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Isolation, molecular identification, and phylogenetic analysis of infectious bronchitis virus from commercial chicken farms in Mekele and Bishoftu, Ethiopia, 2023–2024

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Abstract

Background Avian infectious bronchitis (IB) is a highly contagious respiratory disease that affects the poultry industry globally. The disease is caused by *avian infectious bronchitis virus* (IBV), member of the genus *Gammacoronavirus*. In Ethiopia, IBV has been reported in both commercial and backyard chickens based on clinical observation. The objectives of this study were to isolate the virus, conduct molecular based identification, and phylogenetic analysis of the circulating IBV isolates.

Methods and materials A cross-sectional study was conducted between November 2023 and May 2024 in Mekele and Bishoftu, Ethiopia. A total of 49 clinical samples were collected, comprising 12 tissue samples and 39 pooled swab samples. Of these, 6 samples—specifically, 5 swab samples and 1 tissue sample—tested positive for infectious bronchitis virus (IBV) through virus-specific conventional RT-PCR and real-time PCR. Nested PCR was performed using serotype-specific primers. The purified PCR products, which targeted the spike glycoprotein S1 subunit gene and the 3′ UTR of the IBV, were sequenced, followed by phylogenetic tree analysis.

Results The six positive samples propagated into specific pathogen free embryonated eggs and exhibited characteristic IBV lesions and mortality observed over five consecutive passages. IBV isolates from Bishoftu ($n=4$) and Mekele ($n=2$) were amplified using one-step RT-PCR to target 466 bp of the S1 subunit gene and 433 bp of the 3′UTR. A BLAST search on the S1 partial gene and 3′UTR sequences, nested PCR, and phylogenetic analysis revealed that the present IBV isolates are genetically similar to the Massachusetts serotype. The S1 gene sequences of the five IBV isolates were deposited in GenBank with accession numbers PQ389500 to PQ389504.

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Conclusions This is the first detailed study on IB virus isolation, molecular detection, sequencing, and phylogenetic analysis in Ethiopia. The findings revealed that the outbreaks were caused by the IB virus, which created a serious health risk and economic losses in the chicken industry. To the author's knowledge, this is the first comprehensive study on the isolation and genetic analysis of IBV in Ethiopia. Further research on the economic impact of IBV in chicken production, farm biosecurity, serotyping of circulating IB virus, and vaccine development based on the local serotypes is recommended.

Keywords Avian infectious bronchitis virus, Isolation, Sequencing, Phylogenetic analysis, Chicken, Ethiopia

Introduction

Poultry production is the largest agricultural activity in the world and accounts for about half of all animal protein. Poultry production is Ethiopia's second most populous sector (with approximately 56 million), following cattle production [1]. It is critical for reducing poverty, ensuring food security as a source of meat and eggs, and supporting economic growth in the country. Twenty per cent of the poultry produced in the country is commercially raised. In contrast, the remaining chickens are raised traditionally, exposing them to diseases, predators, a lack of veterinarian care, and other limitations [2].

Avian infectious bronchitis (IB) is one of the most serious acute and transmissible viral diseases in chickens, and the World Organization for Animal Health listed it as a notifiable disease [3]. The disease was first reported in North Dakota, USA 1931 [4]. IB is an airborne disease transmitted horizontally through direct contact and indirectly through fomites, contaminated water, and feed, with transmission through embryonated eggs [5]. Chickens of all ages are susceptible to the IB virus, which targets epithelial cells in the upper and lower respiratory tracts, causing symptoms such as coughing, gasping, respiratory rales, sneezing, and watery nasal discharge; and can affect chickens of all ages [6].

Infectious bronchitis virus affects the reproductive system, resulting in reduced egg quality and production rates and economic losses. The disease damages the kidney's epithelial cells, resulting in urinary tract lesions and nephritis. Mortality rates vary based on the chicken's age, breed, immunological status, virus strain, severe respiratory symptoms, and reproductive problems. The economic burden of this disease on morbidity and control exceeds that of mortality. It has the largest economic impact on chickens worldwide because it lowers egg quality and production efficiency due to poor broiler weight gain, followed by a decrease in feed efficiency [7–9]. The disease is difficult to control because of its rapid dissemination, virus mutation, and lack of cross-protection amongst infectious bronchitis virus strains [8].

Avian infectious bronchitis (IB) is caused by *avian infectious bronchitis virus* (IBV), which belongs to the order Nidovirales, family *Coronaviridae*, subfamily *Coronavirinae*, and genus *Gammacoronavirus*. The coronavirus causing IB disease is an enveloped virus that contains a linear and single-stranded positive-sense RNA genome, which is the largest among the RNA genomes; it is approximately 27.6 kb in size and organized as 5′UTR-1a-1b-S-3a-3b-E-M-5a-5b-N-3′UTR [10–12].

The IBV genome comprises two untranslated regions (UTRs) with poly (A) tails at the ends of its 5′ and 3′ strands; because the 5′ and 3′UTRs contain important structural components, they play a significant role in viral RNA transcription and replication [13, 14]. The conserved 3′UTR of the Avian IBV genome was used for characterization and classification of the strains because these regions are more conserved and less prone to mutation and recombination [15].

The viral genome is divided into two parts that encode structural and non-structural proteins. The 3′ end contains four structural proteins: spike glycoprotein (S), envelope (E), matrix (M), and nucleocapsid (N), and four non-structural secondary proteins: 3a, 3b, 5a, and 5b. The 5′ end of the genome contains two polyproteins, 1a and 1ab, which encode proteins required for RNA replication. The S glycoprotein has two major glycopeptides: S1 at the amino terminus, with about 535 amino acids, and S2 at the carboxyl terminus, with approximately 627 amino acids [10, 13]. The glycopeptides are used to attach the virus to the host cell membrane. The S1 glycoprotein gene is the most important immunogenic component and contains epitopes that neutralize antibodies [10, 13].

In nature, the virus is fragile, mutable, and capable of freely changing its genetic makeup [1, 8, 16]. As a result, several serotypes that are difficult to control with vaccination have emerged, and many of them lack cross-protection. Previously, IBV was described as having seven genotypes and thirty-five (35) lineages [17]. IBV currently has nine genotypes (GI-GIX) across the world and several lineages with pairwise genetic distances of 30% and 13%, respectively [18]. The new classification approach is based on the variability of the IBV's whole S1 sequence. These newly emerged genotypic lineages and strains have different locations and pathogenicity. Because of recombination and mutations caused by substitutions, deletions, and insertions during replication, IBV exhibits continual genetic diversity [8, 13, 18].

In Africa, the Mediterranean basin countries were the first to report cases of IBV. There are currently several IBV lineages identified in Africa, but only GI-26 is

considered endemic to the continent, and is primarily composed of strains from North and West Africa [13, 19]. The IBV 793B serotype was identified in Ethiopia and it is 92–95% related to the French strain FR-94047-94 [20]. Other serotypes reported in Ethiopia include M41, D-274, QX [21], and Massachusetts or mass [22].

The gold standard technique for isolating the virus is to use an embryonated chicken egg as the primary diagnostic tool for IBV infection [3]. Rapid diagnostic testing methods include reverse transcriptase polymerase chain reaction (RT-PCR) and real-time PCR can be used for. Although identifying the S gene is believed to be the gold standard method for IBV classification and characterization, it has disadvantages due to mutation and recombination in the three HVR regions. In addition, the 3′UTR of the IBV genome contains key structural components of the translation and replication of the AIBV and is ideal for molecular diagnostics and strain classification [23–25].

In Ethiopia, there is insufficient molecular evidence to determine the genotypes of the viruses circulating in the country. The commercially available imported vaccines for IBV are Massachusetts and 793B, which match the previously reported IBV strains in Ethiopia. Although imported vaccines are related to previously identified strains in Ethiopia, their efficacy is limited because of the virus's mode of transmission, mutation characteristics, and lack of cross-strain protection. As a result, even when vaccination campaigns are conducted against the virus, outbreaks continue, making it difficult to mitigate the disease in Ethiopia.

Infectious bronchitis is an emerging poultry disease causing significant economic losses in Ethiopia's poultry production system. Hence, the goal of this work was to isolate, molecularly characterize, and perform phylogenetically analysis of the infectious bronchitis virus (IBV) from outbreak samples collected from commercial chicken farms in Ethiopia.

Methods and materials

Study area

The investigation was carried out in Bishoftu and Mekele (Fig. 1). Bishoftu is located around 45 km southeast of Addis Ababa at an elevation of 1900 m above sea level. The average annual temperature is around 18 °C. The area is conducive to chicken production. Mekele is the capital city of the Tigray regional state, which is located 761 km from Addis Ababa. Mekele is situated at 13° 24′ latitude and 39° 25′ longitude, with an elevation of 2200 m above sea level. The average annual rainfall of Mekele is between 579 and 650 mm, with temperatures ranging from 11.8 °C to 29.9 °C. This region has the fourth highest poultry population in Ethiopia [1], with most commercial farms located around Mekele.

Study population and sample size

This study was conducted on chickens that clinically manifest avian infectious bronchitis at various ages (pullets, adults), sex (male, female), breeds (Lohman, Bovans), management (intensive, semi-intensive), and non-vaccinated flocks, based on farm owners' availability and willingness. When chickens manifest IB symptoms such as coughing, sneezing, tracheal raring, and ocular and nasal discharge, the case is considered positive for IB. In the study areas, active outbreaks targeting commercial poultry farms, including broilers and layers with flock sizes ranging from 110 to 4500, were investigated.

Study design

A cross-sectional study was conducted between November 2023 and May 2024 on poultry suspected of having IB disease outbreaks at commercial poultry farms in the study areas of Mekele and Bishoftu, Ethiopia. Chickens from poultry farms with suspected IB cases were purposely sampled.

Sampling technique and sample collection

The samples were collected from eleven chicken farms, eight in Bishoftu and three in Mekele. Swabs from four chicks were pooled in one tube, and the tissue samples from one chicken organ (trachea, lung, or kidney) were pooled in another tube.

After recording clinical and epidemiological data on morbidity, mortality, and vaccination practices, swabs and tissue samples were collected for further laboratory analysis using virology and molecular detection methods. This was done to characterize the circulating IBV strains in the study areas. If there was a single case related to IB occurrence on the farm, it was considered an outbreak, and this information was obtained through interactions with the farm owners and animal health professionals.

During active outbreaks, an antemortem examination was conducted to look for any abnormalities, such as discharge, coughing, sneezing, and tracheal rearing, after which swabs and tissue samples were collected. To collect tissue samples for IBV, suspected chickens were carefully examined for external signs and symptoms of IB infection. The postmortem examination of the chickens was performed according to the standard protocol described in the WOAH manual [3]. Chickens were euthanized humanly through cervical dislocation, postmortem inspections were performed, and tissue samples were collected according to the procedure described by [26].

In total, 49 pooled samples were collected from various IBV outbreaks on commercial poultry farms in Bishoftu and Mekele, as illustrated in Table 3. Thirty-seven swab samples were collected from the upper respiratory tracts of live chickens suspected of having IBV in both study areas. Twelve tissue samples were also collected

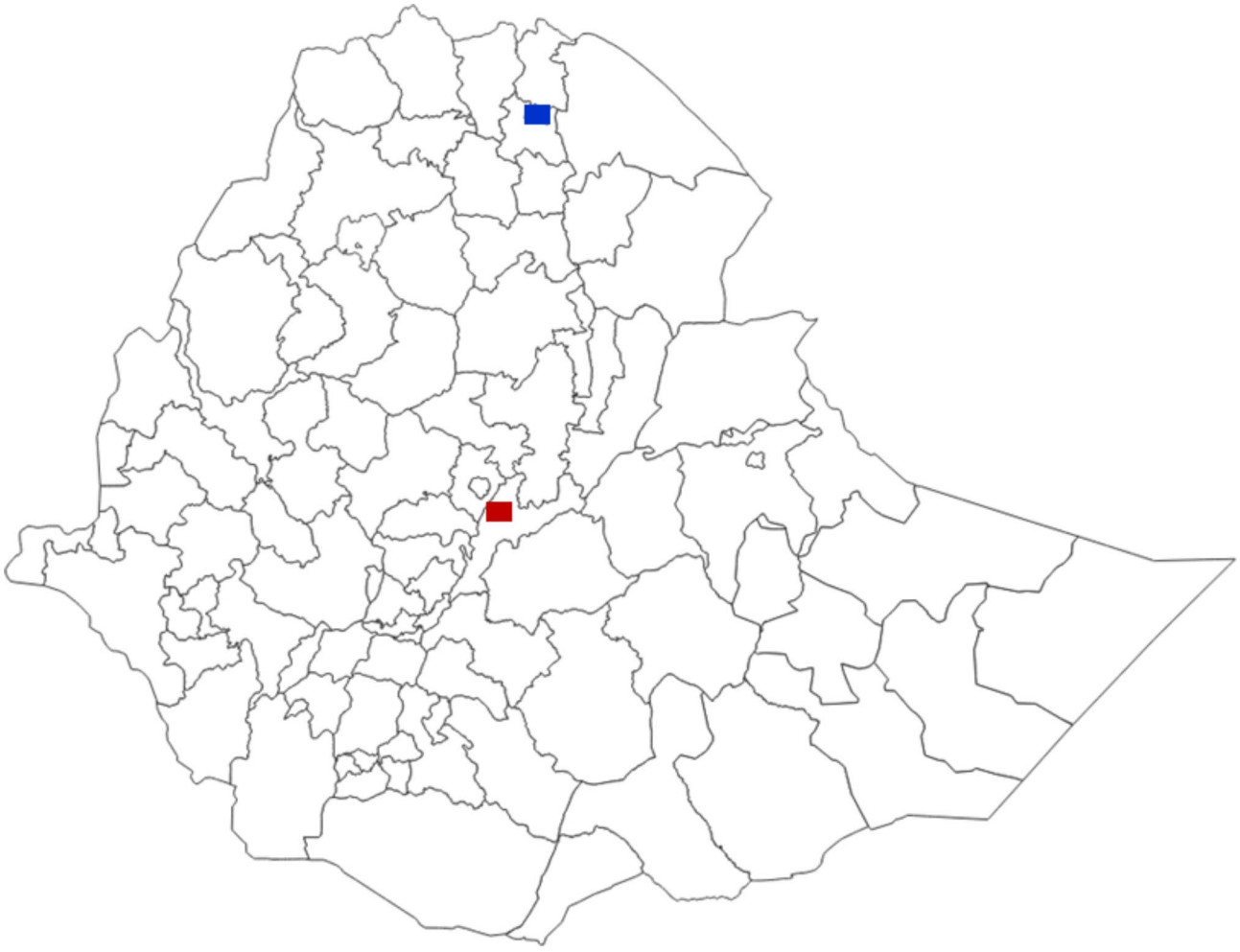

Fig. 1 A map of Ethiopia shows the study areas where suspected infectious bronchitis outbreak samples were collected. Blue represents Mekele, while Red represents Bishoftu. The map was created with QGIS software version 3.10.0-A

and processed from various IBV outbreaks at commercial poultry farms in Bishoftu and Mekele. All collected samples were transported to the National Veterinary Institute (NVI) using virus transport media (VTM) supplemented with 10% antibiotics and labelled with relevant information. The samples were stored at − 80 °C for subsequent laboratory analysis.

Laboratory diagnosis

Sample processing

The swab samples in the VTM were vortexed and clarified by centrifugation at 1500 ×g for 10 min. The suspension was filtered using a 0.45 µm filter syringe (Millipore, USA). The filtrate was stored at − 80 °C for subsequent processing. The tissue samples were processed using WOAH standard operating procedures [3]. The pooled organ samples were thawed at room temperature before being washed with PBS (containing 10% antibiotics) three times in a level two biosafety cabinet. The tissue samples were ground to homogenize with a mortar and pestle and centrifuged at 1500 ×g for 10 min in 9 ml of PBS. To prevent bacterial contamination, supernatants were collected under sterile conditions, filtered through a 0.45 µm filter (Millipore, USA), and stored at − 80 °C until processing [3].

Isolation and titration of virus using an embryonated chicken eggs

Specific pathogen free (SPF) eggs were obtained from the NVI of Ethiopia. The SPF eggs were incubated at 37 °C with 65% humidity. Temperature and humidity were measured daily until the day of sample inoculation. Virus propagation and isolation were performed following the WOAH protocol. The embryonated eggs were disinfected with 70% ethanol, and a hole was made in the center of the eggshell at the top under aseptic conditions. Then, with a 1 ml syringe, 200 µl of filtered inoculum of processed tissue and tracheal swab suspensions were inoculated into the allantoic cavity of 9-days-old embryonated SPF eggs, piercing the chorioallantoic membrane

(CAM) just below the air space for amplification. The control eggs were inoculated with PBS. The hole was sealed with melted candlewax, and the inoculated eggs were incubated at 37 °C. Five eggs were used for each sample, and the progress was monitored daily using a candle after inoculation. Embryos that died within 24 h following inoculation were discarded. At 120 h postinoculation, live and dead embryonated eggs were refrigerated or chilled at 4 °C for four hours. The allantoic fluids were harvested using a pipette and pooled and filtered at each passage to avoid contamination. This procedure was repeated for the five passages. The filtered pooled samples were stored at − 80 °C until molecular analysis [27].

Positive samples were further titrated in embryonated chicken eggs by inoculating serial tenfold serially diluted allantoic fluid. The EID50 was calculated using the Ramkrishnan's formula [28].

Virus propagation and titration in chicken embryo fibroblast culture (CEF)

To culture the IB virus, primary chicken embryo fibroblast cell cultures were prepared from 10-day-old embryonated eggs. An inoculum with an IBV titer of 6.5 ELD50/ml was used from the recovered allantoic fluid. IBV was titrated according to [28] and expressed as 10log (TCID50)/ml using the method described in [29, 30].

Molecular identification of IB viruses

Viral RNA extraction Viral RNA was extracted from collected allantoic fluids of selected IBV isolates and collected swab and tissue samples following the manufacturer instructions of the QIAamp Viral RNA Mini Kit (Qiagen, Germany) and the procedure described previously [31].

Table 1 Real-time and conventional PCR master mix preparation for the amplification of IBV isolates

Type of PCR	Type of reagent	For one reaction
One-step RT–PCR for the 3′UTR amplification	1. RNase free water	4 µl
	2. 5x RT–PCR Buffer	5 µl
	3. 5X Q Solution	5 µl
	4. Primer-AIBV All1-F-Fow-5pm/µl	2 µl
	5′-CAGCGCCAAAACAACAGCG-3′	2 µl
	5. Primer AIBV-Del1-Rev-5pm/µl	1 µl
	5′-CATTTCCCTGGCGATAGAC-3′	1 µl
	6. 10 Mm dNTP mix	5 µl
	7. One-step RT–PCR Enzyme Mix	
	8. Template	
	Total volume	25 µl
One-step RT–PCR for the spike glycoprotein S1 subunit gene amplification	1. RNase free water	8 µl
	2. 5x RT–PCR Buffer	5 µl
	3. 10 Mm dNTP mix	1 µl
	4. Primer-AIBV XCE2-Fow-5pm/µl	2.5 µl
	5′-CACTGGTAATTTTTCAGATGG-3′	2.5 µl
	5. Primer AIBV-XCE2-Rev 5pm/µl	1 µl
	5′-CCTCTATAAACACCCTTGCA-3′	5 µl
	6. One-step RT–PCR Enzyme Mix	
	7. Template	
	Total volume	25 µl

Real-time polymerase chain reaction The extracted genomic viral RNA was tested using real-time PCR to detect IBV [25, 32, 33]. The primers used were IBV Forward primer: 5′-GCTTTTGAGCCTAGCGTT-3′; IBV Reverse primer: 5′-GCCATGTTGTCACTGTCTATT G-3′; and IBV probe: 5′(FAM)-CACCACCAGAACCTG TCACCTC-3′. The prepared master mix had a reaction volume of 25 µL. TaqMan super mix (2 × 12.5 µL), forward and reverse primers (2.5 µL), probes (0.5 µL), enzyme mixture (0.5 µL), nuclease-free water (1.5 µL), and extracted template (5 µL) were added. The mixture was run using a CFX96™ Real-Time PCR System (Bio-Rad) according to the thermal profile of reverse transcription at 50 °C for 30 min for one cycle, one cycle of activation at 95 °C for 15 min, and 40 cycles of denaturation, annealing, and extension at 94 °C for 30 s, 68 °C for 60 s, and 68 °C for 60 s, respectively.

Conventional polymerase chain reaction RT-PCR was carried out using the Invitrogen SuperScript™ III Platinum™ One-Step RT-PCR Kit (Thermo Fisher, Waltham, MA, USA) following the manufacturer's instructions. To detect IBV from the collected allantoic fluid using one-step RT-PCR, a pair of primers, All1-F 5′-CAGCGCCAA AACAACAGCG-3′ and Del1-R 5′-CATTTCCCTGGCG ATAGAC-3′, was used to amplify 433 bp of the conserved region of the 3′UTR. This primer identifies IBV strains that target the most hypervariable region with conserved flanking regions in the IBV genome [15]. The final volume of master mix preparation was 25 µL (Table 1), which included the template. PCR was carried out using a thermocycler with polymerase activation at 50 °C for 30 min, followed by an initial denaturation step at 95 °C for 15 min. Then, 35 cycles of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 45 s were used for denaturation, annealing, and extension, respectively, followed by a 5 min final extension at 72 °C.

To amplify the spike glycoprotein encoding S1 gene of IBV (466 bp), a 25 µl reaction mixture was prepared by adding 8 µl of RNA-free water, 5 µl of 5X one-step RT-PCR buffer, 1 µl of dNTP mixture, 2.5 µl of forward and reverse primers (Table 1), 1 µl of one-step enzyme mixture, and 5 µl of extracted RNA template [34]. The reaction was performed using a thermal cycler (Bio-Rad), with polymerase activation at 50 °C for 30 min and an initial denaturation step at 95 °C for 5 min. Then, 45 cycles of 95 °C for 30 s, 52 °C for 30 s, and 68 °C for 30 s were used, followed by a final extension at 68 °C for 12 min, as shown in Table 1.

Nested PCR After detecting all IBVs using one-step RT-PCR, a nested PCR was conducted to detect the serotypes of Massachusetts, D274, and 4/91 with 295 bp, 217 bp, and 154 bp fragments, respectively (Table 2). The Nested PCR assay serves to supplement the sequencing analysis. To amplify these specific serotypes, primers and protocols described by [34, 35] were used (Table 2). The master mix for nested PCR was 50 µl containing 5 µl of 5x RT–PCR buffer, 2 µl of 10 mM dNTP mixture, 0.5 µl of Taq DNA polymerase, 1 µl of each forward and reverse primer, 1 µl of amplified template, and the remaining RNase-free water. A nested thermocycler was used for 35 cycles of denaturation, annealing, and elongation at 94 °C for 1 min, 48 °C for 2 min, and 72 °C for 90 s, respectively, followed by a 10-minute final elongation at 72 °C.

Agarose gel electrophoresis After PCR, the PCR products were run on a 1.5% agar gel by dissolving and boiling 1.5 g Agarose in 100 ml of 1X TAE Buffer, and then 5 µl of GelRed (Biotium, Inc.) was added and mixed. Five µl of the loading dye was added to each PCR product and mixed well. Each well was loaded with 10 µl of the mixed PCR product and loading dye. A 10 µl 100 bp DNA ladder was used as a reference for band size. The gel was run at 120 V for an hour and the gel image was captured using the gel documentation system (UV transilluminator, UVI TEC, UK) [36]. Positive results for the S1 partial gene and 3′UTR showed a band size of 466 bp and 433 bp, respectively.

Avian infectious bronchitis viruses sequencing and phylogenetic analysis

The positive S1 partial gene and 3′UTR PCR product obtained using conventional PCR was purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and stored at − 20 °C until sequencing. The concentration and purity of the purified PCR products were measured using a microvolume spectrophotometer (NanoDrop™ 2000c, Thermo Fisher Scientific, Waltham, MA, USA). The purified PCR products were mixed with the amplification primers and submitted to a commercial sequencing company (LGC Genomics, Berlin, Germany).

The raw sequence data obtained from the sequencing company were assembled using Staden Package version 3.3 software. The sequences were aligned using BioEdit 7.1.3.0 [37]. The degree of sequence similarity search was conducted using the BLAST program (NCBI). All sequence analyses were conducted in MEGA11 [38]. Multiple sequence alignment was performed using Clustal W. Phylogenetic relationships among the current IB virus isolates and homologous sequences retrieved from the GenBank were determined based on phylogenetic tree constructed using the neighbour-joining algorithm.

Table 2 RT-PCR primers sequence with product size

Target gene	Primer name	Sequence	PCR product Size	References
3′UTR	All 1-Forward Del 1- Revere	5′-CAGCGCCAAAACAACAGCG-3′ 5′-CATTTCCCTGGCGATAGAC-3′	433 bp	Hewson et al., 2009
S1	XCE2 + Forward XCE2-Reverse	5′-CACTGGTAATTTTTCAGATGG-3′ 5′-CCTCTATAAACACCCTTGCA-3′	466 bp	Adzhar et al., 1997
S1	XCE3-Reverse DCE1 + Forward MCE1 + Forward BCE1 + Forward	5′-CAGATTGCTTACAACCACC-3′ 5′-TTCCAATTATATCAAACCAGC-3′ 5′-AATACTACTTTTACGTTACAC-3′ 5′-AGTAGTTTTGTGTATAAACCA-3′	217 bp 295 bp 154 bp	Adzhar et al., 1997

Results

Clinical and pathological observations

During the investigation of IBV outbreaks on chicken farms, suspected chickens were monitored and clinically inspected for infectious bronchitis disease. Several clinical signs were noted during clinical observation, including coughing, tracheal raring, gasping, depression, and poor weight gain (Fig. 2). Gross pathology was done on the clinically diseased chicken during the postmortem examination (Fig. 2), which revealed cloudiness of air sacs with yellow caseous exudates.

On 11 farms, 14,390 chickens were found. Among these flocks, 1,383 chickens showed clinical signs of IBV infection. Of the 1,383 chickens with IB symptoms, 42 died. The morbidity rate, measured as the proportion of sick flocks among the total 14,390 flocks, was 9.61%. The mortality rate, determined as the percentage of deaths among the total 14,390 flocks, was 0.29%. The case fatality rate, or the proportion of deaths among the 1,383 affected flocks, was around 3.04% (Table 3).

Infectious bronchitis virus screening

The field samples were first screened before being isolated and molecularly investigated. IBV molecular detection was performed on a total of 49 pooled samples, including 37 swab samples and 12 pooled tissue samples. Of these, 25 samples were tested using real-time PCR, and three positive samples were detected (Fig. 3). Due to shortage of the real-time PCR reagents, the remaining 24 samples were tested using conventional PCR, and an additional three positive samples were identified. In total, the screening methods identified six IBV-positive

Fig. 2 Chicken pictures taken during clinical manifestation and gross pathological lesion of suspected infectious bronchitis infection. (**A**) tracheal raring, gasping, depression, poor weight gain, (**B**) depression, poor weight gain, and tracheal raring, (**C**) depression, and (**D**) cloudiness of air sacks and yellow caseous exudates cases observed after postmortem examination

samples. Only one of the six positive samples was a tissue sample; the rest were swab samples.

Virus propagation and isolation using embryonated eggs

The six positive pooled samples, which included five swab samples and one tissue sample, were inoculated into 9-day-old embryonated chicken eggs with allantoic sacs for up to five passages (Table 4). During the first and second passages, six of the samples demonstrated a cytopathic effect (CPE) characterized by embryos congestion and haemorrhage, whereas the control samples showed no change or mortality (Fig. 4). Two more samples were inoculated, and inspection of the embryos up to five passages revealed a variety of symptoms, including congestion, haemorrhage, embryo curling, deformation, and

Table 3 Morbidity, mortality, and case fatality rates of infectious bronchitis in the study areas

Area	Farm	No of chickens	No of cases	No of death	Morbidity (%)	Mortality (%)	Fatality (%)	Type and number of pooled samples collected		
								Swab	Tissue	Total
Mekele	Farm 1	830	53	-	6.39	0	0	4	3	7
	Farm 2	300	50	-	16.67	0	0	4	2	6
	Farm 3	110	35	-	31.81	0	0	5	-	5
Bishoftu	Farm 4	2000	105	-	5.25	0	0	2	1	3
	Farm 5	4500	200	15	4.44	0.33	7.5	6	1	7
	Farm 6	3000	200	20	6.66	0.66	10	3	1	4
	Farm 7	1150	575	-	50	0	0	3	-	3
	Farm 8	200 900	15	-	7.5	0	0	1 5	1 -	2 5
	Farm 9	1000	80	2	8.88	0.22	2.5	2	2	4
	Farm 10	400	40	3	4	0.3	7.5	2	1	3
	Farm 11		30	2	7.5	0.5	6.66			
Total	**11**	**14,390**	**1383**	**42**	**9.61**	**0.29**	**3.04**	**37**	**12**	**49**

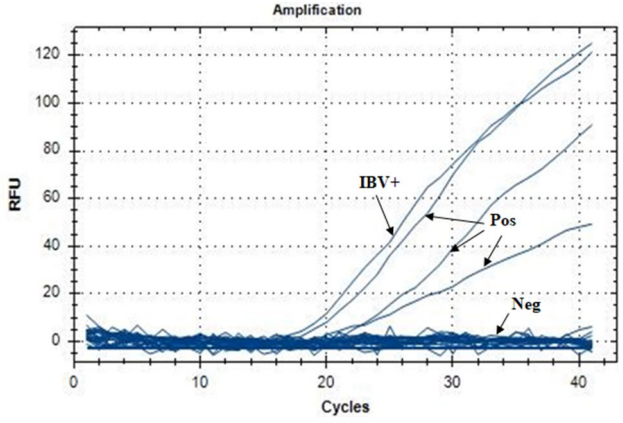

Fig. 3 Real-time PCR result for the infectious bronchitis virus isolates. IBV+: avian infectious bronchitis vaccine positive control; Pos: IBV positive samples; and Neg: negative samples and negative control

stunted growth. The calculated embryo lethal dose for these samples was 6.5 ELD 50/ml (Fig. 5).

Virus propagation using chicken embryo fibroblast cell culture

Positive allantoic fluid samples were filtered through a 0.45 µl minipore filter and inoculated with 0.5 ml of CEF cell culture. Following inoculation, a clear CPE was observed under an inverted microscope at 40× magnification after 4 days post-inoculation and characterized by rounded, aggregated cells and detachment from the flask surface (Fig. 6); however, the control CEF cell culture remained unchanged, as shown in Fig. 6. The IBV isolate showed a titre of 5.7 TCID50/ml in the third passage.

Molecular IBV detection

The S1 gene and 3′UTR of the six IBV positive isolates from the allantoic fluid of embryonated chicken eggs were amplified using one-step RT-PCR. The amplified products revealed a 466 bp region of the IBV S1 gene and a 433 bp region of the 3′UTR, as shown in Fig. 7A and B, respectively. The results showed that all six pooled

Table 4 Virus propagation in embryonated chicken eggs and cytopathic effect across five passages

Sample ID	No of eggs	1st passage (72 h)	2nd passage (72 h)	3rd passage (48 h)	4th passage (48 h)	5th Passage (48 h)
Farm 1 (S)	5	Congestion Haemorrhage	Congestion Haemorrhage	Stunted Growth Curling	Curling Deformation	Stunted Growth Curling
Farm 2 (S)	5	Congestion Haemorrhage	Congestion Bleeding	Stunted Growth Curling	Curling Deformation	Stunted Growth Curling
Farm 6 (T)	5	Congestion Bleeding	Congestion Bleeding			
Farm 9 (S)	5	Congestion Bleeding	Congestion Bleeding			
Farm 10 (S)	5	Congestion Haemorrhage	Congestion Haemorrhage			
Farm 11 (S)	5	Congestion Haemorrhage	Congestion Bleeding			
Total	30 eggs					

S represents the swab samples, and T represents the tissue samples

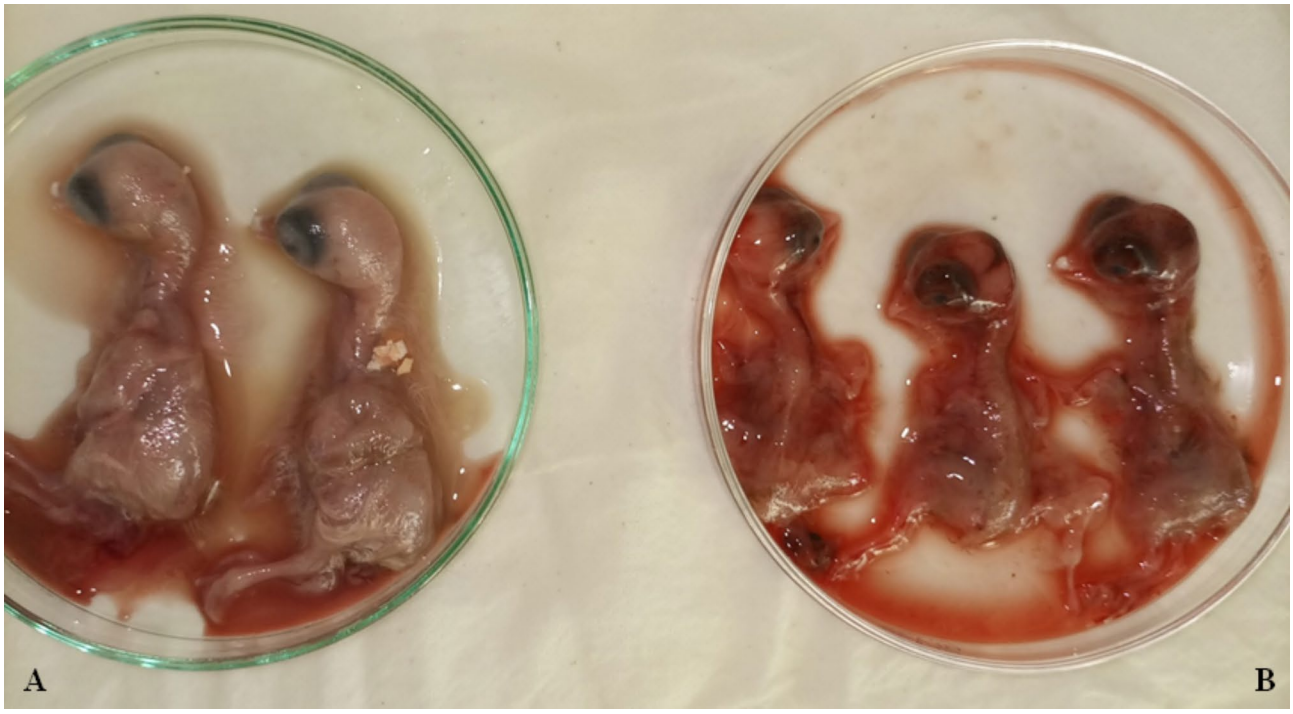

Fig. 4 Isolation of suspected IBV samples with embryonated SPF eggs, passage two. (**A**) normal non-inoculated embryo, and (**B**) bloody and congested embryo following IBV inoculation

samples yielded positive results for both target regions. The nested PCR revealed a positive result for the Massachusetts serotype amplified using the MCE1 primers, with a band size of approximately 295 bp; however, the nested PCR for the 4/91and D274 serotypes was negative.

Sequence and phylogenetic analysis of infectious bronchitis viruses

Six IBV isolates derived from pooled swab samples ($n=5$) and pooled tissue samples ($n=1$) were obtained from Bishoftu ($n=4$) and Mekele ($n=2$). Sequencing analysis was conducted by comparing the alignment of the current outbreak of avian infectious bronchitis virus isolates with sequences retrieved from GenBank. In this investigation, six of the 49 suspected and sampled chickens tested positive for IBV. One of the six positive isolates had a poor chromatogram and was excluded from further sequence analysis. The spike glycoprotein S1 gene of the current five IB virus field isolates were sequenced and deposited in GenBank under accession numbers PQ389500: IBV/Mekele/01/2023, PQ389501: IBV/Bishoftu/01/2024, PQ389502: IBV/Bishoftu/02/2024, PQ389503: IBV/Bishoftu/03/2024, and PQ389504: IBV/Bishoftu/04/2024.

Phylogenetic tree analysis of the spike glycoprotein S1 subunit gene (466 bp) and the 3′UTR (433 bp) sequences of the current infectious bronchitis virus field isolates were conducted using MEGA11 software version 11.0.13. A total of 43 nucleotide sequences, including 5 current IBV isolates of the S1 gene sequence, and 40 homologous sequences retrieved from GenBank, were included in the analysis, as shown in Fig. 8. The mean genetic distance among the five Ethiopian IBV isolates based on the S1 gene sequences was 0.00. The genetic mean distance within the GenBank retrieved IBV isolates was 0.009. The genetic mean distance between the two groups (the current Ethiopian and GenBank retrieved sequences) was 0.0157, indicating a closer relationship among the current Ethiopian isolates with the broader set of GenBank isolates. Phylogenetic tree analysis of the 43 infectious bronchitis viruses based on the spike glycoprotein S1 subunit gene nucleotide sequences indicated in Fig. 8. Additional information on the present IBV isolates S1 partial gene and 3′UTR sequences, as well as agarose gel images can be available in Supplementary File 1.

Discussion

Infectious bronchitis is a highly contagious viral disease of poultry that causes severe respiratory distress and urinary and reproductive loss, resulting in production losses due to poor hatchability, decreased egg production, poor egg quality, and negative economic consequences [7, 39]. The viral genome undergoes continual mutation through deletion, insertion, and recombination. This complicates the prevention and control measure [40, 41]. In Ethiopia, poultry production is the most growing and economically important sector; nonetheless the spread of IB disease is poorly understood, making effective control

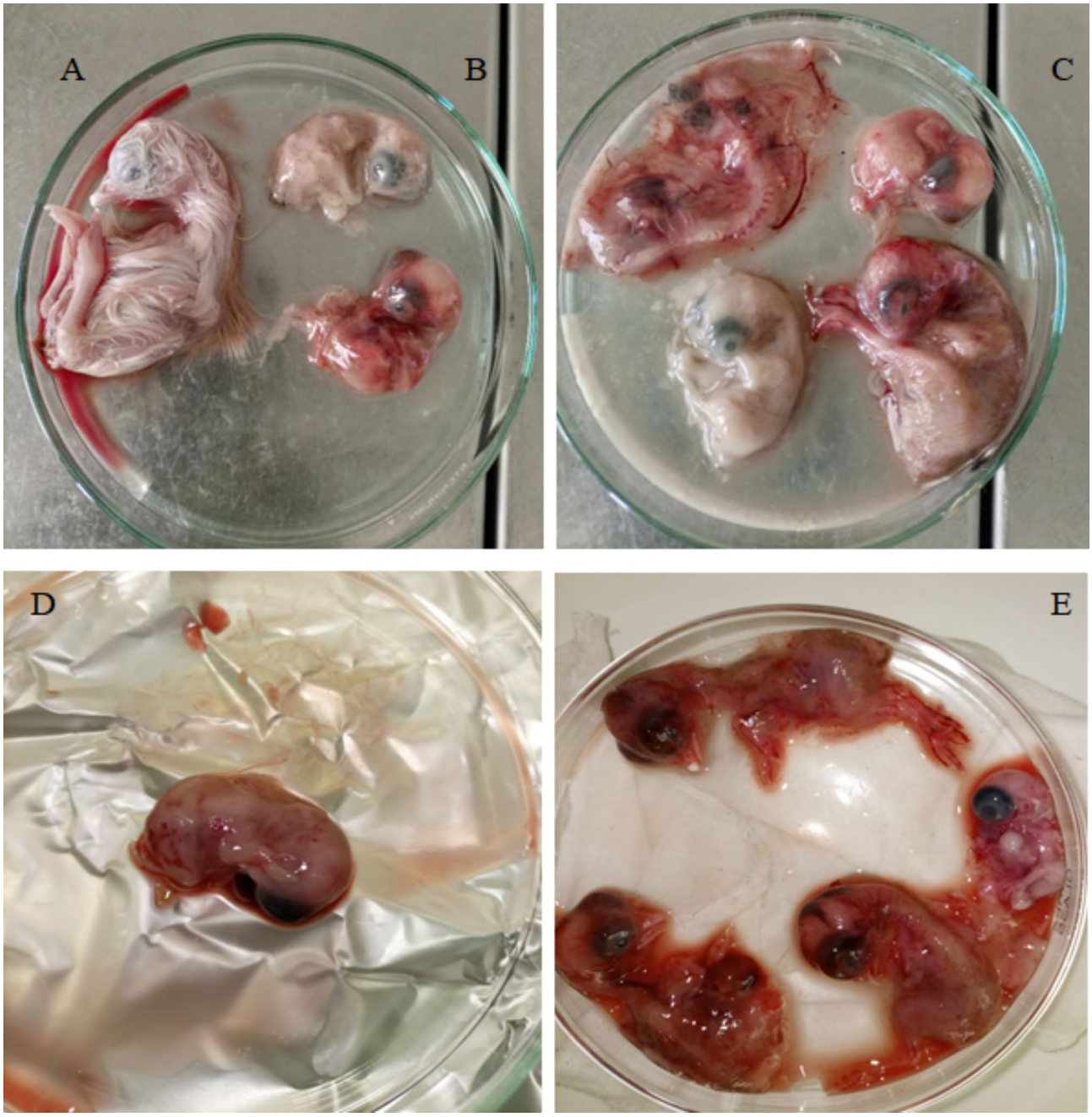

Fig. 5 IBV isolation on embryonated SPF eggs at passages three to five. (**A**) normal embryo, and (**B**, **C**, **D**,and **E**) curled embryo, deformation, and stunted growth after IBV inoculation

measures difficult to implement. The main goal of this study was to investigate the outbreaks and isolate and molecularly characterize the IBV circulating in the study areas. Therefore, the findings provide baseline information for developing and implementing disease prevention and control strategies.

The current outbreak investigation revealed a morbidity and mortality rate of 9.6% and 0.29%, respectively. This result differs from that of [42], who reported morbidity and mortality rates of 85% and 20%, respectively, in 20-day-old chicks in Iraq. During the outbreak investigation, 12.24% of 49 suspected and sampled chickens tested positive using RT-PCR. This percentage is higher than that reported by [43] from the Jima zone, which was 6%. Still, it is significantly lower than the 33.3% reported from 12 swab samples taken from East Shewa and 39% from the Addis Ababa live animal market, as reported by [22, 44], respectively. Differences in IB disease incidence could be attributed to the geographical location of the study areas, farm biosecurity management, infection

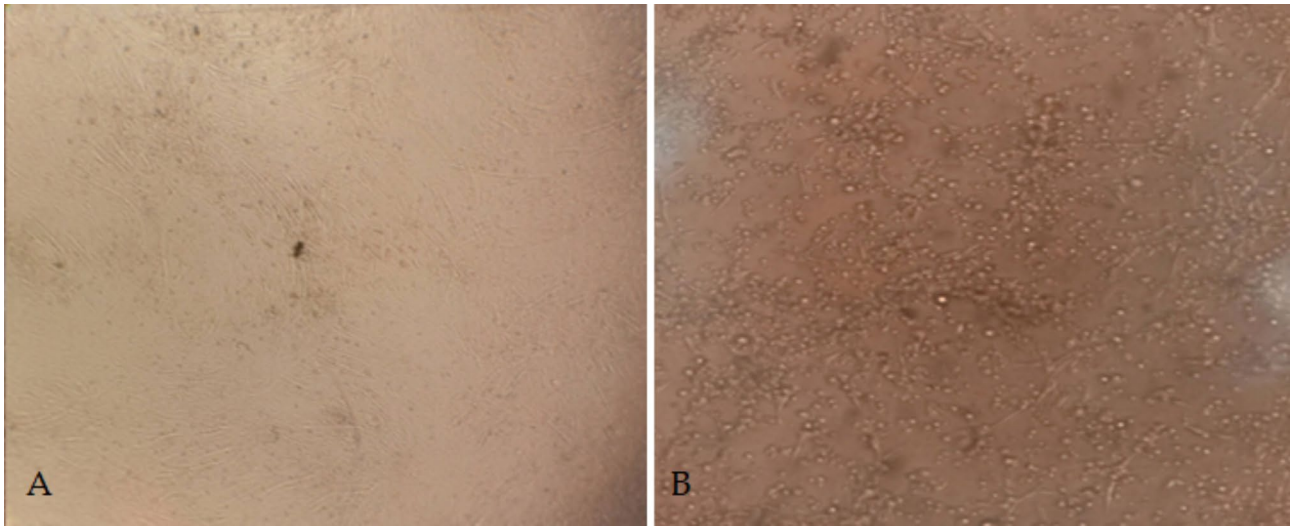

Fig. 6 IBV isolation using CEF cell culture. (**A**) control or non-inoculated CEF cell culture, and (**B**) CPE such as cell rounding, aggregated cells, and cell detachment from the tissue culture flask surface following field IBV inoculation

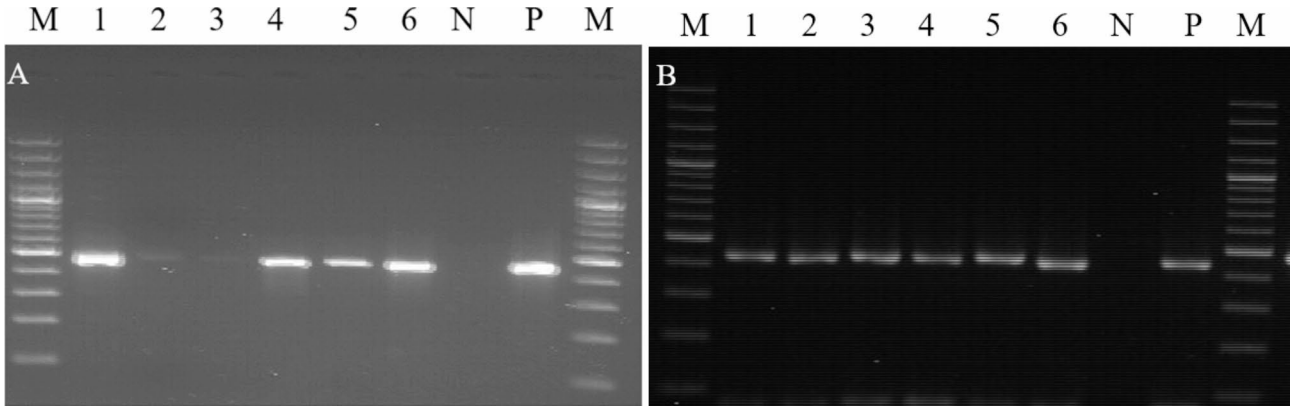

Fig. 7 Conventional PCR-based detection of IB viruses, with an agarose gel image shows the amplification of spike glycoprotein S1 subunit partial gene with a band size of around 466 bp (**A**), and the 3′UTR with a band size of around 433 bp (**B**). Lane M: molecular ladder (GeneRuler™ 100 bp plus DNA Ladder, Thermo Scientific); Lanes 1–6: positive samples; Lane N: negative control; and Lane P: positive control

prevention and control strategies, and other health-related management factors.

This study revealed that depression and respiratory signs such as sneezing, coughing, nasal discharge, and gasping were the most common observed clinical findings among commercial chickens. The findings were consistent with previously reported clinical signs [42, 44–46]. The occurrence of postmortem pathological lesions, such as a fog of air sacs with yellow caseous exudates, was consistent with previous findings of IB virus infections in chickens [46]. Furthermore, similar results have been reported for infections caused by bacterial infections or other viral coinfections [6, 47, 48].

The isolation and propagation of IBV samples using embryonated SPF eggs is a simple and gold-standard method. This study found that the absence of embryo growth or dwarfing/deformation, blood congestion, and curling were indicators of IBV infection, consistent with previous findings [27, 49–51]. Even embryonic changes are not specific to IBV and can be caused by other respiratory viruses, such as pathogenic Newcastle disease virus variants and infectious laryngotracheitis virus [52]. In this investigation, all embryos died 48–72 h after being inoculated with the IB virus. As indicated in Fig. 5, embryos on the left upper side lost their body structure, and embryo deformation, haemorrhage, and mortality were observed from the beginning of the first passage. However, contrary to the current investigations, other researchers have found embryo congestion and haemorrhage beginning with the third passage [27, 49, 51]. These differences could be attributed to co-infection IBV with other viral infections and bacterial complications, or IBV's pathogenic nature could cause them. In the current investigation, the median lethal dose of IBV was 6.5 ELD 50/ml, which was determined based on the total number of dead embryos and surviving embryos at each dilution

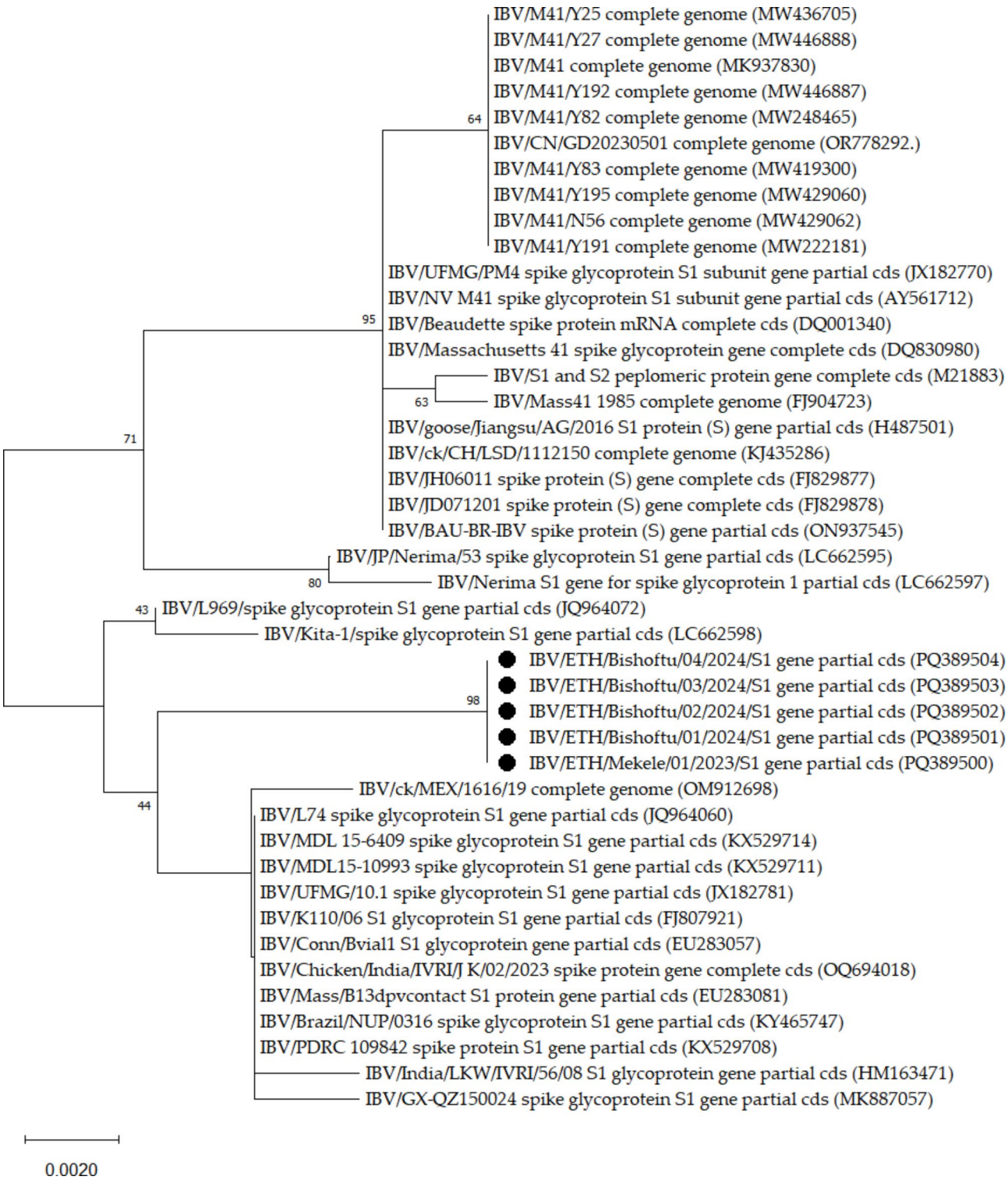

Fig. 8 Phylogenetic tree of infectious bronchitis viruses based on the partial nucleotide sequences (445 bp) of the spike glycoprotein S1 subunit gene. The phylogenetic tree was constructed using 43 IBV nucleotide sequences, which included five Ethiopian field isolates from the current investigation (PQ389500-04, labelled with a circle) and homologous sequences retrieved from GenBank. MEGA11 software was used to compute the neighbor-joining method with the maximum composite likelihood nucleotide substitution model and the pairwise deletion option. The percentage bootstrap scores out of 1000 replicates are displayed next to each branch

[28]. This finding is greater than the velogenic Newcastle disease virus embryo lethal dose reported by [53], which reported a 6.3 ELD50/mL. These results show that the present IBV isolates are highly pathogenic.

The continuous cell line does not support the growth of IBV. However, primary cell cultures derived from chicken embryo fibroblasts supported the growth and isolation of IBV. Cytopathic effects, including rounding and aggregation, as well as detachment of infected cells from the cell surface flask, were observed three days following IBV inoculation. These findings are comparable to those of [27, 53]. The titre recorded in the current study was 5.7 TCID50 in the third passage, which is higher than the titre 4 TCID50 obtained by [27] with the same passage number.

Previous studies have identified and characterized infectious bronchitis viruses by targeting the S1 gene and the 3′-UTR of the IBV [36, 49, 53]. IBV strain characterization is often conducted by amplifying the S1 gene, which is also considered as the gold-standard method. However, this gene comprises the majority of hypervariable regions and is susceptible to mutation and recombination with other strains. Because of their high mutation and recombination rates, new IB virus strains are constantly emerging [18]. The 3′UTR of the IBV genome is a highly conserved region and less prone to mutation and recombination. Furthermore, the 3′UTR contributes to pathogenicity during the replication process [54]. In this study, the harvested allantoic fluid was subjected to RT-PCR targeting the S1 gene and 3′UTR region, and the results confirmed that the disease was caused due to IB viral infection. In addition, the serotypes of IBV were identified using RT-PCR, yielding negative result. This result contradicts with previous reports [20–22, 43]. However, the sequence and phylogenetic analysis revealed that the current isolates were related to Mexican isolates, which are classified as GI-9. As a result, the isolates in the present investigation differ from those previously reported from Ethiopia [20–22, 43].

Ethiopia currently imports 793B and Massachusetts-type vaccines, which closely resemble the previously identified strain. Although these imported strains are comparable to previously identified strains in Ethiopia, they are insufficient for molecular and serological investigations. Because of this situation, as well as the lack of or poor cross-protection among the different serotypes, vaccines may not provide complete protection to vaccinated chickens against circulating virus types, and the nature of the virus's mutation and recombination increases the likelihood of the emergence of a new strain. Previous reports indicate that the outbreak of IBV was caused by field isolates [20–22, 43], which is consistent with the findings of the current investigation. A comparison of multiple sequence alignments of the Ethiopian isolates obtained from Bishoftu isolate three revealed single nucleotide variation at position seven from the other four isolates. In Ethiopia, the circulating IBV variants reported from commercial poultry farms and backyard systems include D274, 793B, Mas, and Qx-, based on antibody testing and RT−PCR methods [20, 21, 43]. However, the present study included genome sequence analysis of the S1 gene and 3′UTR.

A phylogenetic tree was constructed to determine the genetic relationships between the current Ethiopian field isolates and other isolates retrieved from the database. Therefore, the findings provide the first molecular characterization of IBV in Ethiopia, identifying genetically related local viral lineages that differ from those of the strains used in imported vaccines. This study provides useful information for designing an IB vaccine that can match strains of the virus that are already circulating in Ethiopia.

The study's limitations were that representative samples were collected from two outbreak areas (Mekele and Bishoftu) despite the fact that chicken production is practiced throughout the country, and that only a small number of samples were collected. This was due to budget constraints for covering more commercial chicken farms.

Conclusion

This study provides crucial insights into the genotypes of AIBV strains in Ethiopia and highlights the presence of multiple strains. Genetic analysis revealed that the current AIBV isolates in Ethiopia belong to a different lineage, GI-9, which is closely related to the Mexican isolate OM912693 and other Mexican strains, representing the GI-9 (Arkansas type) lineage that has not been previously reported in the country. In conclusion, this information is helpful for the development of more effective AIBV vaccines that can target currently circulating strains in Ethiopia. Based on the findings of this investigation, the following recommendations are proposed: Investigate IBV outbreaks in Ethiopia to better understand their distribution and diversity, serotyping circulating IB viruses to determine their antigenic characteristics and to select potential vaccine candidates, whole-genome sequencing of the IBV field isolates to provide detailed strains characterization, implement stringent farm biosecurity and infection prevention control measures to reduce the risk of IBV transmission and the emergence of new strains, and develop tailored IBV vaccines based on local strains or matching imported vaccines to improve disease prevention and control efforts in Ethiopia's poultry sector.

Supplementary Information

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Supplementary Material 1

Supplementary Material 2

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Author contributions
N.B, E.G: Conceptualization. N.B, H.M, A.L, G.D, E.A, T.T, M.A, T.A, B.B, L.T, K.B, E.G: methodology, formal analysis, writing original draft. E.H, E.G: supervision. N.B, E.H, E.G: writing review and editing. All authors have read and agreed to the published version of the manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
Ethical approval for this study was obtained from the Animal Research Ethical Review Committee of the College of Veterinary Medicine and Agriculture, Addis Ababa University with registration number: VM/ERC/02/13/16/2023. Verbal consent was obtained from the animal owners for their willingness to participate in the study. Sampling was performed following proper animal care.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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