Thesis Ref. No._____

ACTIVE OUTBREAK INVESTIGATION, ISOLATION, AND MOLECULAR CHARACTERIZATION OF INFECTIOUS BRONCHITIS VIRUS FROM POULTRY FARMS IN MEKELE AND BISHOFTU, ETHIOPIA

MSc. THESIS

ADDIS ABABA UNIVERSITY COLLEGE OF VETERINARY MEDICNE AND AGRICULTURE DEPARTMENT OF MICROBIOLOGY, IMMUNOLOGY AND VETERINARY PUBLIC HEALTH

BY

NIGUSU BERHANU KASSAYE

JUNE 2024

BISHOFTU, ETHIOPIA

ACTIVE OUTBREAK INVESTIGATION, ISOLATION, AND MOLECULAR CHARACTERIZATION OF INFECTIOUS BRONCHITIS VIRUS FROM POULTRY FARMS IN MEKELE AND BISHOFTU, ETHIOPIA

MSc. THESIS

A Thesis submitted to the Addis Ababa University College of Veterinary Medicine and Agriculture in partial fulfilment of the requirements for the degree of Master of Science in Veterinary Microbiology

BY

NIGUSU BERHANU KASSAYE

JUNE 2024

BISHOFTU, ETHIOPIA

Addis Ababa University

College of Veterinary Medicine and Agriculture

Department of Microbiology, Immunology and Veterinary Public Health

As MSc research advisors, we here by certify that we have read and evaluated, this thesis prepared under our guidance by Nigusu Berhanu Kassaye entitled **"Active Outbreak Investigation, Isolation, and Molecular Characterization of Infectious Bronchitis Virus from Poultry Farms in Mekele and Bishoftu, Ethiopia**". We recommend that it can be submitted as fulfilling the thesis requirement for the degree of Master of Science in Veterinary Microbiology.

Submitted by: Nigusu Berhanu Kassaye	_____	_____
Dr. Eyob Hirpa (DVM, MSc, Assist. Prof)	_____	_____
Name of Advisor	Signature	Date
Dr. Esayas Gelaye (DVM, MSc, PhD)	_____	_____
Name of Co-advisor	Signature	Date
Professor Bekele Megersa (DVM, MSc, PhD)	_____	_____
Department chairperson	Signature	Date

As member of the Board of Examiners of the MSc, Open Defense Examination, we certify that we have read and evaluated the thesis prepared by Nigusu Berhanu Kassaye entitled: "**Active Outbreak Investigation, Isolation, and Molecular Characterization of Infectious Bronchitis Virus From Poultry Farms in Mekele and Bishoftu, Ethiopia**" and examined the candidate. We recommended that it be accepted as fulfilling the thesis requirement for the degree of Master Science in Veterinary Microbiology.

Mr. Abebe Wirtu	_____	_____
Chairperson	Signature	Date
Dr. Getachew Abichu	_____	_____
External Examiner	Signature	Date
Dr. Debebe Ashenafi	_____	_____
Internal Examiner	Signature	Date

STATEMENT OF THE AUTHOR

First, I declare that this thesis is my authentic work and that all sources of materials used for this thesis have been properly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for MSc degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and it is deposited at the University /College library to be made available to borrowers under rules of the library.

I solemnly state that this thesis is not submitted to any other institution anywhere for the award of any academic certificate, diploma or degree. Brief quotations from this thesis are allowable without special permission provided that accurate acknowledgement of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the College when in his or her judgment the proposed use of the material is in the interest of scholarship. In all other instances, however permission must be obtained from the author.

Name: Nigusu Berhanu Kassaye

Signature: _____

Date of Submission: _____

ACKNOWLEDGEMENTS

First and foremost, I would like to thank the ultimate leaders of all creatures, Almighty God, for his endless mercy and keeping me throughout my life.

I would like to express my heartfelt gratitude to my advisors, Dr. Eyob Hirpa and Dr. Esayas Gelaye, for their intellectual support and constructive advice. I am grateful to the National Veterinary Institute (NVI) for the general support throughout the entire education and research work, the African Union Pan-African Veterinary Vaccine Centre (AU-PANVAC) for the real-time PCR detection, and the Joint FAO/IAEA Center in Vienna, Austria for their assistance with the sequencing of the Infectious Bronchitis virus isolates. I want to extend my deepest gratitude to the research and development directorate for providing me with the necessary facilities and field vehicles during sample collection.

My heartfelt thanks goes to Hawa Mohammed, Dr. Liyuwork Tesfaw, Eyob Asefa, Getaw Derese, Kenaw Birhanu, Abnet Legesse, Dr. Mirtneh Akalu, and Dr. Takele Tesgera for their excellent cooperation and contributions to the virology and molecular aspects of this work. I would also like to appreciate Dr. Esayas Gelaye for his constructive comments on data analysis and other valuable inputs. I want to extend my special appreciation to my beloved family to their general support and kind hospitalities in every aspect.

Finally, I want to thank all my friends for their great support and inspiration throughout my studies, particularly Misganaw Taso, Megenasa Balcha, and Dr. Sagni Keno.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

AIBV	Avian Infectious Bronchitis Virus
BLAST	Basic Local Alignment Search Tool
CAM	Chorioallantoic Membrane
CEF	Chicken Embryo Fibroblast
CPE	Cytopathic Effect
ELD	Embryo Lethal Dose
M	Matrix or Membrane protein
MEGA	Molecular Evolutionary Genetics Analysis
MUSCLE	Multiple Sequence Comparison by Long Expectation
N	Nucleocapsid protein
NCBI	National Center for Biotechnology Information
NSP	None Structural Proteins
NVI	National Veterinary Institute
ORF	Open Reading Frame
PBS	Phosphate Buffered Saline
RDRP	RNA Dependent RNA polymerase
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
S	Spike glycoproteins
SPF	Specific Pathogen Free
TCID	Tissue Culture Infective Dose
UTR	Untranslated Region
VTM	Viral Transport Media
WOAH	World Organization for Animal Health

ABSTRACT

Avian Infectious Bronchitis (AIBV) is a highly contagious respiratory disease that affects the poultry industry globally. In Ethiopia, AIBV has been reported in both commercial and backyard chickens. The currently used vaccine effectiveness is limited due to a lack of cross-strain protection and outbreaks continue to make mitigating the disease in Ethiopia difficult. Therefore, the objectives of this study were to isolate, genomic, and phylogenetic analysis of circulating field AIBV. A cross-sectional study was conducted from November 2023 to May 2024 in Mekele (eighteen samples) and Bishoftu (thirty-one samples) cities. Twelve tissue and thirty-seven pooled swab samples were collected, and six out of forty-nine samples (five swab samples and one tissue sample) tested positive for AIBV using real-time PCR and conventional RT-PCR. The six samples propagated into embryonated eggs and exhibited characteristic AIBV lesions and mortality over five consecutive passages. All the six isolates originating from Bishoftu (n=4) and Mekele (n=2), were amplified targeting 466 bp of the S1 gene and 433 bp of the 3'UTR using one-step RT-PCR. The purified PCR product of the five isolates targeting the 3´UTR region was sequenced and analyzed using bioinformatics tools. The sequence alignment of Ethiopia's five isolates revealed a similar sequence except for one isolate (Bishoftu/03/2024) showed a single nucleotide change (A: C) resulting in amino acid change Glutamine(Q) to Proline (P). The phylogenetic analysis demonstrated the genetic distance was lower among the newly reported isolates (0.001) compared to the broader set of GenBank isolates (0.01), indicating a closer evolutionary relationship between the current local isolates and the Mexican isolates. Therefore, the findings identify genetically related local viral lineages that differ from strains used in imported vaccines. It also indicates that outbreaks were caused by infection with the IB virus which is creating a serious health risk in the poultry industry. Further research on the economic impact of AIB in poultry production, serotyping of circulating AIB viruses, and vaccine development based on the local isolates are recommended.

Keywords: *AIBV, Bishoftu, Chicken, Ethiopia, Isolation, Mekele, RT-PCR, Real-time PCR, Sequencing, Phylogenetic analysis*

1. INTRODUCTION

Poultry production is the main agricultural activity in the world, which covers almost half of animal protein. In Ethiopia poultry production is the second most populated (with around 56 million) after cattle (Zeleke, 2017). Because it is critical for reducing poverty, ensuring food security as a source of meat and eggs, and supporting economic growth in the country. Twenty percent of the poultry produced in the country is raised commercially, while the remaining chickens are raised traditionally which exposes them to various diseases, predators, a lack of veterinarian care, and other limitations (Zeleke, 2017; Birhan *et al.*, 2021).

Avian infectious bronchitis (AIBV) is one of the most important acute and transmittable viral diseases in chickens, listed as a notifiable disease by the World Organization for Animal Health (WOAH, 2018). The disease was reported in 1931 in North Dakota, USA (Schalk and Hawn, 1931). AIB is an air-borne disease that transmits horizontally through direct contact and indirectly through fomites, contaminated water, and feed, with transmission through embryonated eggs (Pereira *et al.*, 2016). Chickens of all ages are susceptible to the IB virus, which targets epithelial cells in the upper and lower respiratory tracts and causes symptoms such as coughing, gasping, respiratory rales, sneezing, and watery nasal discharge, can affect chickens of all ages (Awad *et al.*, 2014).

The 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, leading to urinary lesions and nephritis with higher mortality rates depending on the chicken’s age, breed, immunological status, virus strain, severe respiratory effects, and reproductive problems. The economic impact of the disease on morbidity and control is greater than that of mortality. It has the most economic impact on chicken worldwide because it reduces egg production by decreasing egg quality and production efficiency due to poor broiler weight increase followed by a decrease in feed efficiency (Cook *et al.*, 2012; Jackwood, 2012; Umar *et al.*, 2016). The disease is difficult to control due to the disease's rapid dissemination, mutation, and a lack of cross-protection among infectious bronchitis virus strains (Jackwood, 2012).

The causative agent of infectious bronchitis is the a*vian infectious bronchitis virus* (AIBV), which belongs to the *Nidovirales* order, family *Coronaviridae*, subfamily *Coronavirinae,* and genus *Gammacoronavirus*. The coronavirus causing AIB disease is an enveloped virus containing linear and single-stranded positive-sense RNA genome, which is the largest among RNA genomes consisting of about 27.6 kb in size and arranged as 5′UTR-1a-1b-S-3a-3b-E-M-5a-5b-N-3′UTR (Cavanagh, 2007; Abro *et al.*, 2012; Ali *et al.*, 2022).

The AIBV genome has two untranslated regions (UTRs) with poly (A) tails at the ends of its 5′ and 3′ strands, because the 5′ and 3′UTRs include important structural components, they have a major role in the transcription and replication of viral RNA (Valastro *et al.*, 2016; Bhuiyan *et al.*, 2021). The conserved 3´UTR region of the AIBV genome was used for the AIBV strain characterization and classification because these regions are more conserved and less prone to mutation and recombination (Hewson *et al.*, 2009).

The viral genome's two sections that code for proteins known to as structural and nonstructural. At the 3′ end there are four structural proteins: spike Glycoprotein (S), Envelop (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 necessary for RNA replication. The S1 and S2 are the two different S glycoprotein components. S1 at the amino-terminal, which has about 535 amino acids, and S2 at the carboxyl terminus, which has around 627 amino acids, are the two major glycopeptides found in the S glycoprotein (Cavanagh, 2007; Valastro *et al.*, 2016). The glycopeptides are used to adhere the virus to the host cell’s membrane. The spikes that are visible projecting through the envelope on the electromicrograph give the virus its corona-like appearance. The distinct amino acid sequences on this glycoprotein called epitopes characterize the virus's serotype. Among the two, the S1 glycoprotein gene is the most important immunogenic component and contains epitopes responsible for neutralizing antibodies (Butcher *et al.*, 2003).

In nature, the virus is fragile, mutable, and capable of freely changing its genetic composition (Butcher *et al.*, 2003; Jackwood, 2012; Gallardo, 2021). As a result, several

serotypes arose that are difficult to control with vaccination, and many of them do not provide cross-protection. Previously, AIBV was reported to have seven genotypes and thirty-five (35) lineages (Ma *et al.*, 2019). Nowadays AIBV has nine genotypes (GI-GIX) across the world, several lineages with a pairwise genetic distance of 30% and 13%, respectively (Rafique *et al.*, 2024). The new classification approach is based on the variability of the IBV's whole S1 sequence. The location and pathogenicity of these newly emerged genotypic lineages and strains are also distinct. Because of recombination and mutations caused by substitutions, deletions, and insertions during replication, AIBV exhibits continual genetic diversity (Jackwood, 2012; Valastro *et al.*, 2016; Rafique *et al.*, 2024).

In Africa, the Mediterranean basin countries were the first to report cases of AIBV. There are currently several AIBV 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 (Valastro *et al.*, 2016; Bali *et al.*, 2022). Hutton *et al*. (2017) identified the AIBV 793B serotype in Ethiopia, which shares 92% to 95% similarities with the French strain FR-94047-94. Other serotypes like M41, D-274, QX (Tesfaye *et al.*, 2019), and Massachusetts or Mass (Hirbaye *et al.*, 2024) were also reported in Ethiopia. The circulation of the 193B genotype was also reported (Hutton *et al.*, 2017; Tesfaye *et al.*, 2019; Tegegne *et al.*, 2020) in backyard and commercial farms in Oromia Regional State and Southern Nations, Nationalities, and People's Regional State in Ethiopia. The serological detection of AIBV has been also reported at 23.96% (Birhan *et al.*, 2021) in Northwest Ethiopia 74.88% in unvaccinated backyards, and 68.75% in commercial farms in different parts of Ethiopia (Tesfaye *et al.*, 2019).

The gold standard technique for isolating the virus is to use an embryonated egg as the primary method of diagnosing AIBV infection. Continuous cell lines do not support AIBV growth. Reverse transcriptase polymerase chain reaction (RT-PCR) and real-time PCR can be used to get rapid diagnostic testing. Even if, identification of the S gene is believed to be the gold standard method of AIBV classification and characterization it has its disadvantages regarding mutation and recombination in the three HVR regions. In addition, the 3′UTR region of the AIBV genome contains valuable structural components

that are important for the translation and replication of the AIBV virus and ideal for molecular diagnostics and strain classification (Williams *et al.*, 1993; Masters, 2006; J and S, 2015).

In Ethiopia, there is a lack of sufficient molecular data to determine the genotypes of the virus circulating in the country. The imported commercially available vaccines for AIBV are Massachusetts and 793B, which matched with the previously reported AIBV strains in Ethiopia. Although the imported vaccines are related to the previously identified strains in Ethiopia, their effectiveness is limited due to the virus's mode of transmission, mutational characteristics, and lack of cross-strain protection. As a result, even if vaccination campaigns are conducted against the virus, outbreaks continue to occur, and it is difficult to mitigate the disease in Ethiopia.

General objectives

- Address the knowledge gap on the genotypes of AIBV circulating in Ethiopia through isolation, molecular detection, sequencing, and phylogenetic analysis of the AIBV strains in the study areas.

Specific objectives:

- Isolate infectious bronchitis virus from chickens exhibiting clinical signs of AIB disease in Bishoftu and Mekele.
- Molecular detection of the isolated AIBV
- Sequence and phylogenetic analysis of the circulating infectious bronchitis virus in the study area.

2. LITERATURE REVIEW

Avian infectious bronchitis (AIB) is an acute and highly contagious disease that affects chickens' respiratory, renal, and reproductive systems. As a result, there was a decrease in egg production, a decrease in body weight, and chicken losses. The avian coronavirus species, which is a member of the *Gammacoronavirus* genus and the *Coronaviridae* family, is the causal agent of infectious bronchitis (Lin and Chen, 2017). The AIBV first emerged in the United States in 1930 (Schalk and Hawn, 1931). Several researchers have described the virus's isolation and identification from embryonated eggs as well as the development of characteristic lesions in the growing embryos. These days, the virus is widespread and found in almost every part of the globe. Certain variants appear to be limited to a certain area and only last shortly, while others continue and expand (Cavanagh, 2007; Bande *et al.*, 2017).

2.1. Etiology of the Infectious Bronchitis Infection

Avian infectious bronchitis (AIB) infection is one of the most important viral diseases of poultry in terms of economic impact, which is caused by a virus that belongs to the group three family *Coronaviridae*, *Nidovirales* order (Valastro *et al.*, 2016; Legnardi *et al.*, 2020). Generally, Coronaviruses (CoVs) are often divided into four categories: Group one coronaviruses (CoV) or *alpha coronaviruses* include human CoV-NL63, human CoV-229E, swine transmissible gastroenteritis virus (TGEV), feline infectious peritonitis virus (FIPV), and canine coronavirus (CCoV). The second group of *beta coronaviruses* (CoV) includes bovine CoV (BCoV), mouse hepatitis CoV (MHV), COVID-19, MERS-CoV, and severe acute respiratory syndrome CoV (SARS-CoV). *Gamma coronaviruses* or Group-3 and Group-4 or *delta coronaviruses* are typically linked to all types of poultry but are most commonly found in hens, pheasants, and galliformes. They are mostly caused by avian coronaviruses, such as the turkey coronavirus (TCoV) and the infectious bronchitis virus (AIBV) (Abdel-Moneim, 2017).

2.1.1. Morphology and genomic organization of the virus

The virus has a diameter of about 120 nm, a round to pleomorphic shape, and a surface covered in spikes. It is a positive sense RNA single-strand genome measures about 27,000 nucleotides or 27.6 kb in size. The usual AIBV genome's structural organization is expressed by the 5′UTR-1a-1b-S-3a-3b-E-M-5a-5b-N-3′UTR (Figure 1). The 3′ end of the structural protein coding area contains four proteins: S, E, M, and N. Two contributing genes, ORF3 and ORF5, are also present, each of which expresses two non-structural supporting proteins, 3a, 3b, 5a, and 5b, respectively (Cavanagh, 2007; Valastro *et al.*, 2016; Abdel-Moneim, 2017; Bhuiyan *et al.*, 2021). The 5′ part of the genome contains the two non-structural proteins, 1a and 1ab, both of which constitute nearly three-quarters of the genome and have a length of about 20 kb. The RNA production, transcription, RNA proofreading, RNA caps creation, and viral replication all depend on the 15 proteins present in the NSPs. Additionally, they impair the host immune system response (Bhuiyan *et al.*, 2023).

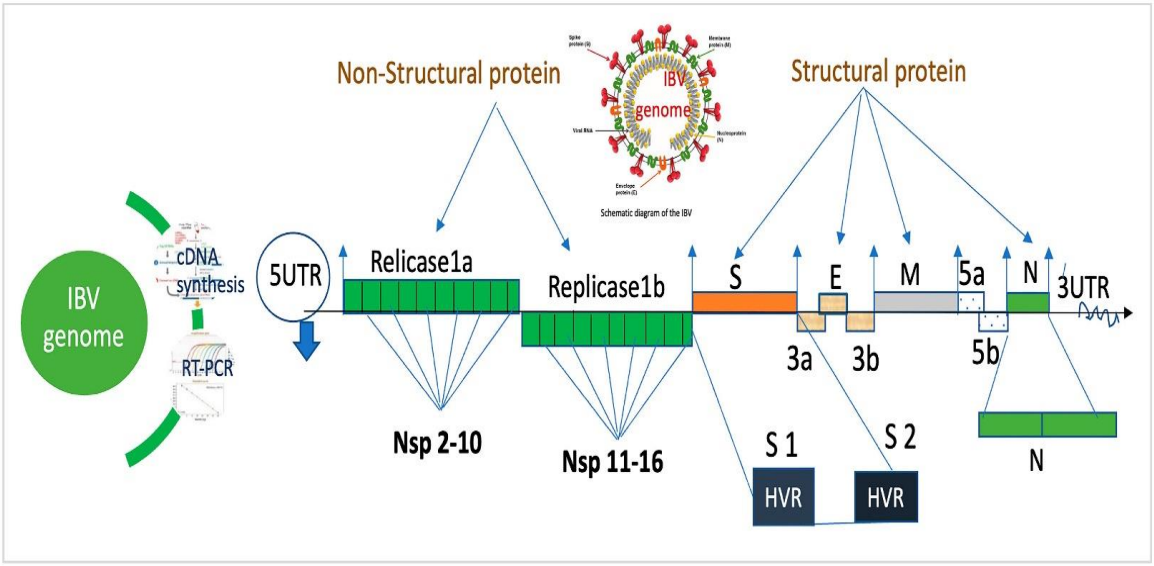


Figure 1: Genomic structure of AIBV with different structural and nonstructural viral genes. (Bhuiyan *et al.*, 2021)

2.1.2. Infectious bronchitis viral proteins

The structural protein-coding segment and the nonstructural protein-coding segment are the two protein-coding segments found in the AIBV. The four essential proteins found in the structural segment are S, E, M, and N. As opposed to this, the nonstructural section comprises 5a, 5b, 3a, and 3b (Cavanagh, 2007; Abdel-Moneim, 2017).

Characteristics of structural proteins: The AIBV genome's structural proteins, S, E, M, and N (Figure 2), make for one-third of the virus's overall structure. On the surface of the virus envelope is the petal-shaped S glycoprotein, which has a length of around 20 nm. The S protein is essential for both the host immune system and the viral attachment. The two different S glycoprotein components are known as S1 (90 kDa), which has an average N-terminal amino acid content of 535, and S2 (84 kDa), which has an estimated C-terminal region amino acid content of 625 (Cavanagh, 2007; Gallardo, 2021). IBV serotypes differ by 20–25% and occasionally by up to 50% of the amino acid sequence. The S1 glycoprotein subunit of the AIBV structure contains three HVR, which are responsible for the virus's variety and ability to evade immune protection. The amino acid positions of HVR 1, HVR 2, and HVR 3 are 38–67, 91–141, and 274–387, respectively. A little change in the nucleotide sequence of the S gene will generate a new AIBV serotype (Jackwood and de Wit, 2013; Abdel-Moneim, 2017; Saadat *et al.*, 2017).

The E protein, which is tiny and ranges in size from 9 to 12 kDa, has a role in the assembly of viruses. The primary responsibilities of the E protein are to assemble, develop, and release the virus from the host cell via the viral envelope. The M structural protein ranges in size from 22 to 25 kDa and is widely present in the virus virion (Ujike & Taguchi, 2015). This structural protein's primary function is to keep the virus in its original shape. It also helps with virus assembly and is necessary for the packaging of viral RNA into nucleocapsids. When it attaches to viral RNA during virion formation, the N protein which is a phosphoprotein that is widely distributed and ranges in size from 50 to 60 kDa is formed. This structural protein is involved in the packaging of viruses, correcting the folding of newly generated viral genomes and causing infected cells to undergo apoptosis (Jayaram *et al.*, 2005; Abdel-Moneim, 2017; Legnardi *et al.*, 2020).

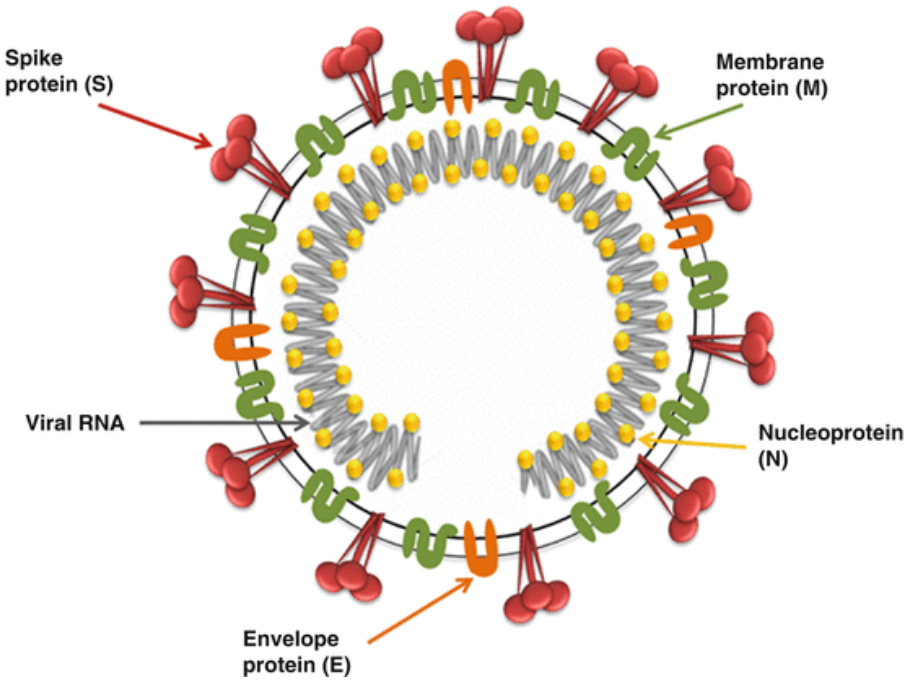


Figure 2: Infectious bronchitis virus morphology with its structural proteins. (Abdel-Moneim, 2017)

Characteristics of nonstructural proteins: Nonstructural proteins are present on each side of the UTR. The 3´UTR region of the AIBV contains the most important structural components that are important for the replication and translation of the virus (Masters, 2006). The pathogenicity of the AIBV virus is changed when the viral replication efficiency is altered during the attainment of the 3´UTR end of the N gene and the 3´UTR region (Liu *et al.*, 2013). On the 3´side, two non-structural accessory proteins 3a, 3b, 5a, and 5b are expressed by ORF3 and ORF5, respectively. At the 5′ end of the genome two non-structural proteins 1a and 1b are encoded, which together make up two-thirds of the genome and are around 20 kb in length. Fifteen (15) proteins (NSP2-NSP16) found in the NSPs are essential for the replication of viruses, RNA synthesis, transcription, RNA proofreading, creation of RNA caps, and control of the host immune response. Though most of these proteins' specific roles are still unknown, in cell culture, some of them are referred to as accessory proteins as they are not required for virus replication (Perlman *et al.*, 2008).

2.1.3. Replication and transcription of infectious bronchitis virus

The cytoplasm is the site of the virus replication cycle (Figure 3). The first stage is for the protein known as S to bind the virion to cellular receptors on the host plasma membrane. The fusion of the virus and the cell membrane occurs in pH-dependent or pH-independent ways at the cell surface and in endosomal vesicles, respectively. Following its release into the cytoplasm, the N-coated viral RNA uncoated itself to make itself available for transcription and translation (Hartenian *et al.*, 2020).

The majority of the genome, ORFs 1a and 1b are translated into a big polyprotein, 1ab, via a ribosomal frameshift mechanism after being released into the cytoplasm. This process results in functional nonstructural proteins (nsp2–16), which start virus replication. The genomic RNA (gRNA), following translation into polyprotein or RNA replicas transcriptase complex, serves as a template for the synthesis of negative-sense RNA species (Tan *et al.*, 2012).

The irregular addition of minus-strand RNA synthesis appears to be the most appropriate model of coronavirus transcription when it comes to producing coronavirus subgenomic mRNA. In this proposed model subgenome-length negative strand, positive sense subgenomic mRNAs are directly transcribed from these subgenome-length negative-strand RNAs, after which RNA is created in a discontinuous transcription using the genome as a template. Thus, the subgenome-length negative strand is the only source from which subgenomic mRNAs can be produced (Williams *et al.*, 1993; Sawicki and Sawicki, 2005). The AIBV genome is replicated continuously by negative-strand RNA. Thus, discontinuous transcription additionally involves genomic RNA replication. For genomic RNA replication, 5' and 3' UTR sequences for secondary RNA structures are necessary in addition to the replicase gene (Tan *et al.*, 2012; Hartenian *et al.*, 2020).

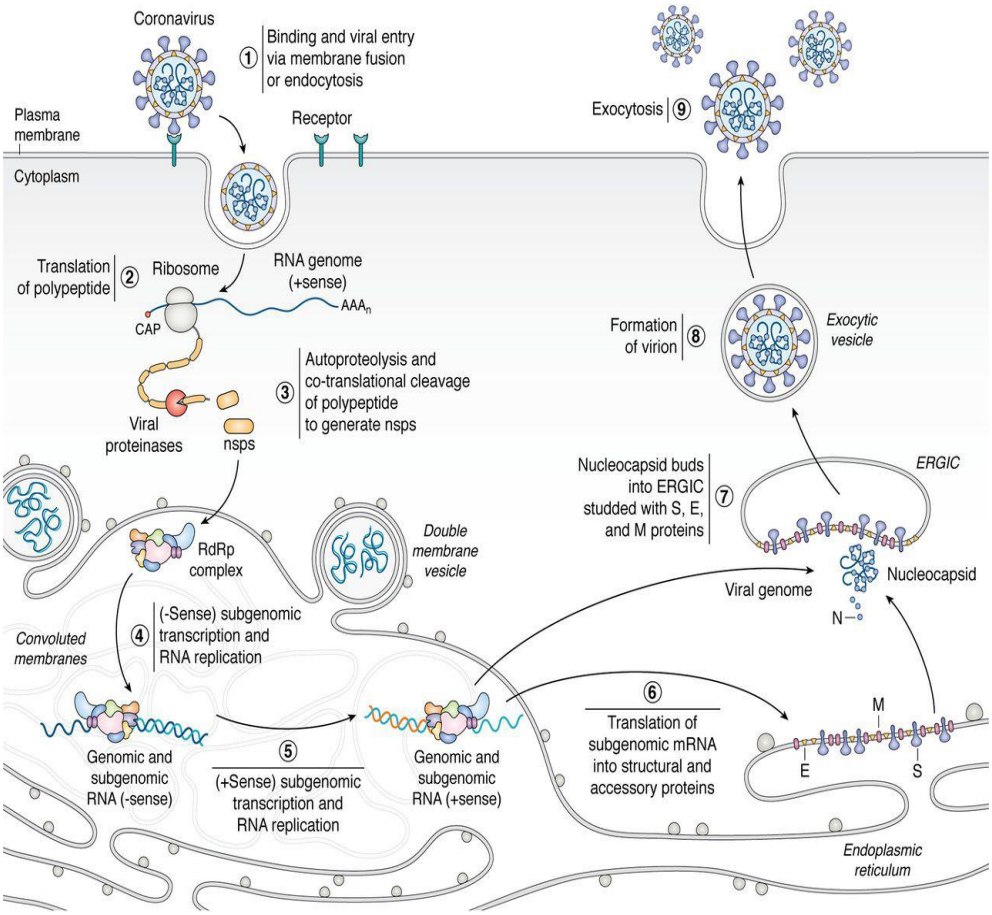


Figure 3: The AIB viral replication life cycle. (Hartenian *et al.*, 2020)

Additionally, the N is necessary for effective viral RNA production. The genome-sized transcripts are packaged into progeny virions (Perlman *et al.*, 2008; Zúñiga *et al.*, 2010). The movement of virus particles follows the intracellular assembly and budding of AIBV via a functioning Golgi stack and their eventual release from the host cells via the exocytic pathway. When the viral buddings form intracellular membranes, a lipoprotein envelope containing E, M, and M surrounds the virus nucleocapsid (Ujike and Taguchi, 2015). In general, the replication of infectious bronchitis virus controlled by RDRP produced by the genomic RNA translation. The Sub-genome length of negative-sense RNA is produced when the polymerase attach-attaches with the 3´UTR end of the viral genome translation initiated primarily.

2.1.4. Evolution and genotype diversity of infectious bronchitis virus

The AIB virus's genetic diversity enables it to adapt to a wide range of environmental situations. The virus is mutated throughout its entire genome, despite the spike glycoprotein gene hosting the majority of these changes (Legnardi *et al.*, 2020). Owing to the S gene changes new serotypes and variations emerged as a result. The S1 glycoprotein sequences of the AIBV serotypes vary by about 20–25%, and occasionally by as much as 50% since they are always changing. This variable has an impact on how well distinct virus strains cross-protect each other. The S1 amino acid sequence may change as a result of multiple passaging in chicken embryos during AIBV adaptation (Saadat *et al.*, 2017).

Viral RNA-dependent RNA polymerase (RdRp), found in RNA viruses like AIBV, mutates from nucleotide insertion, deletion, or point mutation due to a lack of polymerase proofreading activity during replication and genomic recombination, in contrast to the high fidelity of DNA polymerase (Montassier, 2010; Jackwood, 2012; Legnardi *et al.*, 2020). Recombination is the process by which two or more viruses infect the same cell. RdRp can regenerate with the field virus through repeated use of live attenuated vaccines to control AIBV, as well as by fragmenting free from the original template strand and converting to a new template from a different virus that shares sequence similarities in the crossover site. As shown in Figure 4, recombination during AIBV evolution can alter the virus's virulence and lead to the creation of new serotypes (Montassier, 2010; Jackwood, 2012; Moreno *et al.*, 2017).

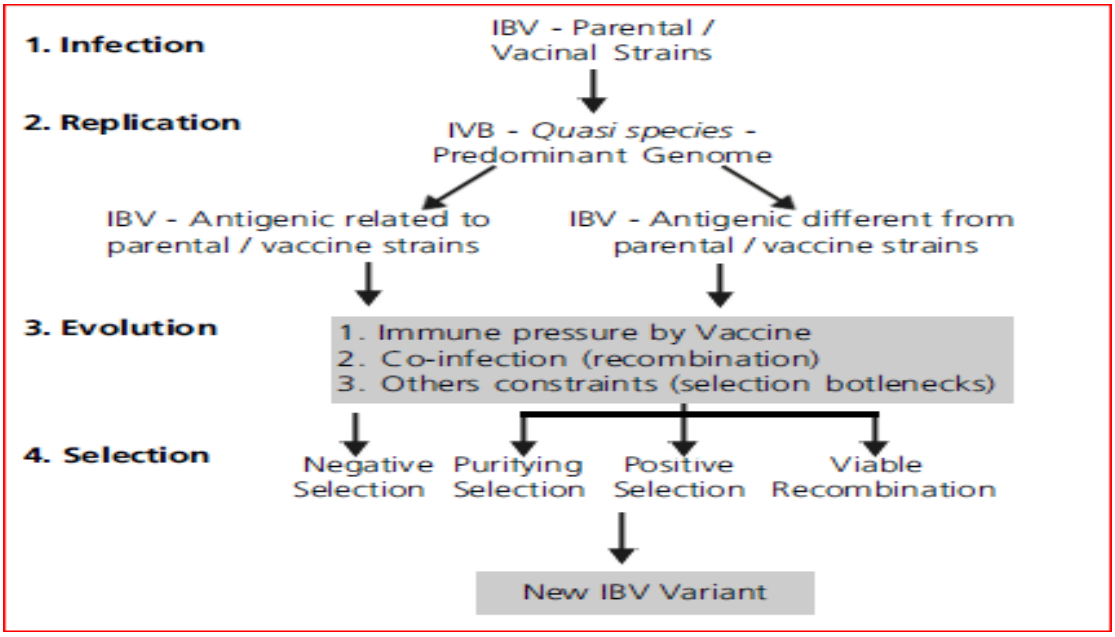


Figure 4: Evolutionary pathway and emergence of new AIBV variants. (Montassier, 2010)

2.2. Epidemiology and Geographical Distribution

Nine genotypes (GI-GIX) and numerous lineages have been identified worldwide since the 1930s when AIBV was originally discovered. These regions include Africa, Asia, America, Australia, and Europe, where an extensive poultry industry has grown (Rafique *et al.*, 2024). The main AIBV serotypes, as indicated in (Figure 5) are dispersed worldwide and include Mass-type, 4/91 (793B or CR88)-like, D274-like (D207, D212 or D1466, D3896), D3128, QX-like, and Italy-02. After the Massachusetts strain was identified, the Connecticut (conn) strain was found in America in the 1970s. Antigenically, this strain differed from the Massachusetts-type strain (Sjaak de Wit *et al.*, 2011). In the USA, reports of AIBV strains like Delaware, SE17, Massachusetts, and Connecticut have been reported (Bande *et al.*, 2017). There is just one AIBV genotype, GIV-1, and the Delaware variant (DE or DE072) is one of these variants that was found in commercial flocks that had a serious respiratory infection. (Lee *et al.*, 2001).

Several serotypes were discovered in Europe in the late 1970s. In Western Europe, the 793B serotype, which was classified under GI-13, was dominant (Bande *et al.*, 2017). Other AIBV serotypes that cause a decrease in egg production include D181 (GII-2) and

the virus that is present in the eye, lung, and trachea (Molenaar *et al.*, 2020). In Western Europe D1466 (GII-1) found locally for a long time and PA/1220/98 (GVIII-1) have been reported (Domanska-Blicharz *et al.*, 2017). Apart from the highly significant serotype QX (GI-19), neuropathogenic variants 2 (GI-23), D274 (GI-12), and Beudette (GI-1) have also been documented (Franzo *et al.*, 2014; Lisowska *et al.*, 2017; Moreno *et al.*, 2017; Fischer *et al.*, 2020).

In Asia numerous AIBV variants including 4/91 and QX (GI-19), which is a significant serotypes dispersed worldwide outside of the United States and Australia (Valastro *et al.*, 2016). The non-infectious genotype GVI-1 was identified in chickens found in China and isolated in South Korea and Japan (Jang *et al.*, 2018; Ma *et al.*, 2019). The other isolated AIBV strains from China, including the IO636/1 isolate, were categorized as new genotype GII. Currently, Colombia has found the sole genotypes of GVI-1 reported in Asia (Ramirez-Nieto *et al.*, 2022).

Only a small percentage of AIBV strains in Australia cause respiratory illnesses; the majority, like the T strain, are nephropathogenic. The GIII-1 lineage was initially discovered in 1988 during an outbreak in Australia. Q3/88 (GIII-2) and N1/88 (GIII-1) isolates of AIBV were found during these outbreaks, but their antigens and genetic makeup set them different from other AIBV variants (Valastro *et al.*, 2016). There have also been reports of other AIBV serotypes in Australia, including NI/03 (GV-1), VicS (GI-6), and N1/62 (GI-5). Due to their geographic location, several AIBV serotypes are restricted in Australia but not in other places (Ignjatovic *et al.*, 2006; Valastro *et al.*, 2016; Quinteros *et al.*, 2021).

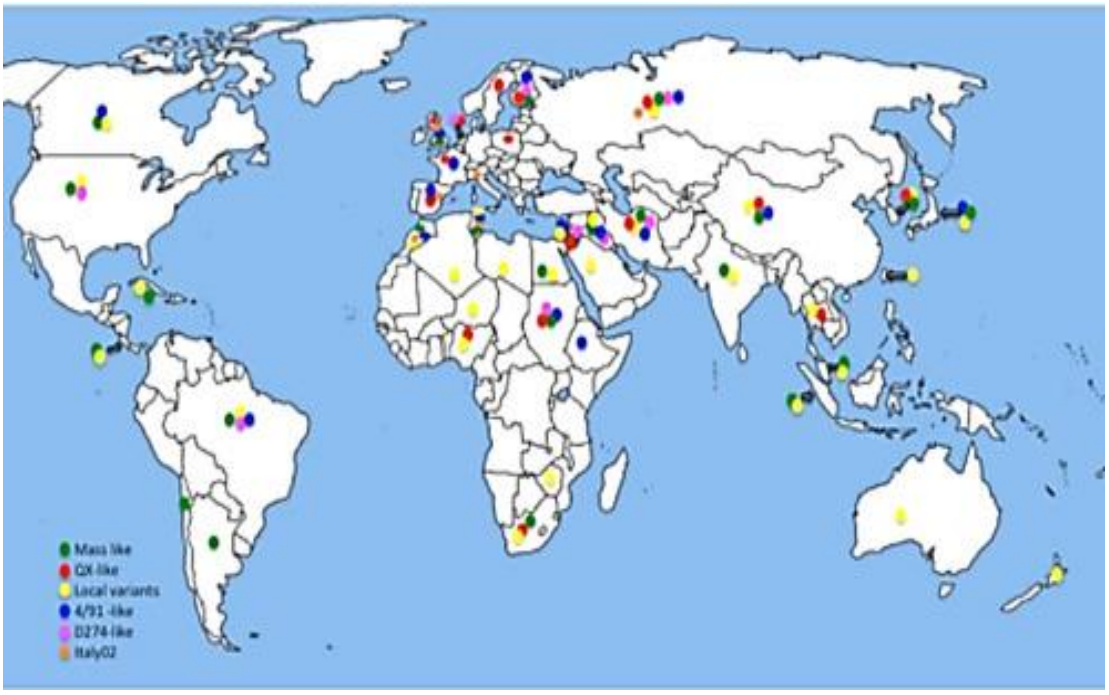


Figure 5: Geographical distribution of major AIBV serotypes. (Bande *et al.*, 2017)

In Africa AIBV first appeared in southern Africa in the early 1980s. AIBV genotypes have already been identified for 13 lineages, however GI-26, which is present in North and West Africa, has been confirmed to be unique to the continent (Valastro *et al.*, 2016; Bande *et al.*, 2017). GI-1 (many African countries), GI-12 (Nigeria), GI-13 (Algeria, Ethiopia, Morocco, South Africa, and Sudan), GI-14 (Cameroon and Nigeria), GI-16 (Côte d'Ivoire and Nigeria), GI-19 (Algeria, Ghana, Nigeria, South Africa, and Zimbabwe), GI-23 (Egypt and Nigeria), and several lineages from Algeria, Tunisia, Libya, and Ethiopia are among the other African AIBV lineages that are only grouped in genotype GI (Valastro *et al.*, 2016; Rafique *et al.*, 2024). Sporadic AIB outbreaks brought on by the Mass AIBV serotypes in the commercial chicken industries of several African countries. There are non-vaccine local variants in Africa, such as QX-like strains and Italy 02, which were first discovered in China and Europe, respectively, in addition to the well-known vaccine serotypes Mass and 4/91 (Sjaak de Wit *et al.*, 2011; Bande *et al.*, 2017).

The Ethiopian serotype 793B, which is classified under GI-13, was initially documented in 1917 by Hutton *et al*, (2017) In addition, its circulation was reported (Tesfaye *et al.*, 2019; Tegegne *et al.*, 2020) in backyard and commercial farms found in Oromia regional state and Southern Nations, Nationalities and People's Regional Sate. Other serotypes named M41, D274, and Qx are also reported (Tesfaye *et al.*, 2019). The incidence, molecular details, and isolation of the infectious bronchitis virus are generally lacking in appropriate knowledge.

2.3. Host Range and Susceptibility

The condition known as infectious bronchitis affects a wide variety of avian species. The AIBV can infect any type of bird, including parrots, pigeons, turkeys, and ducks. However, the virus's natural hosts are domestic poultry (Gallus gallus) and pheasants (Phasianus spp.) (Cavanagh, 2007; Awad *et al.*, 2014), and there are antigenic similarities between the two. In poultry, AIB morbidity is far higher than mortality (Jackwood, 2012). Renal infection increases the mortality rate of AIB disease in poultry more than respiratory infection does. The host's age, immune condition, genetic makeup, and environmental stressors all influence chicken's vulnerability to a virus (Butcher *et al.*, 2003; Bande *et al.*, 2017; Gallardo, 2021).

2.4. Transmission

The infectious bronchitis virus is extremely contagious and spreads quickly after a short incubation period. Airborne transfer of respiratory tract infections from infected flocks to vulnerable ones is widespread when they are present. The disease spreads both directly through contact and indirectly through fomites, contaminated feed and water, polluted litter, contaminated farm equipment, and signs of vertical transmission (Butcher *et al.*, 2003; Awad *et al.*, 2014; Pereira *et al.*, 2016). The use of live attenuated vaccines and wild bird migration are the two additional ways that the disease is spread (Cavanagh, 2005). The transmission rate of the disease is determined by the host's age, immunological status, and viral concentration. Most vulnerable flocks exhibit clinical

symptoms, and the virus can persist in the cecal tonsils for several weeks before excreting in feces after the host recovers (Butcher *et al.*, 2003; Khataby *et al.*, 2016).

2.5. Pathogenesis and Clinical Manifestation

The pathogenesis of the disease varies depending on the host's immunity, age group, virus strain type, and dose. The respiratory system is the main organ that the AIBV attacks. However, certain strains affect the chicken's reproductive, renal, and digestive systems (Cavanagh, 2007; Jackwood, 2012; Khaltabadi Farahani *et al.*, 2020). The incubation period of AIBV is 18–36 hours, depending on the dose and inoculation technique. AIBV-exposed chickens show clinical symptoms within 24–48 hours, and these symptoms last for approximately seven days (Butcher *et al.*, 2003; Woo *et al.*, 2012). AIBV mostly replicates in epithelial cells and cells that secrete mucus in the upper respiratory system. AIBV infection symptoms that affect the respiratory system include coughing, tracheal rales, sneezing, nasal discharge, and gasping. AIBV infection can cause poor weight gain, depression, and eye discharge. Mortality is rare but morbidity is significant unless there are immune-suppressive viruses or secondary bacterial complications involved. The clinical disease often lasts for seven days (Butcher *et al.*, 2003; Steyn *et al.*, 2020).

While neuropathogenic strains of the avian infectious bronchitis virus infect and damage the kidneys by causing nephritis with a high mortality rate within 6 to 16 days of post-infection, other serotypes of the virus mostly affect the respiratory tracts (Awad *et al.*, 2014; Promkuntod, 2016; Steyn *et al.*, 2020). In addition, the AIBV multiplies in the oviduct, alimentary tract, cecal tonsils, and cloaca of the cecum (Cavanagh, 2007; Bande *et al.*, 2017). Egg quality may be reduced by AIBV strains that infect reproductive tract cells, resulting in soft-shelled, malformed, rough, and watery yolks. Moreover, the AIBV irreversibly damages young chickens' oviducts, causing them to develop false layers forever (Butcher *et al.*, 2003; Sjaak de Wit *et al.*, 2011).

2.6. Diagnostic Methods

2.6.1. Clinical and differential diagnosis of infectious bronchitis

The diagnosis of the disease depends on clinical respiratory symptoms such as coughing, sneezing, gasping, and tracheal raring. Even though such clinical signs are essential for diagnosing AIB, it is challenging to distinguish it accurately from other respiratory pathogens such as mycoplasma (Mg), avian influenza (AI), infectious laryngotracheitis (ILT), infectious coryza (IC), and Newcastle disease (ND). Compared to AIB and ILT, infectious laryngotracheitis has a lower morbidity rate and is associated with bloody vomiting. In comparison to AIB, there are more cases of Newcastle disease, a viral respiratory disease that causes neurological symptoms as well as severe and fatal cases of avian influenza (Bhuiyan *et al.*, 2021).

2.6.2. Laboratory diagnosis

Isolation and virus propagation: Passaging of suspected samples in the specific pathogen-free (SPF) embryonated chicken eggs is one of the gold standard test methods (Cook *et al.*, 2012). The collected field samples which were screened by real-time PCR inoculated around three to five passages in 9-11 days old SPF embryonated eggs to increase the concertation of the virus. Embryo dwarfism, curling, hemorrhages, and death are some effects shown after inoculation of AIBV. The virus is also cultured and isolated in Primary cell lines like chicken embryo fibroblasts (CEF). Cytopathic effects like rounding of cells, syncytium formation, and detachment of cells from the flask surface were observed after inoculation of the AIBV (WOAH, 2018).

Molecular diagnosis: Reverse transcriptase polymerase chain reaction (RT-PCR) is the most reliable and sensitive method, which can be used to test, field samples and propagated allantoic fluids. To detect all AIBV strains outbreaks the test can be performed by using primers targeting the conserved 3′UTR and the S glycoprotein of AIBV (Williams *et al.*, 1993; J and S, 2015; WOAH, 2018).

2.7. Status of Infectious Bronchitis in Ethiopia

In Ethiopia, AIB disease was reported for the first time in 2017 on a farm located in Bishoftu (Hutton *et al.*, 2017). The first AIBV serotype reported was serotype 793B. After the first report Tesfaye *et al*, (2019) additionally reported new serotypes named M41, D-274, and Qx of AIB, and for the second time serotype 793B. Totaly four-seroprevalence investigations were reported across the country by different researchers. Tesfaye *et al*, (2019) reported seroprevalence of 68.75% and 74.75% in commercial and backyard chickens, respectively. In central Ethiopia and Northwest Ethiopia, a seroprevalence of 97.96% (Shiferaw *et al.*, 2022) and 23.96% (Birhan *et al.*, 2021) was reported. Similarly Hirbaye *et al* (2024) report 97.96 in East Shewa, central Ethiopia. The commercial vaccines imported into Ethiopia are Ibird, which contains 793B (GI 13), and Bron 120, which contains Massachusetts H120 (GI 1) and Brone120. Even though such imported vaccines are similar to the previously reported strain in Ethiopia, they are not adequate due to the virus's mode of transmission, nature of mutation, and lack of cross-protection among strains.

2.8. Control Measures and Prophylaxis

Infectious Bronchitis virus especially new variants are very difficult to control because the available commercial vaccines are different from the existing variants due to continuous mutation. Therefore, control strategies were developed to reduce the impact of the disease. Even if vaccination has its limitations to control AIB disease, live attenuated vaccines are the most important control measures (Jackwood, 2012; Bhuiyan *et al.*, 2021). To increase the protective immunity of AIB vaccination schedules should include revaccination with one different from the primary vaccine strain and the use of multivalent vaccines designed for the local strains (Awad *et al.*, 2014). Live vaccines are usually applied in the hatchery at day-old chickens and immunity develops within three weeks of vaccination. Inactivated AIB virus vaccine, subunit vaccine, which is purified from S1 glycoprotein, also provides immune protection against AIBV infection. Additionally, a vector vaccine was developed by expressing the S1 glycoprotein gene

using fowl adenovirus, and fowl poxvirus in chickens, and provided good protection. Farm biosecurity measures and production management are necessary to reduce the opportunity for field exposure to the variant AIBV (Butcher *et al.*, 2003; Franzo *et al.*, 2016; Gallardo, 2021).

3. MATERIALS AND METHODS

3.1. Study Area

The study was conducted in Bishoftu and Mekele (Figure 6). Bishoftu is a vibrant city in the Oromia region of Ethiopia, serving as an important industrial, transportation, and tourism hub for the country. Bishoftu is located approximately 45 kilometers southeast of the capital city of Addis Ababa, situated in the Great Rift Valley region at an elevation of around 1,900 meters above sea level. The average annual temperature is around 18°C, and the area receives moderate rainfall throughout the year. Due to this, the area is good for poultry production and most poultry production is reared in and around this study area.

Mekele is the capital city of Tigray regional state, located 761 kilometers from Addis Ababa. Mekele is situated at 13° 24′ latitude and 39° 25′ longitude, with an elevation ranging from 2,000 to 2,200 meters above sea level. The average annual rainfall in Mekele is between 579 and 650 mm, with temperatures ranging from 11.8°C to 29.9°C. The region is fourth in poultry population in Ethiopia, (CSA, 2021) and the majority of commercial farms are found around Mekele.

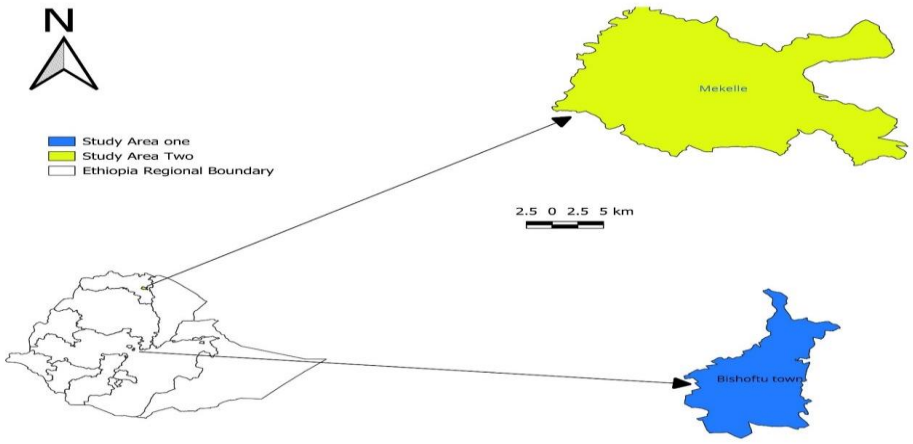


Figure 6: Map of the study areas. Prepared using GIS software. The blue color is Bishoftu and the yellow is Mekele.

3.2. Study Population and Sample Size

The study was conducted on chickens that clinically manifest AIB disease in all ages (pullets, adults), sex (male, female) breeds (Lohman, Bovans), management (Intensive, Semi-intensive), and non-vaccinated flocks, based on the availability and willingness of farm owners. When chickens manifest AIB symptoms such as coughing, sneezing, tracheal raring, and ocular and nasal discharge, the case is considered positive for AIB. In the study areas, active outbreaks were investigated targeting commercial poultry farms including broilers and layers with flock sizes ranging from 110 to 4500. A total of 49 pooled swabs and tissue samples were collected aseptically from 160 suspected AIB chickens (Table 5).

3.3. Study Design

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

3.4. Sampling Technique, Sample Collection and Transportation

Samples were collected from eleven poultry farms, eight of which were located in Bishoftu and three in Mekele. For the swab samples, the sample from four chicks was pooled in one tube, while the tissue sample from one chicken organ (trachea, lung, and kidney) was pooled in one tube.

After recording clinical and epidemiological data about morbidity, mortality, and vaccination practices, swabs and tissue samples were collected for further laboratory analysis using virology and molecular detection techniques. This was done to characterize the circulating AIBV strains in the study areas. If there was one case on the farm related to IB incidence, it was considered an outbreak, and this information was gathered through interaction with the farm owners and animal health professionals.

During active outbreaks, an antemortem examination was conducted to look for any abnormalities such as discharges, coughing, sneezing, and tracheal rearing, and then both swab and tissue sample was collected. To collect tissue samples for AIBV, suspected chickens were carefully examined for external signs and symptoms of AIB infection. Post-mortem examination of chickens was performed according to the standard protocol described in the WOAH manual. Chickens were euthanized through cervical dislocation post-mortem examinations were performed and tissue samples were taken according to the procedure described by Jagne and Buckles (2021).

In total, forty-nine pooled samples were collected from various AIBV outbreaks in commercial poultry farms from Bishoftu and Mekele, as shown in Table 5. Thirty-seven swab samples were collected from the upper respiratory tract of live chickens suspected of IBV from both study areas. Twelve tissue samples were also collected and processed from various AIBV outbreaks of commercial poultry farms in Bishoftu and Mekele. All collected samples were transported to the National Veterinary Institute (NVI) using a virus transport media (VTM) containing 10% antibiotics and labeled with relevant information. The samples were stored at -20°C until the AIB virus isolation and other molecular characterization processes were complete.

3.5. Laboratory Diagnosis

3.5.1. Sample Processing

The swab samples in VTM were vortexed and clarified by centrifugation at 1500 rpm for 10 minutes. The suspension was then filtered through a 0.45µm filter syringe (Millipore USA). The filtrate was stored at -70°C for further processing. The tissue samples (Appendix 1) are processed by WOAH standard operating procedures. The pooled organ samples were thawed at room temperature, and representative pooled tissue was washed with PBS (containing 10% antibiotics) three times in the level two biosafety cabinet. The tissue samples were ground to homogenize using a pestle and mortar and centrifuged at 1500 rpm for 10 minutes in the presence of 9ml of PBS. The supernatants were collected

under sterile conditions, and filtered through a 0.45 μm filter (Millipore USA) to prevent bacterial contamination, and stored in -20 refrigerators until processing (WOAH, 2018).

3.5.2. Virus propagation and isolation in an embryonated egg

Specific pathogen-free (SPF) eggs were obtained from the NVI of Ethiopia. The SPF eggs were incubated at 37°C and 65% humidity. Temperature and humidity were monitored 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 punctured from the middle of the eggshell at the top under aseptic conditions. Then, using a 1 ml syringe, 200μl of filtered inoculum of tissue processed and tracheal swab suspensions was inoculated into the allantoic cavity of 9-day-old embryonated SPF eggs, piercing the chorioallantoic membrane (CAM) just below the air space for amplification (Appendix 6). For the control egg was inoculated with PBS. The hole was sealed with melted candlewax, and the inoculated eggs were incubated at 37°C. For each sample, five eggs were used, and the progress was monitored daily using a candle after inoculation. The embryo died within 24 hr after inoculation was discarded. After 120 hours post-inoculation, live and dead embryonated eggs were refrigerated or chilled at 4°C for four hours. The allantoic fluids were harvested (Appendix 3) using a pipette and then pooled and filtered in each passage to avoid contamination. This procedure was repeated for five passages. The filtered pooled samples were frozen at –20°C until molecular analysis (Atta and Allawe, 2018).

A lethal dose of Avian Infectious Bronchitis Virus in Fertilized Eggs by ELD50: After candling the eggs and marking the inoculation site, five eggs were assigned per dilution. Using PBS as diluent, tenfold serial dilutions were prepared using passage three-allantoic fluid. The eggs were then disinfected with alcohol and punctured by drilling on the marked site. Then, 200μl allantoic fluid was inoculated. After inoculation, the hole in each egg was sealed with melted candle wax. Incubate the eggs at 37°C with 60–65% humidity. Candle the eggs after 24 hr of incubation. Embryo mortality that occurred within 24 hours post-inoculation was considered nonspecific and the eggs were discarded. After 120 hr incubation of post-inoculation, the egg was candled to examine

dead embryos and lesions, which were considered positive for the infection. The ELD_{50} was calculated using the Reed and Muench method formula: 10 ELD_{50}/ml end point dilution = log10 of dilution showing mortality next above 50% - (difference of logarithms × logarithm of dilution factor). In addition, the difference of logarithms = [(mortality at dilution next above 50%)-50%] / [(mortality next above 50%)-(mortality next below 50%)]. This was done based on the cumulative number of dead and surviving embryos at each dilution (Ramakrishnan, 2016).

3.5.3. Virus propagation in chicken embryo fibroblast cell culture (CEF)

Primary cell culture was prepared using 10 days old embryonated eggs (Appendix 2). After discarding the growth media, 0.5 ml of the recovered allantoic fluid was used as an inoculum and added to the monolayer CEF cell culture. After an hour of incubation at 37°C to allow the virus to attach to the cells, maintenance media was supplied to the cells in the same way as the control cell culture. Every day, an inverted microscope is used to check for any signs of a viral cytopathic effect (CPE) in the cell culture. The infected cell culture was frozen at -20°C, then thawed and propagated for two passages (Atta and Allawe, 2018).

Titration of Avian Infectious Bronchitis Virus in CEF cell culture by TCID50: Titration of AIBV using CEF was done by preparing tenfold serial dilutions of the CEF in titration medium after preparing the primary cell (Appendix 2). Fill the wells of column 12 of the 96-well plate with 100 µl/well titration medium which is negative control and fill each well with 50 µl/well titration medium and 50 µl prepared serial dilution with a multichannel pipette. The 96 well plates were incubated at 37°C for 4 days. After 4 days of incubation, score all wells for AIBV-specific CPE using a microscope. The virus titers in the sample, expressed as 10log (TCID50)/ml are calculated using the method described by Spearman and Karber (Kärber, 1931; Lei *et al.*, 2021) using the following formula: $\text{Titer}=(x0- d/2 + d\sum^{r} n)$. In which: x0: logarithm of the inverse value of the lowest dilution at which all embryos are positive, d: logarithm of the dilution factor (d=1 when using tenfold serial dilutions), n: number of eggs used per dilution, r: number of positive eggs at that dilution.

3.5.4. Molecular Laboratory

Virus RNA Extraction: Viral RNA was extracted from the collected allantoic fluids of selected IBV isolates following the instructions of the QIAamp Viral RNA Mini Kit (Qiagen, Germany). The viral RNA extraction procedure of steps was employed (Appendix 4) (Parris *et al.*, 2022).

Real-Time PCR: The collected swab samples and processed tissue samples were tested using real-time PCR. Because real-time PCR is a rapid and effective method for detecting (J and S, 2015; Batra *et al.*, 2017; Icochea *et al*., 2023). The primers used were IBV5′ Forward primer 5′-GCTTTTGAGCCTAGCGTT-3′; AIBV5′ Reverse primer 5′-GCCATGTTGTCACTGTCTATTG-3′; and AIBV probe 5′(FAM)-CACCACCAGAACCTGTCACCTC-3′. The total prepared master mix reactions was 25 µL. TaqMan super mix 2X 12.5 µL, 2.5 µL forward and reverse primer, 0.5 µL probe, 0.5 µL enzyme mix, 1.5 µL Nuclease free water, and 5 µL extracted template were added. The prepared mixture run using CFX96™ Real-Time PCR System (BioRad) according to the thermal profile of reverse transcription at 50°C for 30 minutes for one cycle, one cycle activation step at 95°C for 15 minutes, and 40 cycles of denaturation, annealing, and extension step at 94°C for 30 sec, 68°C for 60 sec and 68°C for 60 sec, respectively.

Conventional Polymerase Chain Reaction: The RT-PCR was performed using the Invitrogen SuperScript™ III Platinum™ One-Step RT-PCR Kit (Thermo Fisher, Waltham, MA, USA) following the manufacturer's instruction. To detect AIBV from the collected allantoic fluid using conventional one-step RT-PCR, a pair of primers All1-F 5´-CAGCGCCAAAACAACAGCG-3´ and Del1-R 5´-CATTTCCCTGGCGATAGAC-3´ used to amplify 433 bp of the conserved region of 3´UTR. This primer identifies AIBV strains targeting the most hypervariable region with conserved flanking regions in the AIBV genome (Hewson *et al.*, 2009). The final volume of master mix preparation (Table 1) was 25 µL including the template. The PCR was carried out using a thermocycler, with polymerase activation at 50°C for 30 minutes, followed by an initial denaturation step of 95°C for 15 min for one cycle each. Then 35 cycles of 94°C for 45 sec, 55°C for 45 sec, and 72°C for 45 sec for denaturation, annealing, and extension respectively with a final extension of 72°C for 5min.

Table 1: One-step RT-PCR master mix preparation for 3′UTR amplification

Ser. No	Type of reagent	For one reaction
1	RNase free water	4µl
2	5x RT-PCR Buffer	5 µl
3	5X Q Solution	5 µl
4	Primer-AIBV All1-F -Fow-5pm/µl 5′-CAGCGCCAAAACAACAGCG-3′	2 µl
5	Primer AIBV- Del1-Rev-5pm/µl 5′-CATTTCCCTGGCGATAGAC-3′	2 µl
6	10Mm dNTP mix	1 µl
7	One-step RT-PCR Enzyme Mix	1 µl
8	Template	5 µl
	Total volume	**25 µl**

To amplify the Spike protein gene of IBV, a 25 µl reaction mixture was prepared by adding 8µl of RNA-free water, 5µl of 5X one-step RT-PCR buffer, 1µl dNTPs mix, 2.5µl of forward and reverse primer, 1µl of one step enzyme mix, and 5µl of extracted RNA template (Adzhar *et al.*, 1997). The reaction was carried out using a Thermal Cycler (BioRad) with polymerase activation at 50°C for 30 min, and an initial denaturation step of 95°C for 5 min. Then 45 cycles of 95°C for 30 sec, 52°C for 30 sec, and 68°C for 30 sec, followed by a final extension of 68°C for 12 min, as shown in Table 2.

Table 2: One-step RT-PCR master mix preparation for Spike protein gene amplification

Ser. No	Type of reagent	For one reaction
1	RNase free water	8µl
2	5x RT-PCR Buffer	5 µl
3	10Mm dNTP mix	1 µl
4	Primer-AIBV XCE2-Fow-5pm/ µl 5′-CACTGGTAATTTTTCAGATGG-3′	2.5 µl
5	Primer AIBV-XCE2-Rev 5pm/ µl 5′-CCTCTATAAACACCCTTGCA-3′	2.5 µl
6	One-step RT-PCR Enzyme Mix	1 µl
7	Template	5 µl
	Total volume	**25 µl**

Nested PCR: After conducting one-step RT-PCR for the detection of all AIBV we employed nested PCR to detect the specific serotypes of Massachusetts, D274, and 4/91

with 295bp, 217bp, and 154bp fragments, respectively (Table 3). To amplify these specific serotypes we used primers and protocols described by Adzhar *et.al,* (1997*)* and Roussan *et.al,* (2008) (Table 3). The master mix reaction of nested PCR prepared a total volume of 50 µl containing 5 µl of 5x RT-PCR Buffer, 2 µl of 10Mm dNTP mix, 0.5 µl of TaqDNA polymerase, 1 µl of each forward and reverse primers, 1 µl of amplified template and the rest RNase free water. The nested thermocycler was conducted for 35 cycles with denaturation, annealing, and elongation steps with 94°C for 1 minute, 48°C for 2 minutes, and 72°C for 90 seconds, respectively, and additional final elongation of 72°C for 10 minutes.

Table 3: RT-PCR primer sequences

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

Agarose Gel Electrophoresis: After the PCR run, the PCR products were run on 1.5% agar gel by dissolving and boiling 1.5gm of agarose in 100 ml of 1X TAE Buffer, then 5µl of GelRed (Biotium, Inc.) was added and mixed. The mixture was poured into a gel casting tray with an inserted comb and allowed to solidify at room temperature. When the gel completely solidified, the combs were removed carefully and placed in an electrophoresis chamber filled with 1X TAE buffer. Then, 5µl Loading dye was added to each PCR product, and mixed well. Then load 10 µl mixed PCR product and loading dye in each well. The 10 µl of 100 bp DNA ladder was used as a reference for band size. The gel was run at 120 V for one hour, observed under a UV trans-illuminator, and a photograph was captured using the Gel documentation system (Lee *et al.*, 2012). The

band size of positive results for the 3´UTR gene and the S gene is around 433bp and 466bp, respectively.

3.6. Sequencing of Avian Infectious Bronchitis Viruses

The positive PCR product from conventional PCR was purified (Appendix 5) using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) and stored at -20°C until sequencing. NanoDrop™ 2000/2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) was used to measure the concentration and purity of the purified PCR products (Table 6). Six purified PCR products were sent to LGC Genomics (Berlin, Germany) together with the amplification forward and reverse primers for commercial sequencing of the 3´UTR gene using the Sanger sequencing method.

3.7. Phylogenetic Analysis

Bioinformatics software was used to evaluate the raw sequence data after editing to generate the consensus sequence. The nucleotide sequences of the 3´UTR region of the IBV isolates were assembled the bad sequence was masked/trimmed using the Staden Package software and the clean nucleotide sequences were aligned using BioEdit software.

Sequence similarity search was conducted using the Basic Local Alignment Search Tool (BLAST) at the National Centre for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/). The nucleotide sequences of AIBV strains 3´UTR gene were retrieved from the NCBI database. The sequences were aligned using the MUSCLE algorithm in MEGA7 (Kumar *et al.,* 2016) using default parameters. Following alignment, the sequences were manually trimmed to obtain equal size.

Phylogenetic tree analyses were performed using MEGA 7 software (Kumar *et al.,* 2016) using the neighbor-joining approach with 1000 bootstrap replicates. Gaps were treated as complete deletion options.

3.8. Data Management and Analysis

The data obtained from all investigations were entered and stored in a Microsoft Office Excel spreadsheet 2010 for simple descriptive analysis to summarize data of the field survey and laboratory investigation.

3.9. Ethical Consideration

Ethical clearance for the study was provided by the Animal Research Ethical Review Committee of the College of Veterinary Medicine and Agriculture, Addis Ababa University (Appendix 7). The significance of this research was evaluated from ethical perspectives, applicability, originality, and technical competence points of view. Finally, approval was granted with a minute number (VM/ERC/03/16/024) and Reference number (VM/ERC/02/13/16/2024). Verbal consent was obtained from animal owners for their willingness to participate in this study. Sampling was done following proper animal care.

4. RESULTS

4.1. Clinical and Pathological Observation

During the outbreak investigation of AIBV in poultry farms, suspected poultry was monitored and clinically inspected for infectious bronchitis disease. AIBV is characterized clinically by respiratory signs such as coughing, tracheal rales, sneezing, nasal discharge, gasping, ocular discharge, depression, and poor weight gain. However, during the outbreak investigation, various clinical symptoms were observed, including coughing, tracheal raring, sneezing, depression, and poor weight gain (Figure 7). During the post-mortem examination, the gross pathological examination was observed (Figure 7D) on the clinically diseased chicken that revealed cloudiness of air sacs with yellow caseous exudates on tissue samples found from Bishoftu.

Figure 7: Clinical manifestation and Gross pathological lesion of AIBV observed in affected poultries. (A) Tracheal raring, gasping, depression, poor weight gain, (B)

Depression, poor weight gain, tracheal raring, (C) Depression, (D) Cloudiness of air sacks and yellow caseous exudates cases observed after post-mortem examination.

Table 4: Morbidity, mortality, and case fatality rate of IB in the study areas

Area	Farm	No of chickens	No of Cases	No of Death	Morbidity (%)	Mortality (%)	Fatality (%)
Mekele	Farm 1	830	53	-	6.39	0	0
	Farm 2	300	50	-	16.67	0	0
	Farm 3	110	35	-	31.81	0	0
Bishoftu	Farm 4	2000	105	-	5.25	0	0
	Farm 5	4500	200	15	4.44	0.33	7.5
	Farm 6	3000	200	20	6.66	0.66	10
	Farm 7	1150	575	-	50	0	0
	Farm 8	200	15	-	7.5	0	0
	Farm 9	900	80	2	8.88	0.22	2.5
	Farm 10	1000	40	3	4	0.3	7.5
	Farm 11	400	30	2	7.5	0.5	6.66
Total	**11 Farms**	**14390**	**1383**	**42**	**9.61**	**0.29**	**3.04**

Where; S: swab samples, T: tissue samples.

In a total of 11 farms, 14,390 poultry flocks were found. Of these flocks, 1,383 chicken flocks exhibited clinical symptoms of AIBV. Out of the 1,383 chicken flocks with AIB symptoms, 42 flocks died. The morbidity rate, calculated as the proportion of sick flocks out of the total 14,390 flocks, was 9.61%. The mortality rate, calculated as the proportion of deaths out of the total 14,390 flocks, was 0.29%. The case fatality rate, which is the proportion of deaths among the 1,383 affected flocks, was approximately 3.04%. Additionally, 49 samples were collected from the affected flocks for isolation and molecular characterization of the AIBV (Table 4).

Table 5: List of samples collected from IB suspected chickens

City	Farm	No of chickens	Type and number of pooled samples		
			Swab	Tissue	Total
Mekele	Farm 1	830	4	3	7
	Farm 2	300	4	2	6
	Farm 3	110	5	-	5
Bishoftu	Farm 4	2000	2	1	3
	Farm 5	4500	6	1	7
	Farm 6	3000	3	1	4
	Farm 7	1150	3	-	3
	Farm 8	200	1	1	2
	Farm 9	900	5	-	5
	Farm 10	1000	2	2	4
	Farm 11	400	2	1	3
Total	**11 Farms**	**14390**	**37**	**12**	**49**

4.2. Infectious Bronchitis Virus Screening

The field samples were initially screened before being isolated and molecular investigated. A total of 49 pooled samples, comprising 37 swab samples and 12 pooled tissue samples, were subjected to AIBV molecular detection. Of these, 25 samples were screened by real-time PCR, and 3 positive samples were detected (Figure 8). The remaining 24 samples were tested by conventional PCR, and an additional 3 positive samples were recorded. In total, the screening methods detected 6 AIBV-positive samples. Out of these 6 positive samples, only 1 was a tissue sample, while the others were swab samples.

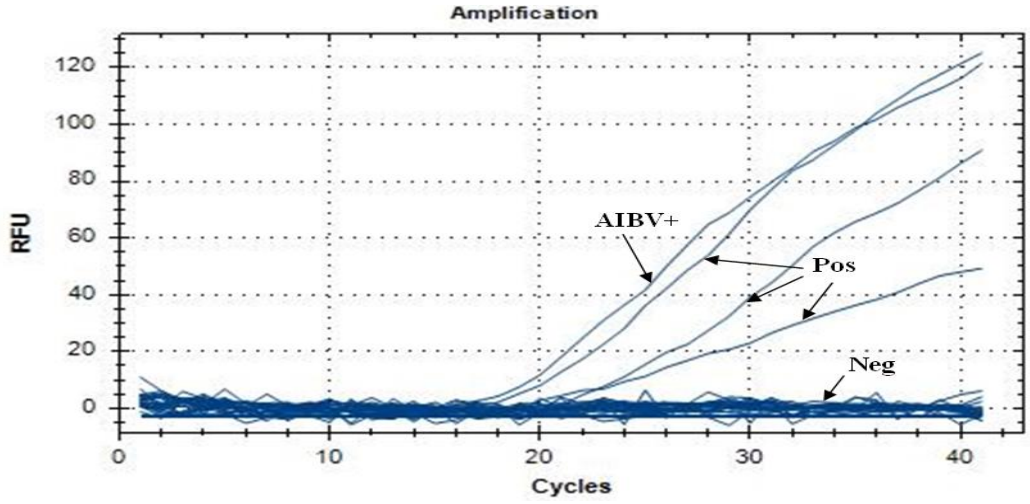


Figure 8: Real-time PCR Result. AIBV+: Avian Infectious bronchitis vaccine positive control; Pos: positive samples; and Neg: negative samples and negative control.

4.3. Virus Propagation and Isolation on Embryonated Egg

The 6 positive pooled samples, comprising 5 swab samples and 1 tissue sample were inoculated into 9-day-old embryonated chicken eggs with allantoic sacs for up to 5 passages (Table 6). During the first and second passages, 6 of the samples exhibited a cytopathic effect (CPE) characterized by congestion and bleeding in the embryos, while the control samples displayed no change or mortality (Figure 9). Additionally, two other samples were inoculated, and examination of the embryos up to five passages revealed several symptoms, including congestion, hemorrhage, embryo curling, deformation, and stunted growth. The calculated embryo lethal dose for these samples was 6.5 ELD 50/ml.

Table 6: Virus propagation in embryonated chicken eggs and cytopathic effect across five passages

Sample No	No of Eggs	1st passage (72hr)	2nd passage (72hr)	3rdpassage (48hr)	4th passage (48hr)	5th Passage (48hr)
Farm 1 (S)	5	Congestion Hemorrhage	Congestion Hemorrhage	Stunted Growth Curling	Curling Deformation	Stunted Growth Curling
Farm 1 (S)	5	Congestion Hemorrhage	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 11 (S)	5	Congestion Hemorrhage	Congestion Hemorrhage			
Farm 11 (S)	5	Congestion Hemorrhage	Congestion Bleeding			
Total	**30 Eggs**					

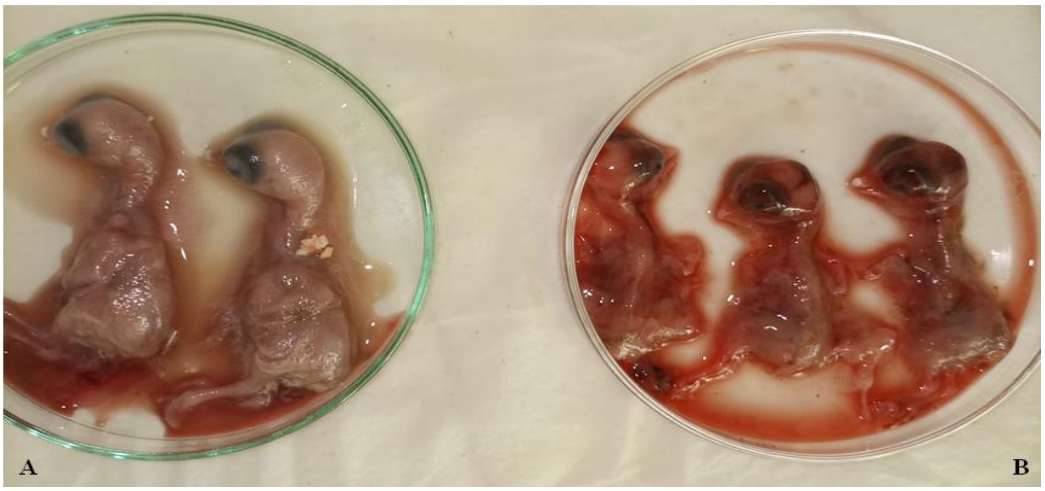


Figure 9: Isolation of IBV on embryonated egg passages one and two. A: normal embryo, B: congested with blood embryo after inoculation.

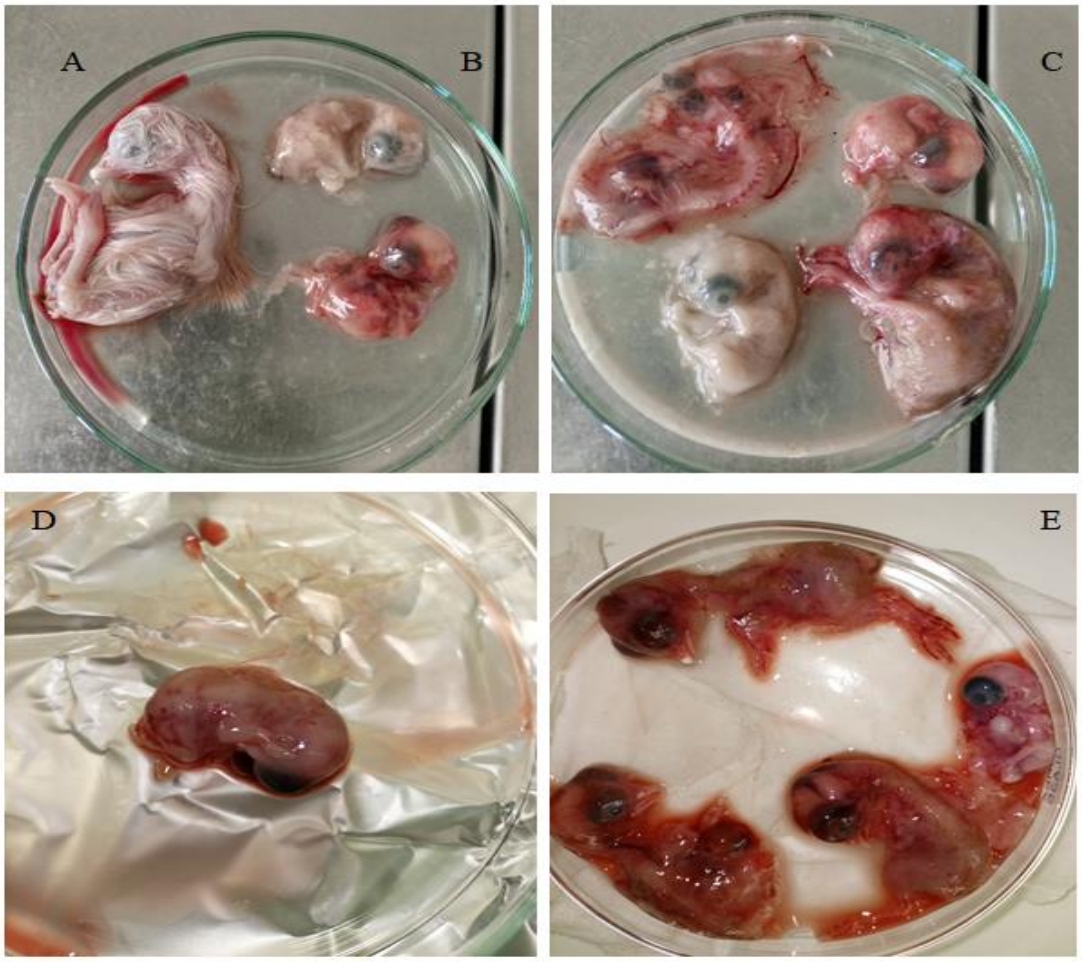


Figure 10: Isolation of AIBV on embryonated egg from passage three to five. A: normal embryo; B, C, D, and E: curling embryo, deformation, stunned growth of embryo after inoculation of IBV normal embryo.

4.4. Virus Propagation in Embryo Fibroblast Cell Culture

Positive samples of allantoic fluids were filtered through a 0.45 µl minipore filter and inoculated with 0.5 ml on CEF cell culture. Following inoculation, a clear CPE was observed under an inverted microscope with a 40x magnification after 4 days of post-inoculation, and characterized by rounded, aggregated cells and detachment from the flask surface (Figure 11 B); however, the control CEF cell culture remained unchanged as shown in Figure 11 A. The AIB isolate had a titer of 5.7 TCID50 in 1 ml in the third passage.

Figure 11: Isolation of AIBV using CEF cell culture. (A) control or non-inoculated CEF cell culture, and (B) CPE such as rounding of cells, aggregated cells, and detachment from the flask surface after inoculation of field AIBV.

4.5. Molecular AIBV Detection Result

The 3' UTR and S genes of the 6 positive AIBV isolates from the embryonated chicken egg allantoic fluids were amplified using a one-step RT-PCR. The amplified products were visualized by gel electrophoresis, targeting a 466 bp region of the AIBV S1 gene and a 433 bp region of the 3'UTR as shown in Figure 12 and Figure 13, respectively. The results showed that all six pooled samples yielded positive results for both target regions, with the positive samples originating from the collected allantoic fluid of specific-pathogen-free (SPF) eggs inoculated with 5 swab samples and 1 tissue sample.

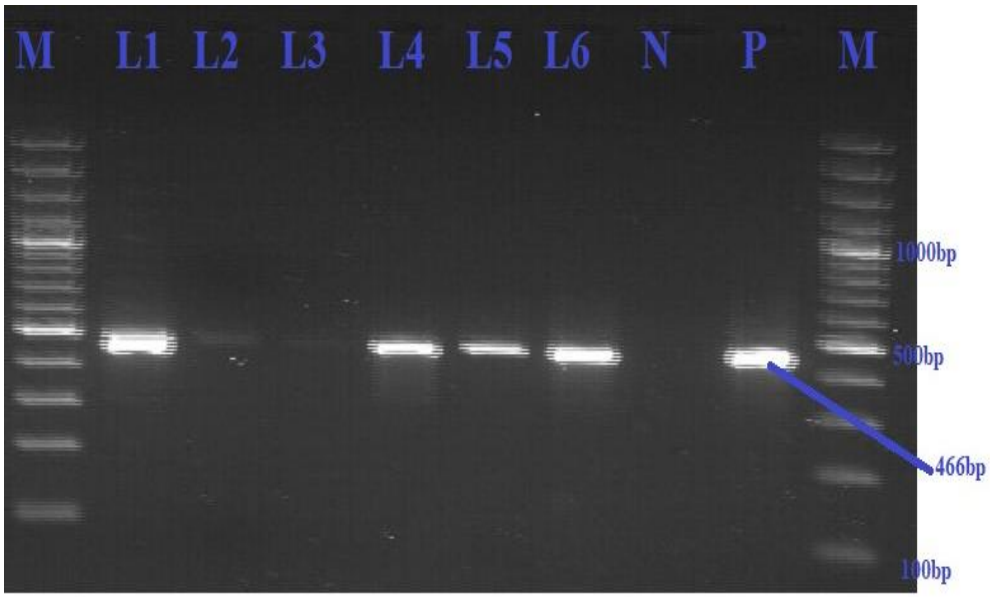


Figure 12: Conventional PCR agarose gel showing the amplification of the S gene with 466bp band size. M: molecular ladder (GeneRuler™ 100bp plus DNA Ladder, Thermo Scientific), Lanes L1, L4, L5, and L6 are strong positive samples; Lanes L2 and L3 are weak positive samples; Lane N: negative control; and Lane P: positive control.

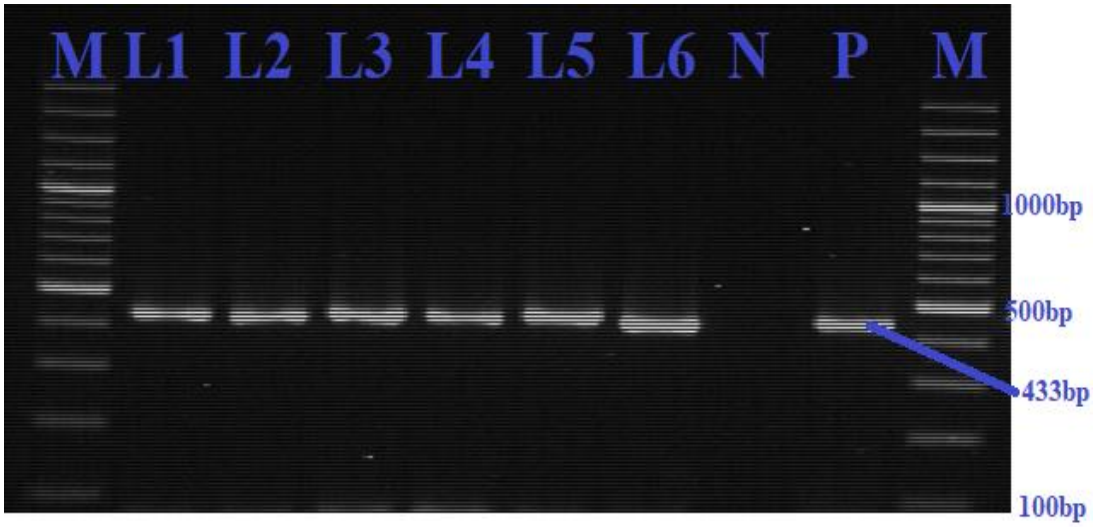


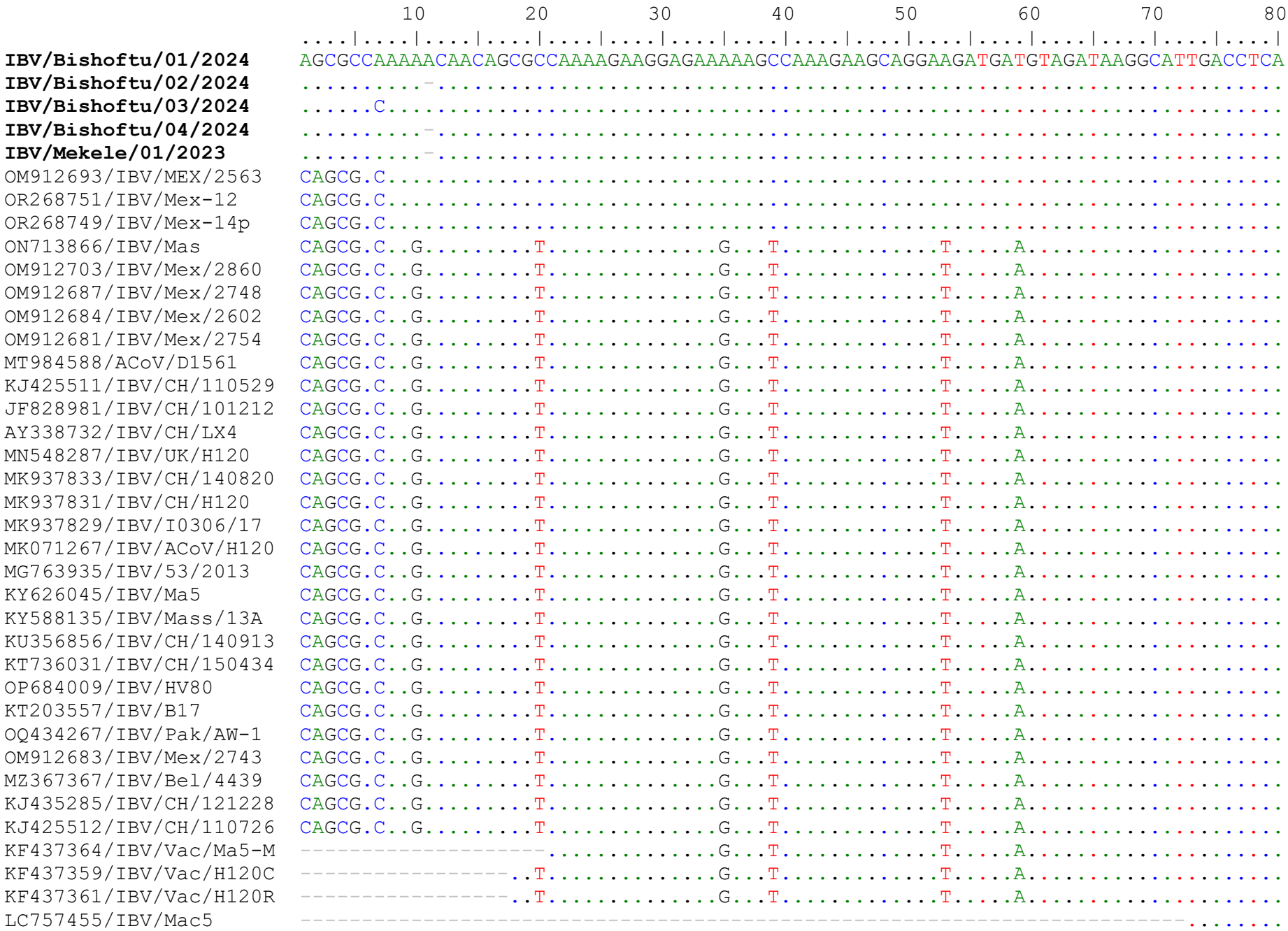
Figure 13: Conventional PCR agarose gel showing the amplification of 3′UTR with 433bp band size. M: molecular ladder (GeneRuler™ 100bp plus DNA Ladder, Thermo Scientific), Lanes 1-6: IBV positive samples, Lane N: negative control, and Lane P: positive control.

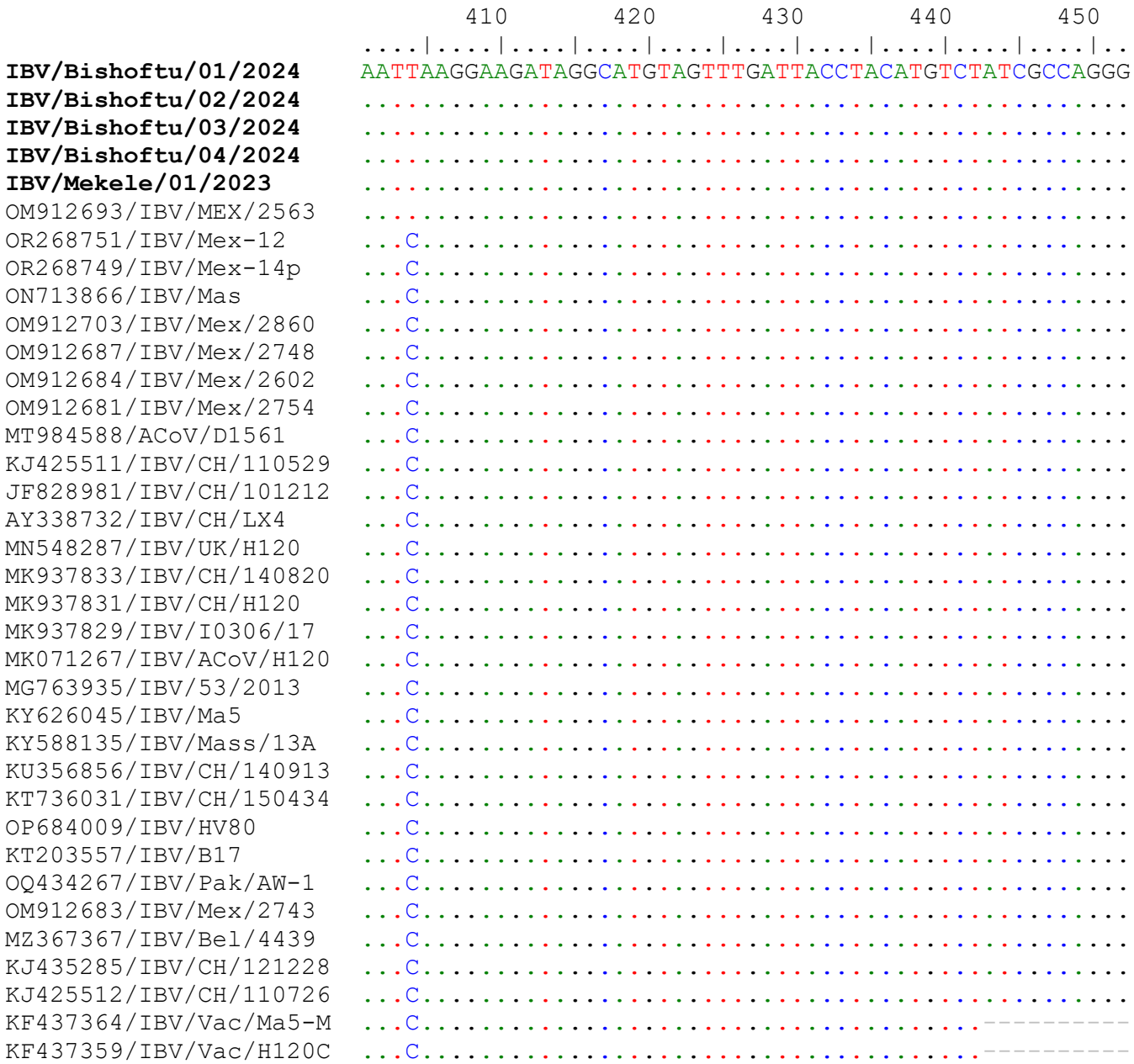
Table 7: Concentration and purity of the purified PCR products

S/N	Sample ID	Nucleic Acid Conc.	Unit	A260	A280	A260/280	260/230	Sample Type	Factor
1	Blank	-1.1	ng/µl	-0.016	-0.016	1.38	0.02	DNA	50
2	IBV-1 Mekele	47.9	ng/µl	0.958	0.509	1.88	1.52	DNA	50
3	IBV-2 Mekele	39.5	ng/µl	0.789	0.539	1.46	1.08	DNA	50
4	IBV-1 Bishoftu	35	ng/µl	0.7	0.37	1.89	-2.74	DNA	50
5	IBV-2 Bishoftu	45.2	ng/µl	0.905	0.496	1.82	1.41	DNA	50
6	IBV-3 Bishoftu	34.4	ng/µl	0.688	0.369	1.87	-2.1	DNA	50
7	IBV-4 Bishoftu	46.1	ng/µl	0.922	0.506	1.82	1.2	DNA	50

4.6. Sequence Analysis Result of Avian Infectious Bronchitis Viruses

Six AIBV isolates were obtained from Bishoftu (n=4) and Mekele (n=2), derived from pooled swab samples (n=5) and a pooled tissue sample (n=1). The concentration and purity of the purified PCR products prepared for sequencing were measured as shown in Table 7. The sequencing analysis was conducted by comparing the alignment of the current outbreak of avian infectious bronchitis virus isolates with sequences retrieved from GenBank. The 3'UTR sequences of five isolates from the current outbreaks were analyzed, with one of the sequenced isolates having a poor chromatogram value and thus not aligned with the other isolates. All five aligned current Ethiopian AIBV sequences were highly similar to one another, with only one isolate (AIBV/Bishoftu/03/2024) having a single nucleotide substitution of 'A' to 'C' at position 7, as shown in Figure 14. Similarly, a single nucleotide polymorphism (SNP) resulted in a change in the amino acid Glutamine (Q) with Proline (P) at position 3 (Figure 15). The current isolates displayed a high alignment similarity score ranging from 97.82% to 99.78%, and their alignment similarity score with other isolates ranged from 90.80% to 99.83%.


10 20 30 40 50 60 70 80
IBV/Bishoftu/01/2024 AGCGCCAAAAACAACAGCGCCAAAAGAAGGAGAAAAAGCCAAAGAAGCAGGAAGATGATGTAGATAAGGCATTGACCTCA
IBV/Bishoftu/02/2024
IBV/Bishoftu/03/2024
IBV/Bishoftu/04/2024
IBV/Mekele/01/2023
OM912693/IBV/MEX/2563
OR268751/IBV/Mex-12
OR268749/IBV/Mex-14p
ON713866/IBV/Mas
OM912703/IBV/Mex/2860
OM912687/IBV/Mex/2748
OM912684/IBV/Mex/2602
OM912681/IBV/Mex/2754
MT984588/ACoV/D1561
KJ425511/IBV/CH/110529
JF828981/IBV/CH/101212
AY338732/IBV/CH/LX4
MN548287/IBV/UK/H120
MK937833/IBV/CH/140820
MK937831/IBV/CH/H120
MK937829/IBV/I0306/17
MK071267/IBV/ACoV/H120
MG763935/IBV/53/2013
KY626045/IBV/Ma5
KY588135/IBV/Mass/13A
KU356856/IBV/CH/140913
KT736031/IBV/CH/150434
OP684009/IBV/HV80
KT203557/IBV/B17
OQ434267/IBV/Pak/AW-1
OM912683/IBV/Mex/2743
MZ367367/IBV/Bel/4439
KJ435285/IBV/CH/121228
KJ425512/IBV/CH/110726
KF437364/IBV/Vac/Ma5-M
KF437359/IBV/Vac/H120C
KF437361/IBV/Vac/H120R
LC757455/IBV/Mac5


410
420
430
440
450
IBV/Bishoftu/01/2024 AATTAAGGAAGATAGGCATGTAGTTTGATTACCTACATGTCTATCGCCAGGG
IBV/Bishoftu/02/2024
IBV/Bishoftu/03/2024
IBV/Bishoftu/04/2024
IBV/Mekele/01/2023
OM912693/IBV/MEX/2563
OR268751/IBV/Mex-12
OR268749/IBV/Mex-14p
ON713866/IBV/Mas
OM912703/IBV/Mex/2860
OM912687/IBV/Mex/2748
OM912684/IBV/Mex/2602
OM912681/IBV/Mex/2754
MT984588/ACoV/D1561
KJ425511/IBV/CH/110529
JF828981/IBV/CH/101212
AY338732/IBV/CH/LX4
MN548287/IBV/UK/H120
MK937833/IBV/CH/140820
MK937831/IBV/CH/H120
MK937829/IBV/I0306/17
MK071267/IBV/ACoV/H120
MG763935/IBV/53/2013
KY626045/IBV/Ma5
KY588135/IBV/Mass/13A
KU356856/IBV/CH/140913
KT736031/IBV/CH/150434
OP684009/IBV/HV80
KT203557/IBV/B17
OQ434267/IBV/Pak/AW-1
OM912683/IBV/Mex/2743
MZ367367/IBV/Bel/4439
KJ435285/IBV/CH/121228
KJ425512/IBV/CH/110726
KF437364/IBV/Vac/Ma5-M
KF437359/IBV/Vac/H120C

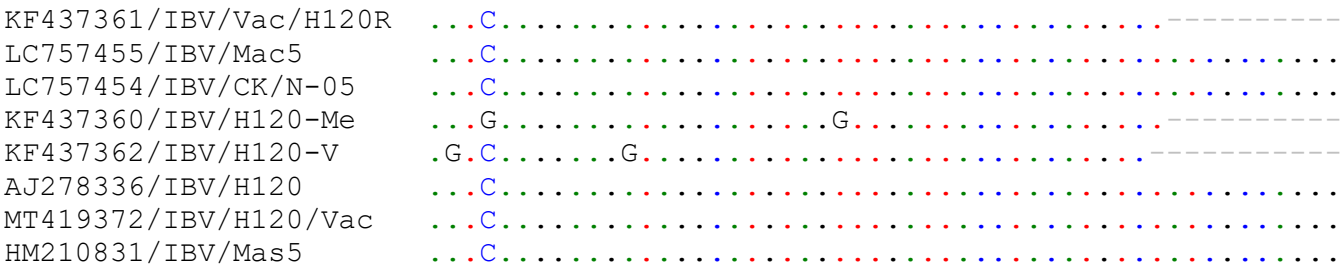


Figure 14: Plot identity of the 3´UTR nucleotide sequences of avian infectious bronchitis viruses. Avian Infectious Bronchitis virus of the current isolates (indicated in bold) in comparison with the previously sequenced infectious bronchitis viruses retrieved from the GenBank database. Identical nucleotides are indicated as dots.

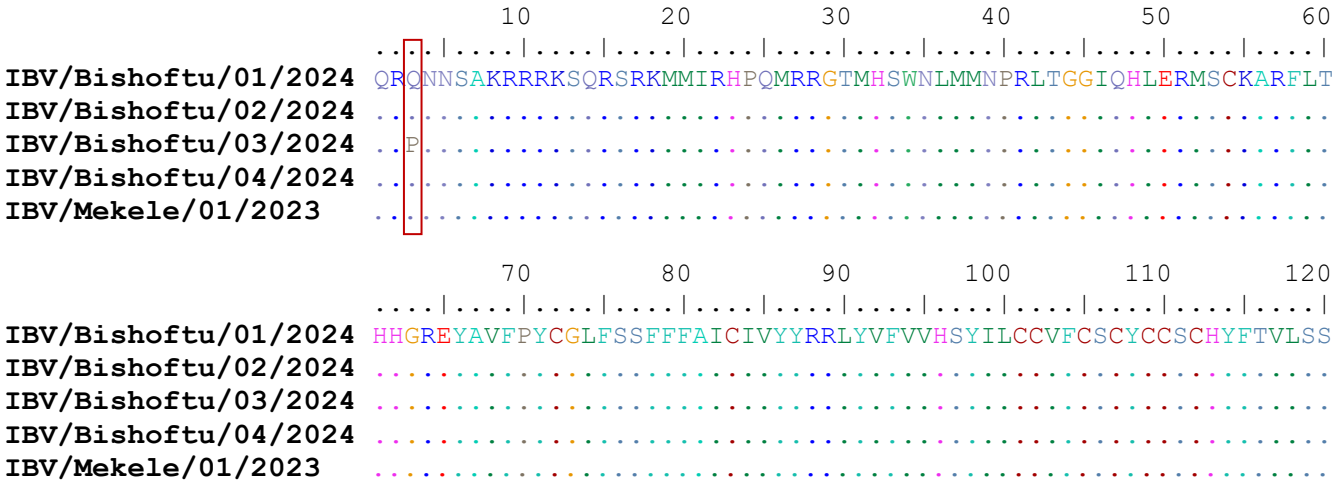


Figure 15: Plot identity of the 3´UTR amino acid sequences of the current five Ethiopian field infectious bronchitis. A single amino acid change Q:P was observed at residue position 3 in the AIBV/Bishoftu/03/2024 isolate. Identical amino acids are indicated as dots.

4.7. Phylogenic analysis of Avian Infectious Bronchitis virus isolates

The phylogenic tree analysis of avian infectious bronchitis virus isolates was conducted using MEGA7 software. A total of 45 sequences, including 5 current isolate sequences and 40 sequences retrieved from GenBank were included in the analysis as shown in Figure 16. The current isolates share the same clade with the Mexican isolates of OM912693, OR268751, OR268749, and OR268752. Therefore, the current Ethiopian isolates are closely related to that Mexican isolates (OM912693) that represent GI-9 with a percentage identity of 95.39%. The current isolate is also related to other Mexican isolates (OR268751 and OR268749) with percentage identity of 94.54% and 94.32%, respectively. However, the current five Ethiopian isolates were more distantly related to the Pakistan isolate and Jordan isolate of Massachusetts. In this study, the current Ethiopian AIBV outbreak isolates were shown closely related to each other. The genetic mean distance within the five Ethiopian AIBV isolates is 0.001. The genetic mean distance within the GenBank retrieved AIBV isolates is 0.01. The genetic mean distance between the two groups (the current Ethiopian and GenBank retrieved sequences) is 0.079 indicating a closer relationship among the current Ethiopian isolates compared to the broader set of GenBank isolates.

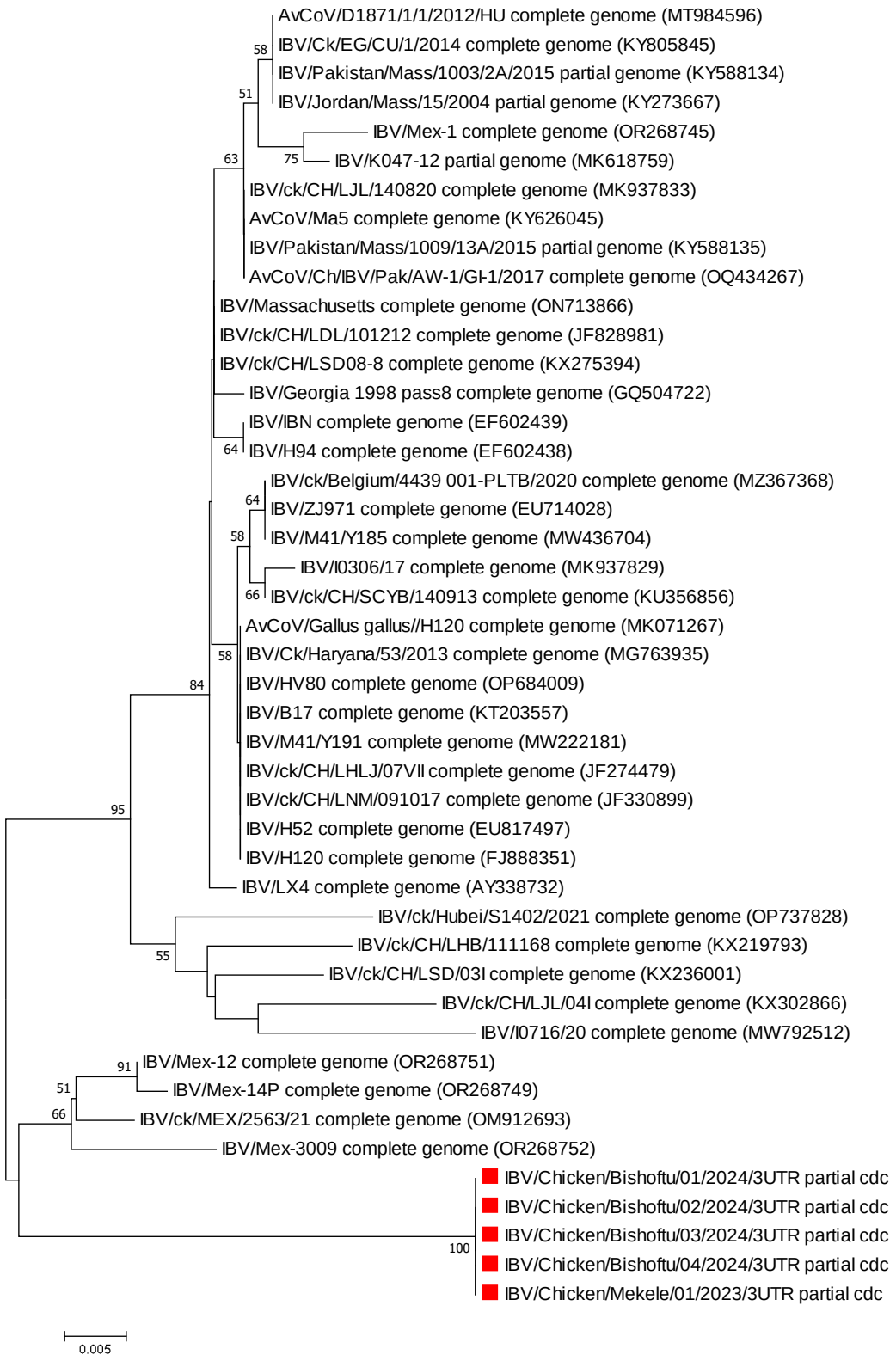


Figure 16: Phylogenetic tree analysis of the 45 infectious bronchitis viruses based on the 3´UTR nucleotide sequences. The tree was constructed using five field Ethiopian isolates

from the current investigation (labeled with a red rectangle) and homologous sequences retrieved from Gene Bank. MEGA7 software is used to compute the neighbor-joining method with the maximum composite likelihood nucleotide substitution model and the pairwise deletion option. The percentage bootstrap scores greater than 50% (out of 1000 replicates) are displayed next to each branch.

5. DISCUSSION

Infectious bronchitis is a highly contagious viral disease of poultry causing severe respiratory distress, and urinary and reproductive loss, leading to production losses due to poor hatchability, decreased egg production, and poor egg quality, and negative economic consequences (Cook *et al.*, 2012; Ayim-Akonor *et al.*, 2018). The viral genome undergoes continual mutation through deletion, insertion, and recombination. This makes the control measure difficult (Montassier, 2010; Yilmaz *et al.*, 2016). In Ethiopia, poultry production is the most growing and economically important sector, while the spread of the IB disease is not well known which makes implementing effective control measures difficult. The main purpose of this study was to investigate the outbreaks, isolate, and molecularly characterize the AIBV circulating in the study areas. Therefore, the findings provide baseline information for the development and implementation of disease control strategies.

The current outbreak investigation revealed a morbidity and mortality rate of 9.6% and 0.29%, respectively. This result is dissimilar to Hasan *et al.* (2020) who reported morbidity and mortality rates of 85% and 20% respectively in 20-day-old chickens in Iraq. During the outbreak investigation, 12.24% of the chickens tested positive in RT-PCR from 49 suspected and sampled chickens. This is higher than the report of Tegegne *et al.* (2020) from the Jima zone which was 6% but significantly lower than 33.3% from 12 swab samples taken from East Shewa and 39% from the Addis Ababa live animal market, as reported by Hirbaye *et al*. (2024) and Teklemariam *et al*. (2022), respectively. The differences in IB disease prevalence could be attributed to the geographical location of the study areas, farm biosecurity management, and infection prevention and control strategies, and other factors.

The results of this study revealed that the observed clinical findings among examined commercial chickens were depression, and respiratory signs such as sneezing, coughing, nasal discharges, and gasping. These findings were consistent with previously recorded clinical signs (Hassan *et al.*, 2016; Lebdah *et al.*, 2017; Mahmoud *et al.*, 2019; Tekelemariam *et al.*, 2022). The presence of postmortem pathological lesions, such as a fog of air sacs with yellow caseous exudates, was consistent with previous reports of IB

virus infections in chickens (Mahmoud *et al.*, 2019). Additionally, comparable findings on infections caused by bacterial infections or other viral coinfection were reported (Boroomand *et al*., 2012; Awad et al., 2014; Kong *et al*., 2021).

Isolation and propagation of AIBV samples in embryonated SPF eggs is a simple and gold-standard method. In this study, stunned of embryo growth or dwarfing/deformation, congestion with blood, and curling were detected as signs of the existence of AIBV, which was consistent with the previous findings (Patel *et al.*, 2015b; Bande *et al.*, 2016; Atta & Allawe, 2018; Al-Jallad *et al.*, 2020). Even if the embryonic changes are not specific to the AIBV and can be caused by other respiratory viruses such as pathogenic Newcastle variants and infectious laryngotracheitis (Cavanagh and Naqi, 2003; Taha *et al.*, 2017). In this investigation, all embryos died after 48 to 72 hours of post-inoculation with AIB virus. As indicated in Figure 10C on the left upper side, embryos completely lose their body structure, and embryo deformation, hemorrhage, and mortality are observed from the beginning of the first passage. However, other researchers have documented embryo congestion and hemorrhage beginning with the third passage, in contrast to the current investigations (Patel *et al.*, 2015b; Atta and Allawe, 2018; Al-Jallad *et al.*, 2020). These differences may result from AIBV co-infection with other viral diseases and bacterial complications, or they may be due to the pathogenic nature of the AIBV itself. In the current study, the median lethal dose of the AIBV has scored 6.5 ELD 50/ml which was done based on the cumulative number of dead embryos and surviving embryos at each dilution (Ramakrishnan, 2016). This finding is greater than the velogenic Newcastle disease virus embryo lethal dose reported by Husseium *et al.* (2023*)* who reported 6.3 ELD50/mL. This data reveals that the present AIBV isolates are highly pathogenic.

The continuous cell line does not support the growth of the AIBV. However, the primary cell culture prepared from the chicken embryo fibroblast cell culture supported the growth and isolation of AIBV. Cytopathic effects were observed following three days of AIBV inoculation, including rounding and aggregated cells, as well as detachment of the infected cells from the cell surface flask. These findings are similar to the report of (Atta and Allawe, 2018; Hussein *et al.*, 2018). The titer achieved in the current study was 5.7

TCID50 in the third passage, which is greater than the titer 4 TCID50 obtained by Atta and Allawe (2018) with an identical passage number.

Previous studies have identified and characterized infectious bronchitis viruses by targeting the S gene and the 3´UTR conserved region of the AIBV (Adzhar *et al.,* 1997; Patel *et al.,* 2015; Hussein *et al.,* 2018). AIBV strain characterization is generally done by amplifying the S gene, which is also considered the gold-standard method. However, this gene contains most hypervariable regions and is exposed to mutation and recombination with another stain. Because of the rapid mutation and recombination rates, new strains of the AIB virus are constantly emerging (Rafique *et al.,* 2024). In contrast, the 3´UTR region is a highly conserved region of the AIBV genome that is less prone to mutation and recombination. As a result, amplifying the 3´UTR region is rapid for outbreak investigation of IBV and applied to classify and characterize AIBV strains (Williams *et al.*, 1993; J and S, 2015). In addition to this, the 3´UTR region contributes to pathogenicity during the replication process (Liu *et al.*, 2013). In this study, the harvested allantoic fluids were detected by RT-PCR targeting the S and 3´UTR regions and confirmed the infection was due to the AIB virus. In addition, the serotype of AIBV identification by RT-PCR was conducted and a negative result was obtained. This result does not agree with the previous reports (Hutton *et al.*, 2017; Tesfaye *et al.*, 2019; Tegegne *et al.*, 2020; Hirbaye *et al.*, 2024). However, the sequence phylogenic analysis indicates that the current isolates were related to the Mexican isolates, which grouped under GI-9. Therefore, taking this into account the isolates of the current study are different from the previously reported Ethiopian isolates.

Currently in Ethiopia, 793B and Massachusetts-type vaccines imported that are closely resemble the previously identified strain in Ethiopia. Even though these imported vaccinations are similar to the previously identified strain in Ethiopia, they are insufficient and the molecular and serological investigations are limited. Because of this situation and the existence of no or poor cross-protection among the different serotypes; the vaccines may not provide complete protection to the vaccinated chickens against the circulating virus types and the virus's nature of mutation and recombination increases the likelihood of the emergence of a new strain. The previous reports indicate that the

outbreak of IBV was from field isolate (Hutton *et al.*, 2017; Tesfaye *et al.*, 2019; Tegegne *et al.*, 2020; Hirbaye *et al.*, 2024) which is similar to the current study. In this study, six of the 49 suspected and sampled chickens tested positive. One out of six positive isolates had a bad chromatogram and was not aligned with the other five isolates.

A comparison of multiple sequence alignment of the Ethiopian isolates obtained from Bishoftu isolate three showed a single nucleotide variation at position seven from the other four isolates. In Ethiopia, the circulating AIBV variants reported from commercial poultry farms and backyard systems were D274, 793B, Mas, and Qx- like by using the RT- PCR targeting the S gene of IBV, in contrast to the current study, which targeted additional 3´UTR region (Hutton *et al.*, 2017; Tesfaye *et al.*, 2019; Tegegne *et al.*, 2020). In the current investigation, the 3´UTR gene sequences of five outbreak isolates were analyzed and were found to be comparable to each other, with alignment similarity ranging from 97.82% to 99.78%. This finding was similar to the finding of Pattel *et al*. (2015). In contrast, alignment similarity scores from gene bank isolates range from 90.8% to 99.83%. The nucleotide sequence alignment of the 3'UTR of the five new Ethiopian IBV isolates revealed a single nucleotide polymorphism (SNP) A: C in the IBV/Bishoftu/03/2024 isolate. The SNP changed the amino acid sequence (Q:P), substituting Glutamine (Q) with Proline (P).

The phylogenic tree was constructed to understand the evolutionary relationship between the Ethiopian current field isolates and other isolates retrieved from the database. The current isolates were closely related to the Mexican isolate OM912693, which represents GI-9 (Arkansas type) (Kariithi *et al.*, 2022) with a percentage identity of 95.39%. The current isolate is also related to other Mexican isolates, OR268751 and OR268749 (Marandino *et al.*, 2023), with percentage identity of 94.54% and 94.32%, respectively. Therefore, the findings provide the first molecular characterization of AIBV in Ethiopia, identifying genetically related local viral lineages that differ from strains used in imported vaccines This study provides good information for designing an AIB vaccine that can match strains of the virus that are already circulating in Ethiopia.

6. CONCLUSION AND RECOMMENDATIONS

This study provides crucial insights into the genotypes of AIBV in Ethiopia and demonstrates the presence of multiple strains. The genetic analysis indicates 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 the currently circulating strains in Ethiopia. Based on the findings of this study, the following recommendations are proposed:

- ✓ Conduct outbreak investigations to better understand the distribution and diversity of AIBV strains in Ethiopia
- ✓ Serotype the circulating AIB viruses to determine their antigenic characteristics and to select suitable vaccine candidates
- ✓ Full-genome sequencing of the AIBV isolates for detail characterization of the strains.
- ✓ Implement stringent farm biosecurity measures, such as quarantine, disinfection, and movement control, to reduce the risk of AIBV transmission and new strain emergence.
- ✓ Develop tailored AIBV vaccines based on local strains or match with imported vaccines to improve disease prevention and control efforts in Ethiopia’s poultry sector.

7. REFERENCES

Abdel-Moneim, A. S. (2017). Coronaviridae: Infectious bronchitis virus. *Emerging and Re-emerging Infectious Diseases of Livestock*, 133-166.

Abro, S. H., Renstrom, L. H., Ullman, K., Belak, S., & Baule, C. (2012). Characterization and analysis of the full-length genome of a strain of the European QX-like genotype of infectious bronchitis virus. *Arch Virol*, ***157***(6), 1211-1215. https://doi.org/10.1007/s00705-012-1284-0

Adzhar, A., Gough, R. E., Haydon, D., Shaw, K., Britton, P., & Cavanagh, D. (1997). Molecular analysis of the 793/B serotype of infectious bronchitis virus in Great Britain. *Avian Pathol*, ***26***(3), 625-640. https://doi.org/10.1080/03079459708419239

Al-Jallad, T., Kassouha, M., Salhab, M., Alomar, A., Al-Masalma, M., & Abdelaziz, F. (2020). Molecular characterization of isolated infectious bronchitis viruses from affected vaccinated broiler flocks in Syria. *BMC Vet Res*, ***16***(1), 449. https://doi.org/10.1186/s12917-020-02672-1

Ali, A., Ojkic, D., Elshafiee, E. A., Shany, S., El-Safty, M. M., Shalaby, A. A., & Abdul-Careem, M. F. (2022). Genotyping and In Silico Analysis of Delmarva (DMV/1639) Infectious Bronchitis Virus (IBV) Spike 1 (S1) Glycoprotein. *Genes (Basel)*, ***13***(9), 1-19. https://doi.org/10.3390/genes13091617

Atta, R., & Allawe, A. B. (2018). Isolation and sequencing of field isolates of Avian infectious bronchitis virus in Iraq. *J Entomol Zool Stud*, *6*, 529-540.

Awad, F., Chhabra, R., Baylis, M., & Ganapathy, K. (2014). An overview of infectious bronchitis virus in chickens. *World's Poultry Science Journal*, ***70***(2), 375-384. https://doi.org/https://doi.org/10.1017/S0043933914000385

Ayim-Akonor, M., Obiri-Danso, K., Toah-Akonor, P., & Sellers, H. S. (2018). Widespread exposure to infectious bronchitis virus and Mycoplasma gallisepticum in chickens in the Ga-East district of Accra, Ghana. *Cogent Food & Agriculture*, ***4***(1), 1439260. https://doi.org/10.1080/23311932.2018.1439260

Bali, K., Kaszab, E., Marton, S., Hamdiou, S. H., Bentaleb, R. K., Kiss, I., Palya, V., & Bányai, K. (2022). Novel Lineage of Infectious Bronchitis Virus from Sub-

Saharan Africa Identified by Random Amplification and Next-Generation Sequencing of Viral Genome. *Life (Basel)*, ***12***(4). https://doi.org/10.3390/life12040475

Bande, F., Arshad, S. S., Omar, A. R., Bejo, M. H., Abubakar, M. S., & Abba, Y. (2016). Pathogenesis and Diagnostic Approaches of Avian Infectious Bronchitis. *Adv Virol*, *2016*, 1-11. https://doi.org/10.1155/2016/4621659

Bande, F., Arshad, S. S., Omar, A. R., Hair-Bejo, M., Mahmuda, A., & Nair, V. (2017). Global distributions and strain diversity of avian infectious bronchitis virus: a review. *Anim Health Res Rev*, ***18***(1), 70-83. https://doi.org/10.1017/s1466252317000044

Batra, A., Maier, H. J., & Fife, M. S. (2017). Selection of reference genes for gene expression analysis by real-time qPCR in avian cells infected with infectious bronchitis virus. *Avian Pathol*, ***46***(2), 173-180. https://doi.org/10.1080/03079457.2016.1235258

Bhuiyan, M. S. A., Amin, Z., Bakar, A., Saallah, S., Yusuf, N. H. M., Shaarani, S. M., & Siddiquee, S. (2021). Factor Influences for Diagnosis and Vaccination of Avian Infectious Bronchitis Virus (Gammacoronavirus) in Chickens. *Vet Sci*, ***8***(3), 1-25. https://doi.org/10.3390/vetsci8030047

Bhuiyan, M. S. A., Sarker, S., Amin, Z., Rodrigues, K. F., Saallah, S., Shaarani, S. M., & Siddiquee, S. (2023). Infectious Bronchitis Virus (Gammacoronavirus) in Poultry: Genomic Architecture, Post-Translational Modifications, and Structural Motifs. *Poultry*, ***2***(3), 363-382. https://doi.org/https://doi.org/10.3390/poultry2030027

Birhan, M., Temesgen, M., Shite, A., Berhane, N., Bitew, M., Gelaye, E., Abayneh, T., & Getachew, B. (2021). Seroprevalence and Associated Risk Factors of Infectious Bronchitis Virus in Chicken in Northwest Ethiopia. *ScientificWorldJournal*, *2021*, 1-10. https://doi.org/10.1155/2021/4553890

Boroomand, Z., Asasi, K., & Mohammadi, A. (2012). Pathogenesis and tissue distribution of avian infectious bronchitis virus isolate IRFIBV32 (793/B serotype) in experimentally infected broiler chickens. *ScientificWorldJournal*, *2012*, 1-7. https://doi.org/10.1100/2012/402537

Butcher, G. D., Shapiro, D. P., & Miles, R. D. (2003). Classical and Variant Avian Infectious Bronchitis Virus Strains: VM127/PS039, 5/2002. *EDIS*, ***2003***(16), 1-5. https://doi.org/https://doi.org/10.32473/edis-ps039-2002

Cavanagh, D. (2005). Coronaviruses in poultry and other birds. *Avian Pathol*, ***34***(6), 439-448. https://doi.org/10.1080/03079450500367682

Cavanagh, D. (2007). Coronavirus avian infectious bronchitis virus. *Vet Res*, ***38***(2), 281-297. https://doi.org/10.1051/vetres:2006055

Cavanagh, D., & Naqi, S. (2003). Infectious bronchitis. In *Diseases of poultry* (Vol. 11, pp. 118-135).

Cook, J. K., Jackwood, M., & Jones, R. C. (2012). The long view: 40 years of infectious bronchitis research. *Avian Pathol*, ***41***(3), 239-250. https://doi.org/10.1080/03079457.2012.680432

CSA. (2021). Federal Democratic Republic of Ethiopia: Central Statistical Agency: Agricultural Sample Survey. ***2***(589), 1-199.

Domanska-Blicharz, K., Lisowska, A., Pikuła, A., & Sajewicz-Krukowska, J. (2017). Specific detection of GII-1 lineage of infectious bronchitis virus. *Lett Appl Microbiol*, ***65***(2), 141-146. https://doi.org/10.1111/lam.12753

Fischer, S., Klosterhalfen, D., Wilms-Schulze Kump, F., & Casteel, M. (2020). Research Note: First evidence of infectious bronchitis virus Middle-East GI-23 lineage (Var2-like) in Germany. *Poult Sci*, ***99***(2), 797-800. https://doi.org/10.1016/j.psj.2019.10.031

Franzo, G., Naylor, C. J., Lupini, C., Drigo, M., Catelli, E., Listorti, V., Pesente, P., Giovanardi, D., Morandini, E., & Cecchinato, M. (2014). Continued use of the IBV 793B vaccine needs reassessment after its withdrawal led to the genotype's disappearance. *Vaccine*, ***32***(50), 6765-6767. https://doi.org/10.1016/j.vaccine.2014.10.006

Franzo, G., Tucciarone, C. M., Blanco, A., Nofrarías, M., Biarnés, M., Cortey, M., Majó, N., Catelli, E., & Cecchinato, M. (2016). Effect of different vaccination strategies on IBV QX population dynamics and clinical outbreaks. *Vaccine*, ***34***(46), 5670-5676. https://doi.org/10.1016/j.vaccine.2016.09.014

Gallardo, R. A. (2021). Infectious bronchitis virus variants in chickens: evolution, surveillance, control and prevention. *Austral journal of veterinary sciences*, ***53***(1), 55-62.

Hartenian, E., Nandakumar, D., Lari, A., Ly, M., Tucker, J. M., & Glaunsinger, B. A. (2020). The molecular virology of coronaviruses. *J Biol Chem*, ***295***(37), 12910-12934. https://doi.org/10.1074/jbc.REV120.013930

Hasan, II, Rasheed, S. T., Jasim, N. A., & Shakor, M. K. (2020). Pathological effect of infectious bronchitis disease virus on broiler chicken trachea and kidney tissues. *Vet World*, ***13***(10), 2203-2208. https://doi.org/10.14202/vetworld.2020.2203-2208.

Hassan, K. E., Shany, S. A., Ali, A., Dahshan, A. H., El-Sawah, A. A., & El-Kady, M. F. (2016). Prevalence of avian respiratory viruses in broiler flocks in Egypt. *Poult Sci*, ***95***(6), 1271-1280. https://doi.org/10.3382/ps/pew068

Hewson, K., Noormohammadi, A. H., Devlin, J. M., Mardani, K., & Ignjatovic, J. (2009). Rapid detection and non-subjective characterization of infectious bronchitis virus isolate using high-resolution melt curve analysis and a mathematical model. *Arch Virol*, ***154***(4), 649-660. https://doi.org/10.1007/s00705-009-0357-1

Hirbaye, G., Tola, E. H., Moje, N., & Sori, T. (2024). Molecular and Serological Investigation of Infectious Bronchitis Virus in the East Shewa, Central Ethiopia. *Veterinary Medicine: Research and Reports*, *15*, 81-90. https://doi.org/10.2147/VMRR.S452153

Hussein, M. A., Sabbar, A. A., & Khammas, E. J. (2018). Isolation and identification of infectious bronchitis virus and experimental infection in broilers. (10), 290-302.

Husseiun, R. S., Dyary, H. O., & Saeed, N. M. (2023). Determination of median embryo lethal dose for a velogenic Newcastle disease virus isolated in Sulaimani/Iraq. *Basrah Journal of Veterinary Research*, ***22***(4), 1-14.

Hutton, S., Bettridge, J., Christley, R., Habte, T., & Ganapathy, K. (2017). Detection of infectious bronchitis virus 793B, avian metapneumovirus, Mycoplasma gallisepticum, and Mycoplasma synoviae in poultry in Ethiopia. *Trop Anim Health Prod*, ***49***(2), 317-322. https://doi.org/10.1007/s11250-016-1195-2

Icochea, E., González, R., Castro-Sanguinetti, G., Maturrano, L., Alzamora, L., Sesti, L., Chacón, J., & More-Bayona, J. (2023). Genetic Analysis of Infectious Bronchitis Virus S1 Gene Reveals Novel Amino Acid Changes in the GI-16 Lineage in Peru. *Microorganisms*, ***11***(3). https://doi.org/10.3390/microorganisms11030691

Ignjatovic, J., Gould, G., & Sapats, S. (2006). Isolation of a variant infectious bronchitis virus in Australia that further illustrates diversity among emerging strains. *Arch Virol*, ***151***(8), 1567-1585. https://doi.org/10.1007/s00705-006-0726-y

Junfeng, S., and Shengwang, L. (2015). An RT-PCR Assay for Detection of Infectious Bronchitis Coronavirus Serotypes. *Animal Coronaviruses*, *10*, 121-130.

Jackwood, M. W. (2012). Review of infectious bronchitis virus around the world. *Avian Dis*, ***56***(4), 634-641. https://doi.org/10.1637/10227-043012-Review.1

Jackwood, M. W., & de Wit, S. (2013). Infectious bronchitis. *Diseases of poultry*, 139-159.

Jagne, J., & Buckles, E. (2021). How to Perform a Necropsy. *Backyard Poultry Medicine and Surgery: A Guide for Veterinary Practitioners*, 477-503.

Jang, I., Lee, H. J., Bae, Y. C., Park, S. C., Lee, H. S., & Choi, K. S. (2018). Genetic and Pathologic Characterization of a Novel Recombinant TC07-2-Type Avian Infectious Bronchitis Virus. *Avian Dis*, ***62***(1), 109-113. https://doi.org/10.1637/11764-103017-ResNote.1

Jayaram, J., Youn, S., & Collisson, E. W. (2005). The virion N protein of infectious bronchitis virus is more phosphorylated than the N protein from infected cell lysates. *Virology*, ***339***(1), 127-135. https://doi.org/10.1016/j.virol.2005.04.029

Kärber, G. (1931). Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. *Naunyn-Schmiedebergs Archiv für experimentelle Pathologie und Pharmakologie*, ***162***(4), 480-483. https://doi.org/10.1007/BF01863914

Kariithi, H. M., Volkening, J. D., Leyson, C. M., Afonso, C. L., Christy, N., Decanini, E. L., Lemiere, S., & Suarez, D. L. (2022). Genome Sequence Variations of Infectious Bronchitis Virus Serotypes From Commercial Chickens in Mexico. *Front Vet Sci*, *9*, 931272. https://doi.org/10.3389/fvets.2022.931272

Khaltabadi Farahani, R., Ghalyanchilangeroudi, A., Fallah Mehrabadi, M. H., Ghafouri, S. A., & Maghsoudloo, H. (2020). Molecular Monitoring of D1466 Genotype of

Avian Infectious Bronchitis Virus in Iran: A Retrospective Study in 2013-2017. *Arch Razi Inst*, ***75***(2), 163-168. https://doi.org/10.22092/ari.2018.121512.1214

Khataby, K., Fellahi, S., Loutfi, C., & Mustapha, E. M. (2016). Avian infectious bronchitis virus in Africa: a review. *Vet Q*, ***36***(2), 71-75. https://doi.org/10.1080/01652176.2015.1126869

Kong, L., You, R., Zhang, D., Yuan, Q., Xiang, B., Liang, J., Lin, Q., Ding, C., Liao, M., Chen, L., & Ren, T. (2021). Infectious Bronchitis Virus Infection Increases Pathogenicity of H9N2 Avian Influenza Virus by Inducing Severe Inflammatory Response. *Front Vet Sci*, *8*, 824179. https://doi.org/10.3389/fvets.2021.824179

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. *Mol Biol Evol*, ***33***(7), 1870-1874. https://doi.org/10.1093/molbev/msw054

Lebdah, M. A., Hegazy, A. M., Hassan, M. H., & Mohammed, M. (2017). Isolation and molecular characterization of infectious bronchitis virus from broiler chickens, Egypt during 2014-2016. *Zagazig Veterinary Journal*, ***45***(1), 11-18.

Lee, C. W., Hilt, D. A., & Jackwood, M. W. (2001). Identification and analysis of the Georgia 98 serotype, a new serotype of infectious bronchitis virus. *Avian Dis*, ***45***(1), 164-172. https://doi.org/https://doi.org/10.2307/1593024

Lee, P. Y., Costumbrado, J., Hsu, C. Y., & Kim, Y. H. (2012). Agarose gel electrophoresis for the separation of DNA fragments. *J Vis Exp*(62). https://doi.org/10.3791/3923

Legnardi, M., Tucciarone, C. M., Franzo, G., & Cecchinato, M. (2020). Infectious Bronchitis Virus Evolution, Diagnosis and Control. *Vet Sci*, ***7***(2), 1-18. https://doi.org/10.3390/vetsci7020079

Lei, C., Yang, J., Hu, J., & Sun, X. (2021). On the Calculation of TCID(50) for Quantitation of Virus Infectivity. *Virol Sin*, ***36***(1), 141-144. https://doi.org/10.1007/s12250-020-00230-5

Lin, S. Y., & Chen, H. W. (2017). Infectious Bronchitis Virus Variants: Molecular Analysis and Pathogenicity Investigation. *Int J Mol Sci*, ***18***(10), 1-18. https://doi.org/10.3390/ijms18102030

Lisowska, A., Sajewicz-Krukowska, J., Fusaro, A., Pikula, A., & Domanska-Blicharz, K. (2017). First characterization of a Middle-East GI-23 lineage (Var2-like) of infectious bronchitis virus in Europe. *Virus Res*, *242*, 43-48. https://doi.org/10.1016/j.virusres.2017.09.010

Liu, X., Ma, H., Xu, Q., Sun, N., Han, Z., Sun, C., Guo, H., Shao, Y., Kong, X., & Liu, S. (2013). Characterization of a recombinant coronavirus infectious bronchitis virus with distinct S1 subunits of spike and nucleocapsid genes and a 3' untranslated region. *Vet Microbiol*, ***162***(2-4), 429-436. https://doi.org/10.1016/j.vetmic.2012.10.027

Ma, T., Xu, L., Ren, M., Shen, J., Han, Z., Sun, J., Zhao, Y., & Liu, S. (2019). Novel genotype of infectious bronchitis virus isolated in China. *Vet Microbiol*, *230*, 178-186. https://doi.org/10.1016/j.vetmic.2019.01.020

Mahmoud, A., Shahin, A., & Eid, A. (2019). The role of infectious bronchitis virus in respiratory and renal problems in broiler chickens. *Zagazig Veterinary Journal*, ***47***(1), 32-44.

Marandino, A., Mendoza-González, L., Panzera, Y., Tomás, G., Williman, J., Techera, C., Gayosso-Vázquez, A., Ramírez-Andoney, V., Alonso-Morales, R., Realpe-Quintero, M., & Pérez, R. (2023). Genome Variability of Infectious Bronchitis Virus in Mexico: High Lineage Diversity and Recurrent Recombination. *Viruses*, *15*(7). https://doi.org/10.3390/v15071581

Masters, P. S. (2006). The molecular biology of coronaviruses. *Adv Virus Res*, *66*, 193-292. https://doi.org/10.1016/s0065-3527(06)66005-3

Molenaar, R. J., Dijkman, R., & de Wit, J. J. (2020). Characterization of infectious bronchitis virus D181, a new serotype (GII-2). *Avian Pathol*, ***49***(3), 243-250. https://doi.org/10.1080/03079457.2020.1713987

Montassier, H. J. (2010). Molecular epidemiology and evolution of avian infectious bronchitis virus. *Brazilian Journal of Poultry Science*, *12*, 87-96. https://doi.org/https://doi.org/10.1590/S1516-635X2010000200003

Moreno, A., Franzo, G., Massi, P., Tosi, G., Blanco, A., Antilles, N., Biarnes, M., Majó, N., Nofrarías, M., Dolz, R., Lelli, D., Sozzi, E., Lavazza, A., & Cecchinato, M. (2017). A novel variant of the infectious bronchitis virus resulting from

recombination events in Italy and Spain. *Avian Pathol*, ***46***(1), 28-35. https://doi.org/10.1080/03079457.2016.1200011

Parris, D. J., Kariithi, H., & Suarez, D. L. (2022). Non-target RNA depletion strategy to improve the sensitivity of next-generation sequencing for the detection of RNA viruses in poultry. *J Vet Diagn Invest*, ***34***(4), 638-645. https://doi.org/10.1177/10406387221102430

Patel, B. H., Bhimani, M. P., Bhanderi, B. B., & Jhala, M. K. (2015). Isolation and molecular characterization of nephropathic infectious bronchitis virus isolates of Gujarat state, India. *Virusdisease*, ***26***(1-2), 42-47. https://doi.org/10.1007/s13337-015-0248-x

Pereira, C. G., Saraiva, G. L., Vidigal, P. M., Fietto, J. L., Bressan, G. C., Moreira, M. A., de Almeida, M. R., & Júnior, A. S. (2016). Distribution of infectious bronchitis virus strains in different organs and evidence of vertical transmission in natural infection. *Arch Virol*, ***161***(12), 3355-3363. https://doi.org/10.1007/s00705-016-3030-5

Perlman, S., Gallagher, T., and Snijder, E. J. (Eds.). (2008). *Nidoviruses* (pp. xvi-433). Washington, DC: ASM press.

Promkuntod, N. (2016). Dynamics of avian coronavirus circulation in commercial and non-commercial birds in Asia--a review. *Vet Q*, ***36***(1), 30-44. https://doi.org/10.1080/01652176.2015.1126868

Quinteros, J. A., Ignjatovic, J., Chousalkar, K. K., Noormohammadi, A. H., & Browning, G. F. (2021). Infectious bronchitis virus in Australia: a model of coronavirus evolution - a review. *Avian Pathol*, ***50***(4), 295-310. https://doi.org/10.1080/03079457.2021.1939858

Rafique, S., Jabeen, Z., Pervaiz, T., Rashid, F., Luo, S., Xie, L., & Xie, Z. (2024). Avian infectious bronchitis virus (AIBV) review by continent. *Front Cell Infect Microbiol*, *14*, 1-17. https://doi.org/10.3389/fcimb.2024.1325346

Ramakrishnan, M. A. (2016). Determination of 50% endpoint titer using a simple formula. *World J Virol*, ***5***(2), 85-86. https://doi.org/10.5501/wjv.v5.i2.85

Ramirez-Nieto, G., Mir, D., Almansa-Villa, D., Cordoba-Argotti, G., Beltran-Leon, M., Rodriguez-Osorio, N., Garai, J., Zabaleta, J., & Gomez, A. P. (2022). New

Insights into Avian Infectious Bronchitis Virus in Colombia from Whole-Genome Analysis. *Viruses*, ***14***(11). https://doi.org/10.3390/v14112562

Saadat, Y., Bozorgmehri Fard, M. H., Charkhkar, S., Hosseini, H., Shaikhi, N., & Akbarpour, B. (2017). Molecular characterization of infectious bronchitis viruses isolated from broiler flocks in Bushehr province, Iran: 2014 - 2015. *Vet Res Forum*, ***8***(3), 195-201.

Sawicki, S. G., & Sawicki, D. L. (2005). Coronavirus transcription: a perspective. *Curr Top Microbiol Immunol*, *287*, 31-55. https://doi.org/10.1007/3-540-26765-4_2

Schalk, A. F., & Hawn, M. (1931). An apparently new respiratory disease of baby chicks. *Journal of the American Veterinary Medical Association*, *78*, 413-423.

Shiferaw, J., Dego, T., Tefera, M., & Tamiru, Y. (2022). Seroprevalence of infectious bronchitis virus in broiler and layer farms of Central Ethiopia. *BioMed Research International*, *2022*, 1-5.

Sjaak de Wit, J. J., Cook, J. K., & van der Heijden, H. M. (2011). Infectious bronchitis virus variants: a review of the history, current situation and control measures. *Avian Pathol*, ***40***(3), 223-235. https://doi.org/10.1080/03079457.2011.566260

Steyn, A., Keep, S., Bickerton, E., & Fife, M. (2020). The characterization of chIFITMs in avian coronavirus infection in vivo, ex vivo and in vitro. *Genes*, ***11***(8), 1-18.

Taha, Z., Allawe, A., Kadhum, M., & Abbas, A. (2017). Isolation and sequencing of field isolates of infectious laryngeotracheitis virus in Iraq. *J. Entomol. Zool. Stud*, *5*, 882-886.

Tan, Y. W., Hong, W., & Liu, D. X. (2012). Binding of the 5'-untranslated region of coronavirus RNA to zinc finger CCHC-type and RNA-binding motif 1 enhances viral replication and transcription. *Nucleic Acids Res*, ***40***(11), 5065-5677. https://doi.org/10.1093/nar/gks165

Tegegne, D., Deneke, Y., Sori, T., Abdurahaman, M., Kebede, N., Cecchinato, M., & Franzo, G. (2020). Molecular Epidemiology and Genotyping of Infectious Bronchitis Virus and Avian Metapneumovirus in Backyard and Commercial Chickens in Jimma Zone, Southwestern Ethiopia. *Vet Sci*, ***7***(4), 1-11. https://doi.org/10.3390/vetsci7040187

Tekelemariam, T. H., Walkden-Brown, S., Atire, F. A., Tefera, D. A., Alemayehu, D. H., & Gerber, P. F. (2022). Detection of Chicken Respiratory Pathogens in Live Markets of Addis Ababa, Ethiopia, and Epidemiological Implications. *Vet Sci*, ***9***(9). https://doi.org/10.3390/vetsci9090503

Tesfaye, A., Kassa, T., Mesfin, S., Garoma, A., Koran, T., Dima, C., Guyassa, C., Hailu, H., & Teshale, S. (2019). Four serotypes of infectious bronchitis virus are widespread in unvaccinated backyard chicken and commercial farms in Ethiopia. *World J. Vet. Sci*, *1*(1), 1-4.

Ujike, M., & Taguchi, F. (2015). Incorporation of spike and membrane glycoproteins into coronavirus virions. *Viruses*, ***7***(4), 1700-1725. https://doi.org/10.3390/v7041700

Umar, S., Shah, M., Munir, M., Ahsan, U., & Kaboudi, K. (2016). Infectious bronchitis virus: evolution and vaccination. *World's Poultry Science Journal*, ***72***(1), 49-60. https://doi.org/ https://doi.org/10.1017/S0043933915002706

Valastro, V., Holmes, E. C., Britton, P., Fusaro, A., Jackwood, M. W., Cattoli, G., & Monne, I. (2016). S1 gene-based phylogeny of infectious bronchitis virus: An attempt to harmonize virus classification. *Infect Genet Evol*, *39*, 349-364. https://doi.org/10.1016/j.meegid.2016.02.015

Williams, A., Wang, L., Sneed, L., & Collisson, E. (1993). Analysis of a hypervariable region in the 3'non-coding end of the infectious bronchitis virus genome. *virus Research*, ***28***(1), 19-27.

WOAH. (2018). Avian Infectious Bronchitis. CHAPTER 3.3.2.OIE Terrestrial Manual. In (pp. 1-9).

Woo, P. C., Lau, S. K., Lam, C. S., Lau, C. C., Tsang, A. K., Lau, J. H., Bai, R., Teng, J. L., Tsang, C. C., Wang, M., Zheng, B. J., Chan, K. H., & Yuen, K. Y. (2012). Discovery of seven novel Mammalian and avian coronaviruses in the genus deltacoronavirus supports bat coronaviruses as the gene source of alphacoronavirus and betacoronavirus and avian coronaviruses as the gene source of gammacoronavirus and deltacoronavirus. *J Virol*, ***86***(7), 3995-4008. https://doi.org/10.1128/jvi.06540-11

Yilmaz, H., Altan, E., Cizmecigil, U. Y., Gurel, A., Ozturk, G. Y., Bamac, O. E., Aydin, O., Britton, P., Monne, I., Cetinkaya, B., Morgan, K. L., Faburay, B., Richt, J. A.,

& Turan, N. (2016). Phylogeny and S1 Gene Variation of Infectious Bronchitis Virus Detected in Broilers and Layers in Turkey. *Avian Dis*, ***60***(3), 596-602. https://doi.org/10.1637/11346-120915-Reg.1

Zeleke, B. (2017). Status and growth trend of draught animals' population in Ethiopia. *Journal of Dairy, Veterinary & Animal Research*, ***6***(1), 238-241.

Zúñiga, S., Cruz, J. L., Sola, I., Mateos-Gómez, P. A., Palacio, L., & Enjuanes, L. (2010). Coronavirus nucleocapsid protein facilitates template switching and is required for efficient transcription. *J Virol*, ***84***(4), 2169-2175. https://doi.org/10.1128/jvi.02011-09

8. LIST OF APPENDICES

Appendix 1: Tissue processing procedure

1. Internal organs like trachea, lungs, and kidney was thawed at room temperature
2. Take 3g from pooled sample and washed with PBS (with 5% antibiotics) three times in the biosafety cabinet level two.
3. The tissue sample was grinded into small pieces by using a pistil and mortar.
4. Then by adding 9 ml of sterile PBS at a pH of 7.2 centrifugation was carried out at 3000 rpm for 10 min at +4°C.
5. The Supernatants of grinded organs were collected under sterile conditions.
6. The virus suspension was filtered through a 0.45 μm (Millipore USA) filter to avoid any bacterial contamination and preserved in -20°C refrigerators until use.

Appendix 2: Primary cell culture preparation

1. By using sterile technique, the embryo was removed by using forceps
2. The extremities of the embryo and the visceral organs were removed
3. Washed by PBS containing antibiotics
4. The tissue fragment was transferred to a glass flask containing a magnetic stirrer bar
5. Then add 50ml pre-warmed working trypsin solution it and put on a stir plate at low speed in a 37°C incubator for 10 minutes
6. Then the supernatant was poured into a beaker containing calf serum with sterile gauze and centrifuged at 1000 rpm for 10 minutes
7. By discarding, the supernatant the remaining pellets were trypsinized by adding 10% minimum essential media (MEM)
8. Pour on tissue flask and incubate at 37°C until the cell is confluent.

Appendix 3: Collection of IBV from infected allantoic fluid eggs

1. Candle the eggs once daily after inoculation. Discard all eggs with embryos that die within the first 24 hours after inoculation
2. Collect allantoic fluid from all eggs with embryos that die >24 h after inoculation and from eggs with embryos that survive through the specified incubation period. Eggs with live embryos following the specified incubation period are refrigerated at 4 °C overnight, before the collection of allantoic fluid
3. Place eggs in an egg flat with the air sack up. Disinfect the portion of the eggshell that covers the air sack, and use sterile forceps to crack and remove the shell over air cell
4. Use forceps to gently dissect through the shell membrane and CAM to expose the allantoic fluid. Use forceps to depress membranes within the allantoic cavity so that allantoic fluid pools around the tip of the forceps. Use a pipette or syringe with a needle to aspirate fluid. Place fluid in sterile, 12 × 75 mm snap-cap tubes, or other vials. Store at −80°C for long storage
5. Examine allantoic fluid for the presence of coronavirus using RT-PCR.

Appendix 4: Virus RNA extraction procedure

1. Pipet 560 µl prepared Buffer AVL containing Carrier RNA in a 1.5 ml micro centrifuge tube
2. Add a 140-µl sample to the buffer AVL carrier RNA in the microcentrifuge tube. Mix by pulse-vortexing for 15 seconds
3. Incubate at room temperature for 10 minutes
4. Briefly centrifuge the tube to remove drops from the inside of the lid
5. Add 560-µl ethanol (96-100%) to the sample and mix by pulse vortexing for 15 seconds. After mixing, briefly centrifuge the tube to remove drops from inside the lid
6. Carefully apply 630 µl of the solution from step 5 to the QIAamp Mini column (in a 2ml collection tube) without wetting the rim. Close the cap, and centrifuge at 8000 rpm for one minute. Place the QIAamp Mini column into a clean 2 ml collection tube, and discard the tube containing the filtrate
7. Carefully open the QIAamp Mini column, and repeat step 6
8. Carefully open the QIAamp Mini column, and add 500 µl Buffer AW1. Close the cap, and centrifuge at 8000rpm for one minute. Place the QIAamp Mini column in a clean 2 ml collection tube, and discard the tube containing the filtrate
9. Carefully open the QIAamp Mini column, and add 500 µl Buffer AW2. Close the cap and centrifuge at full speed (14000rpm) for 3 minutes
10. Place the QIAamp Mini column in a new 2 ml collection tube, and discard the old collection tube with the filtrate. Centrifuge at full speed for one minute
11. Place the QIAamp Mini column in a clean 1.5 ml microcentrifuge tube. Discard the old collection tube containing the filtrate. Carefully open the QIAamp Mini column and add 60 µl Buffer AVE equilibrated to room temperature. Close the cap, and incubate at room temperature for one minute
12. Centrifuge at 8000 rpm for 1 minute to elute the viral RNA from the QIAamp Mini column
13. Then the viral RNA is stored at -80°C until PCR.

Appendix 5: PCR product purification procedure by centrifugation

Processing PCR Amplifications

1. Add an equal volume of Membrane Binding Solution to the PCR amplification

Binding of DNA

2. Insert SV Minicolumn into the Collection tube
3. Transfer the prepared PCR product to the Minicolumn assembly. Incubate at room temperature for one minute
4. Centrifuge at 13000 rpm for one minute. Discard flow through and reinsert Minicolumn into the collection tube.

Washing

5. Add 700 µl Membrane Wash Solution. Centrifuge at 13000 rpm for one minute. Discard flow through and reinsert Minicolumn into the Collection tube
6. Repeat step 5 with 500 µl Membrane Wash Solution. Centrifuge at 13000 rpm for 5 minutes
7. Empty the Collection tube and centrifuge the column assembly for one minute. With the microcentrifuge, the lid is open to allow evaporation of any residual ethanol.

Elution

8. Carefully transfer Minicolumn to a clean 1.5 ml microcentrifuge tube
9. Add 50 µl of Nuclease–Free water to the Minicolumn. Incubate at room temperature for one-minute centrifuge at 13000 rpm for one minute
10. Discard minicolumn and store DNA at -20°C until sequencing.

Appendix 6: Sample collection, egg inoculation, harvesting, and virus titration

Appendix 7: Ethical clearance certificate

አዲስ አበባ ዩኒቨርሲቲ
የእንስሳት ሕክምናና
ግብርና ኮሌጅ
ቢሾፍቱ

ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
Bishoftu

Animal Research Ethical Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/02/13/16/2024

Name of Applicant: **Nigusu Berhanu Kassaye (DVM, MSc student)**

Address: Department of Microbiology, Immunology and Veterinary Public Health, College of Veterinary Medicine and Agriculture, Addis Ababa University

Title of the project: *Active outbreak investigation, isolation and molecular characterization of Infectious Bronchitis Virus in selected areas of Mekele and Bishoftu, Ethiopia*

Date of application:	**December, 2023**
Nature of the project:	**Field investigation**
Target animal species:	**Chicken**
Number of animals involved:	**160**
Study area:	**Mekele and Bishoftu- Ethiopia**

Minutes No. and date of review: **VM/ERC/03/16/024, 16/05/2024**

The Institutional Animal Care and Use Committee of the College of Veterinary Medicine and Agriculture of the Addis Ababa University has reviewed the above research project and unanimously approved the application of **Nigusu Berhanu Kassaye.**

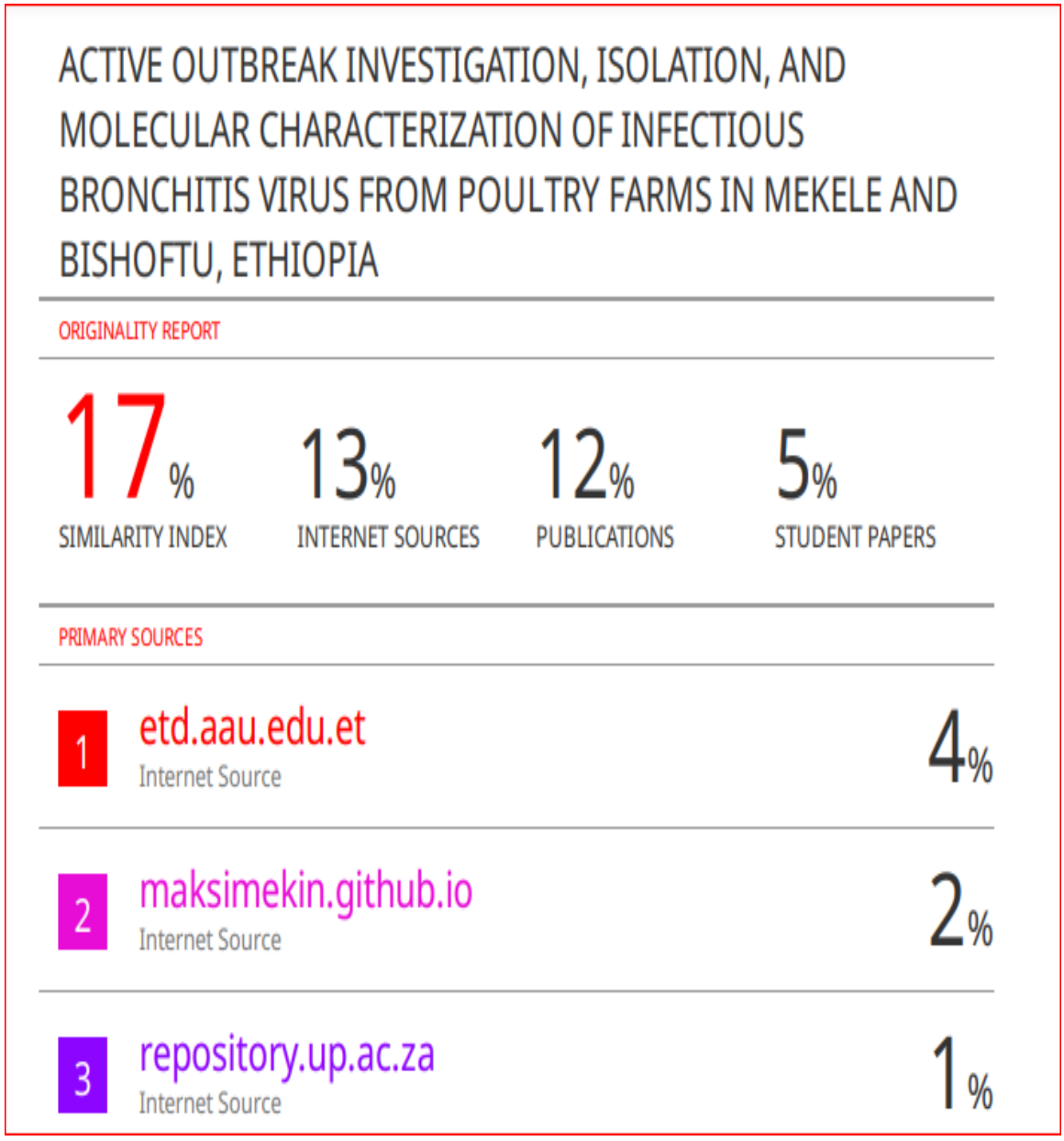
Professor Getachew Terefe (DVM, PhD)
Chairman

Signature

መልሱን በሚጽፉልን ጊዜ ላካከሾን የኛን ዶሴቁጥር ይግለፁልን
Please quote Our Ref. No. When replying

ፋክስ) ስልክ } ፖ.ሣ.ቁ ቢሾፍቱ፣ ኢትዮጵያ
Fax 251-11-4339933 Tel. +251 114338450 P.o.x. Box}34 Bishoftu, Ethiopia

Appendix 8: Plagiarism Report



ACTIVE OUTBREAK INVESTIGATION, ISOLATION, AND MOLECULAR CHARACTERIZATION OF INFECTIOUS BRONCHITIS VIRUS FROM POULTRY FARMS IN MEKELE AND BISHOFTU, ETHIOPIA

ORIGINALITY REPORT

17%	13%	12%	5%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	etd.aau.edu.et Internet Source	4%
2	maksimekin.github.io Internet Source	2%
3	repository.up.ac.za Internet Source	1%