |
| | | 529-531 | CGT>AAA | 237 | A>S | |
| | | 565 | G>C | 239 | Q>E | |
| | | 580 | C>T | 341 | L>conversion deletion | |
| | | 703 | C>T | 342 | Y>X | |
| | | 709 | T>G | 343 | D>X | |
| | | 715 | G>C | 345 | S>X | |
| | | 792 | C>T | 346 | S>P | |
| | | 873 | T>C * | 376 | V>D | |
| | | 993 | C>T | | | |
| | | 1025 | T>A | | | |
| | | (1027-1035) | NNN | | | |
| | | 1036 | C>T | | | |
| | | 1044 | C>T * | | | |
| | | 1127 | A>T | | | |
| | | 1167 | G>T | | | |
| S gene | | 70 | G>C | 24 | V>L | |
| | | 108 | C>T | 113 | I>N | |
| | | 162 | C>T | 143 | H>Y | |
| | | 333 | G>A | 403 | V>X | |
| | | 338 | T>A | 436 | A>S | |
| | | 427 | C>T * | 509 | T>N * | |
| | | 432 | T>A | 547 | Y>X | |
| | | 507 | T>C | 548 | K>N | |
| | | 630 | T>C * | 596 | G>E * | |
| | | 708 | A>G * | 743 | S>A | |
| | | 735 | C>T * | 829 | T>I | |
| | | 807 | A>G * | 904 | V>L | |
| | | 810 | C>T * | 1070 | F>S | |
| | | 816 | C>A | 1111 | T>A | |
| | | 1188 | T>A | 1117 | A>X | |
| | | 1207 | G>R | | | |
| | | 1306 | G>T | | | |
| | | 1440 | T>C | | | |
| | | 1526 | C>A * | | | |
| | | 1584 | C>T * | | | |
| | | 1602 | C>T | | | |
| | | 1605 | C>A * | | | |
| | | 1641 | C>T C>Y | | | *In sanger, only C>T was detected |
| | | 1644 | G>T | | | SNP at nucleotide location from 1642-1644 were not read on sanger |
| | | 1734 | T>C * | | | |
| | | 1770 | T>A * | | | *Indicates changes detected in both NGS and sanger |
| | | 1787 | G>A * | | | |
| | | 1926 | C>T * | | | |
| | | 1953 | G>A * | | | |
| | | 1983 | G>A | | | |
| | | 2085 | T>C | | | |
| | | 2227 | T>G | | | |

| | |
|---|---|
| 2293 | C>A * |
| 2486 | C>T |
| 2679 | C>T * |
| 2710 | G>T |
| 2823 | A>T |
| 2871 | T>C |
| 2922 | A>G |
| 3108 | T>C |
| 3189 | T>C |
| 3209 | T>C |
| 3331 | A>G |
| 3351 | A>W |
| 3468 | T>C |
| 3510 | G>T |
| 3528 | G>A |
| 3585 | T>C |
| 3630 | T>C |
| 3636 | G>T |
| 3711 | A>C |

## Conclusion

In this study, we successfully sequenced the near-complete genome (~32 kb) of bovine coronavirus (BCoV) directly from clinical nasal swab samples collected from multiple farms in northern Jordan. Amplicon-based PCR and targeted Oxford Nanopore Technology (ONT) sequencing were employed for both whole-genome and spike gene analysis. Phylogenetic analysis revealed that the Jordanian BCoV strains clustered within the GIb subgroup.

A total of 22 shared nucleotide-level single-nucleotide polymorphisms (SNPs) and three amino acid-level SNPs were identified through both next-generation sequencing (NGS) and Sanger sequencing in the spike (S) and hemagglutinin-esterase (HE) genes. These findings highlight the effectiveness of ONT as a tool for rapid genomic surveillance of BCoV. Nevertheless, further large-scale studies are required to provide a more comprehensive epidemiological understanding of BCoV in Jordan and the broader Middle East region.

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**Author's Contribution:** Mustafa Ababneh and Mohammad Al-zghoul supervised the research and reviewed the manuscript. Wardeh Qattous conducted lab experiments and managed the workflow and prepared the manuscript, Ibrahim Abualghusein supervised the design of the workflow in both wet-lab and bioinformatic analysis, Hebah A. A. Aboomer and Majid Al-Shannag conducted the wet-lab experiments.

**Generative AI Statement:** The authors declare that no Gen AI/DeepSeek was used in the writing/creation of this manuscript.

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