Thesis Ref. No: _____

ISOLATION AND MOLECULAR DETECTION OF INFECTIOUS BRONCHITIS AND INFECTIOUS LARYNGOTRACHEITIS VIRUSES IN CHICKENS IN MAYA CITY AND SEROPREVALENCE IN SELECTED AREAS OF OROMIA, ETHIOPIA

MSC THESIS

BY

ALMAZ DAWIT RAGASA

ADVISORS:

HIKA WAKTOLE (PhD, Associate Professor)

TAKELE BEYENE (PhD, Associate Professor)

TAKELE TESGERA (PhD)

JUNE, 2026

BISHOFTU, ETHIOPIA

ISOLATION AND MOLECULAR DETECTION OF INFECTIOUS BRONCHITIS AND INFECTIOUS LARYNGOTRACHEITIS VIRUSES IN CHICKENS IN MAYA CITY AND SEROPREVALENCE IN SELECTED AREAS OF OROMIA, ETHIOPIA

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

BY

ALMAZ DAWIT RGASA

JUNE, 2026

BISHOFTU ETHIOPIA

ISOLATION AND MOLECULAR DETECTION OF INFECTIOUS BRONCHITIS AND INFECTIOUS LARYNGOTRACHEITIS VIRUSES IN CHICKENS IN MAYA CITY AND SEROPREVALENCE IN SELECTED AREAS OF OROMIA, ETHIOPIA

Submitted by: Almaz Dawit Ragasa _____ _____

Name of Student Signature Date

Approved for submittal to thesis assessment committee:

Name	Signature	Date
1. Hika Waktole (PhD, Associate Professor) Major Advisor	_____	_____
2. Takele Beyene (PhD, Associate Professor) Co-Advisor	_____	June 11, 2026
3. Takele Tasgera (PhD) Co-Advisor	_____	_____
4. Takele Beyene (PhD) Department Chairperson	_____	June 11, 2026

Addis Ababa University

College of Veterinary Medicine and Agriculture

Department of Biomedical Science.

This is to certify that the thesis prepared by Almaz Dawit Ragasa entitled "**Isolation and Molecular Detection of Infectious Bronchitis and Infectious Laryngotracheitis Viruses in Chickens in Maya City, and Seroprevalence in Selected Areas of Oromia, Ethiopia",** and submitted in partial fulfillment of the requirements for the Master of Science degree in Veterinary Biotechnology, complies with the regulations of the university and meets the accepted standards of originality and quality.

Approved by Board of Examiners

Name	Signature	Date
1. Dr. Mekonen Sorsa	_____	_____
External Examiner		
2. Dr. Samson Leta	_____	_____
Internal Examiner		
3.Dr. Lishan Asefa	_____	_____
Chairperson		
4. Dr. Takele Beyene	_____	_____
Department Chairman		

Final approval and acceptance of the thesis dissertation is contingent upon the submission of its final corrected copy to the candidate's major department

TABLE OF CONTENTS

Contents

STATEMENT OF AUTHOR

I hereby declare that this thesis is my own original work and that all sources used have been properly acknowledged. It has been submitted as partial fulfillment of the requirements for the award of an MSc degree at Addis Ababa University, College of Veterinary Medicine and Agriculture. This thesis has been deposited in the University/College library and may be accessed by borrowers in accordance with the Library's regulations. I solemnly affirm that this thesis has not been submitted to any other institution for the purpose of obtaining any academic degree, diploma, or certificate. Short quotations from this thesis may be used without special permission, provided that the source is properly acknowledged. Requests for longer quotations or for reproduction of this manuscript, in whole or in part, may be approved by the head of the major department or the Dean of the College if they consider the intended use to be in the interest of scholarship. In all other cases, permission must be obtained from the author.

Name:Almaz Dawit

Signature _____

Date of submission: _____

College of Veterinary Medicine and Agriculture, Bishoftu, Ethiopia

ACKNOWLEDGEMENTS

First and foremost, I would like to thank Almighty God, the Creator of all, for His blessings in every aspect of my life, including my academic journey. Next, I would like to express my sincere appreciation to my advisors, Dr.Hika Waktole, Dr.Takele Beyene, and Dr.Takele Tesgera, for their invaluable guidance, intellectual support, constructive comments, and continuous encouragement throughout this research work.

Furthermore, I wish to convey my gratitude to the National Veterinary Institute (NVI), particularly the Research & Development Directorate, for providing the necessary inputs and a suitable environment for carrying out this study successfully. I am highly thankful to Hawa Mohammed, Getu Ayele, Kedir Sherefa, Abnet Legesse, and Dr. Nigusu Berhanu for their contribution to the serology, virology, and molecular biology components of this research.

I also gratefully acknowledge Addis Ababa University, through a thematic research grant led by Dr.Hika Waktole, for its financial support and for admitting me to its graduate study program.Lastly, I am deeply grateful to my family for their tremendous support in all aspects of life—moral, emotional, and financial. I also extend my heartfelt thanks to Haramaya University for the various forms of assistance it has provided and to all my friends for being a source of motivation, encouragement, and inspiration.

LIST OF ABBREVIATIONS/ACRONYMS

bp	base pair
CEF	Chicken Embryo Fibroblasts
CPE	Cytopathic Effect
DNA	Deoxyribonucleic Acid
IBV	Infectious Bronchitis Virus
ICP	Infected Cell Protein
ILTV	Infectious Laryngotracheitis Virus
NVI	National Veterinary Institute.
PBS	Phosphate-Buffered Saline.
PCR	Polymerase chain reaction
RNA	Ribonucleic Acid
RT-PCR	Reverse transcriptase polymerase chain reaction
UTR	Untranslated Region
VTM	Virus Transport Media
WOAH	World Organization for Animal Health

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDIXES

ABSTRACT

The poultry industry in Ethiopia is highly vulnerable to significant economic risks posed by highly pathogenic respiratory viruses such as Infectious Bronchitis (IBV) and Infectious Laryngotracheitis (ILTV) due to their high morbidity and mortality. This cross-sectional study, conducted from November 2025 to May 2026, aimed to isolate and molecularly detect the IBV and ILTV in poultry farms in Maya City and to determine the seroprevalence of these viruses in selected towns of Oromia, Ethiopia. A total of 384 serum samples were randomly collected from selected chickens and analyzed using indirect ELISA to determine seroprevalence. Additionally, 12 pooled clinical oropharyngeal swab samples, representing 60 chickens from Maya City, were tested using one-step RT-PCR targeting the 3' untranslated region for IBV and conventional PCR targeting the ICP4 gene of ILTV. Sam,,,,,mples positive by molecular methods were further subjected to virus isolation using specific pathogen-free embryonated chicken eggs for IBV and primary chicken embryo fibroblasts (CEF) cell culture for ILTV. The overall seroprevalence of IBV was extremely high (99.7%), while ILTV seroprevalence was 17.2%, with the highest proportion observed in Maya City (53.8%). Backyard production systems showed higher ILTV prevalence (34.4%) compared to commercial systems (13.8%).Biosecurity measure were significantly associated with reduced odds of viral positivity. Specifically, the use of farm specific clothing (OR = 0.191; $p < 0.001$) and dedicated footwear (OR = 0.089; $p < 0.001$) acted as strong protective factors against infection.Molecular detection revealed IBV and ILTV rates of 41.7% and 33.3% , respectively. Characteristic embryonic lesions (stunting, curling, and feather dystrophy) were observed following IBV inoculation in embryonated eggs; however, subsequent RT-PCR failed to confirm viral RNA in later passages. ILTV isolation in CEF cell culture produced normal cytopathic effect (CPE) with the main characteristic being cell rounding and syncytium formation.In conclusion, this study demonstrates widespread exposure to IBV and the presence of both IBV and ILT in the study area. The findings emphasize the need for improved surveillance, strengthened biosecurity practices, and further molecular characterization of circulating strains to support effective disease control strategies in Ethiopia's poultry sector.

Key words: *chickens; IBV; ILTV; isolation; molecular detection; seroprevalence*

1. INTRODUCTION

1.1 **Background**

The poultry sector has expanded rapidly and plays a key role in global food security. It contributes over half of the world's animal protein, making it the largest agricultural activity worldwide. In Ethiopia, poultry is the second largest sector after cattle and employs more than 56 million people (Balcha *et al*., 2023; Berhanu *et al*., 2025). As grazing land per person continues to decline, poultry production is viewed as an efficient means of supplying high-quality animal protein to low-income households and smallholders. Due to rapid population growth and changing living conditions, demand for chicken meat and eggs in Ethiopia is likely to rise (Shapiro *et al*., 2015).

The Ethiopian government has come up with plans to improve the poultry industry by introducing exotic breeds and advanced technologies as a way to deal with the problems (Shapiro *et al.,* 2015). The poultry industry in Ethiopia is growing rapidly, but diseases are causing a lot of mortality and morbidity during production, which is a big problem *(*Tesfaye *et al*., 2019). Infectious Bronchitis Virus (IBV) and Infectious Laryngotracheitis Virus (ILTV) significantly affect the production of poultry as there is a decline in the production of eggs along with their quality, while growth rates are also low, resulting in economic losses (Baumberger *et al*., 2025)). These cause direct losses and interact with microorganisms that cause respiratory disease, such as Mycoplasma gallisepticum (Mg) and Mycoplasma synoviae (Ms) (Hutton *et al*., 2017).

Infectious bronchitis in chickens is a viral disease characterized by high morbidity and transmissibility in chickens, which has been included as a notifiable disease by the World Organization for Animal Health (Borić *et al*., 2024). It was first reported in 1931 in North Dakota, United States (Rafique, 2018). This disease is a respiratory one that is transmitted via air and by horizontal transmission, whereas indirect transmission occurs through feed, contaminated water, and fomites. It also transmits through embryonated eggs (Pereira *et al.*, 2016).

The IB virus can affect chickens of any age. It can attack epithelial cells in the upper and lower respiratory tracts and cause signs like coughing, gasping, respiratory rales, sneezing, and watery nasal discharge (Awad *et al.*, 2014).

The technology used for the isolation of the virus uses a chicken embryo egg, and this is the main technology used for diagnosing IBV infections (Shiferaw *et al*., 2022). Antibody-based methods are used to check how strong the immune response is or to identify how IBV has spread in the past. Hemagglutination inhibition (HI), viral neutralization (VN), and enzyme–linked immunosorbent assay (ELISA) are some of the serological tests that are available (Legnardi *et al*., 2020). Conversely, ELISA is regarded as an immunoassay test, which causes a fast response and high antibody titer (Shiferaw *et al*., 2022). Reverse transcriptase polymerase chain reaction (RT-PCR) and real-time PCR are two rapid diagnostic tests(Berhanu *et al*., 2025).

To prevent and control the spread of the infectious bronchitis virus, better farm management and making a vaccine from the area where the virus is spreading are the best ways. It is not recommended to administer an attenuated live vaccine for an IBV strain that has not yet been identified in that region, as the virus can rapidly evolve and recombine with other strains, potentially facilitating the emergence of a new pathogenic strain. The best choice is vaccination that can provide cross-protection against several infectious bronchitis virus genotypes (Ike *et al*., 2021).

Infectious Laryngotracheitis was initially identified in 1925 and was caused by a pneumotropic virus belonging to the genus Iltrovirus, subfamily Alphaherpesvirinae, family Herpesviridae (Tesfaye *et al*., 2019). Clinical symptoms of ILT infection typically manifest six to twelve days post natural infection by the virus. Signs of severe illness can include hemorrhagic conjunctivitis, discharge from the eyes, nasal discharge, and breathing problems like rales, gasping, and the expulsion of blood stained mucus. The disease is characterized by gross lesions primarily localized in the upper respiratory tract and sinuses, accompanied by severe morbidity, considerable mortality, and reduced egg production (Mo & Mo, 2025; Gowthaman *et al*., 2020).

For ILT diagnosis, several serological tests have been used, including fluorescent antibody technique (FTA), indirect immunofluorescence (IIF), enzyme- linked immunosorbent assay (ELISA), serum neutralization (SN), and agar gel immunodiffusion (AGID), but they typically exhibit lower sensitivity. Using conventional polymerase chain reaction (PCR) or real-time PCR to find DNA has become a common way to find viruses. Real-time PCR is widely used because it's faster, more sensitive, more reproducible, and less likely to cause carryover contamination (Zhao *et al*., 2013).

1.2. Problem Statements

Despite the growing significance of the poultry industry and demand for poultry products in Ethiopia, there is great potential risk to suffer large economic loss from respiratory viral disease. Among this infectious bronchitis virus and infectious laryngotracheitis virus, which pose major threat to the poultry production system (Tesfaye et al., 2019).). Infections caused by these two viruses result in high mortality rate, low egg production, and economic burden among the farmers. The problem with controlling and preventing the spread of these two diseases is the lack of knowledge of the epidemiology of these viruses, including accurate molecular identification and regional seroprevalence of IBV and ILTV. Therefore, the current study intended to determine the seroprevalence of IBV and ILTV among commercial chickens in Maya City, Sebeta, Waliso, and Eteya, and isolate and identify them molecularly in Maya City, Oromia, Ethiopia.

1.3. Research Questions

- What is the Seroprevalence rate for IBV and ILTV among chickens in the selected study areas?
- Are IBV and ILTV present in commercial poultry farms within the selected study areas using molecular detection method?
- Are IBV and ILTV successfully isolated from commercial poultry farms within the selected study areas?

1.4. Significance of the study

This study gives updated isolation and molecular data on ILTV and IBV circulating in Maya City and contributes to knowledge on the seroprevalence of IBV and ILTV in Maya City, Sebeta, Eteya, and Waliso towns, Oromia, Ethiopia, where recent surveillance data are limited. Seroprevalence is important to determine the exposure of poultry to previous viruses, while molecular and virus isolation to identify current circulating viruses. The studies are used to support improved poultry health management by providing strong disease surveillance and guiding effective vaccination strategies.

1.5. Objectives

1.5.1 General objective.

Isolation and molecular detection of Infectious Bronchitis Virus (IBV) and Infectious Laryngotracheitis Virus (ILTV) in selected poultry farms in Maya City, as well as to determine the seroprevalence of both viruses in selected poultry farms in Maya City, Sebeta, Waliso, and Eteya towns.

1.5.2 Specific objectives.

- To determine the seroprevalence of IBV and ILTV in the poultry farms of Maya City, Sebeta, Waliso and Eteya.
- To detect IBV and ILTV from commercial chickens in Maya city using molecular techniques.
- To isolate IBV and ILTV by embryo and primary cell culture, respectively.

2. LITERATURE REVIEW

2.1. Etiology of IB and Molecular Biology of Infectious Bronchitis Virus (IBV)

Avian infectious bronchitis is a significant acute and transmissible viral disease in chickens, recognized as a notifiable disease by the World Organization for Animal Health (Borić *et al*., 2024). It is caused by a member of the genus Coronavirus and the family Coronaviridae, within the Nidovirales order (Cook *et al*., 2012). Coronaviruses are typically categorized into four groups: alpha, beta, gamma, and delta (Zhao *et al*., 2014). Alpha and beta coronaviruses are restricted to mammals, illustrating the host specificity of coronaviruses. Delta coronaviruses can infect wild birds and some animals, but gamma coronaviruses only infect birds (Aziz, 2020). Important avian coronaviruses include turkey coronavirus and infectious bronchitis, which are major causes of disease in poultry (Abdel-Moneim, 2017).

This virus measures approximately 120nm in size, is spherical to pleomorphic in shape, and has one spike on its surface. This virus consists of a single-stranded genome that has positive-sense RNA that is approximately 27,000 nucleotides/27.6kb long. The typical organization of the genome structure of the AIBV is shown by the sequence 5'UTR-1a-1b-S-3a-3b-E-M-5a-5b-N-3′UTR. Four proteins occur on the 3'end of the structural protein coding region: S, E, M, and N. Two genes, ORF3 and ORF5, have two non-structural supporting proteins named 3a, 3b, 5a, and 5b (Abdel-Moneim, 2017; Bhuiyan *et al.*, 2021).

The IBV genome has two untranslated regions (UTRs) located at the 5' and 3'ends, with a poly (A) tail present at the 3'end. These UTRs are very important for the transcription and replication of viral RNA because they have important structural parts (Valastro *et al*., 2016; Bhuiyan *et al*., 2021). Because these parts are more stable and less likely to mutate and recombine, the conserved 3'UTR region of the IBV genome was used to identify and classify AIBV strains (Hewson *et al*., 2009).

On the 5’ end of the virus, there are 2 non-structural proteins, 1a and 1ab, accounting for almost three-quarters of the genome and measuring 20 kb in size. There are fifteen proteins involved in the function of non-structural proteins. These are required in processes such as RNA synthesis, transcription, proofreading, RNA caps formation, and replication of the virus. They also weaken the immune system of the host (Bhuiyan *et al*., 2023).

The S1 glycoprotein is the most important part of the immune system and has epitopes that stop antibodies from working (Berhanu *et al*., 2025). The genotype of Infectious bronchitis virus strains was determined using RT-PCR with the analysis of nucleotide sequences of the S1 gene, then DNA sequencing (Abozeid, 2023). Membrane (M) and Envelope (E) are the other surface proteins that help the virus change shape and assemble. The M protein forms complexes with the N protein in addition to E and S. The N protein directly binds genomic RNA to form a helical ribonucleoprotein complex and may also be involved in RNA packaging (Fehr and Perlman, 2015). Figure 1 shows how this genome is organized.

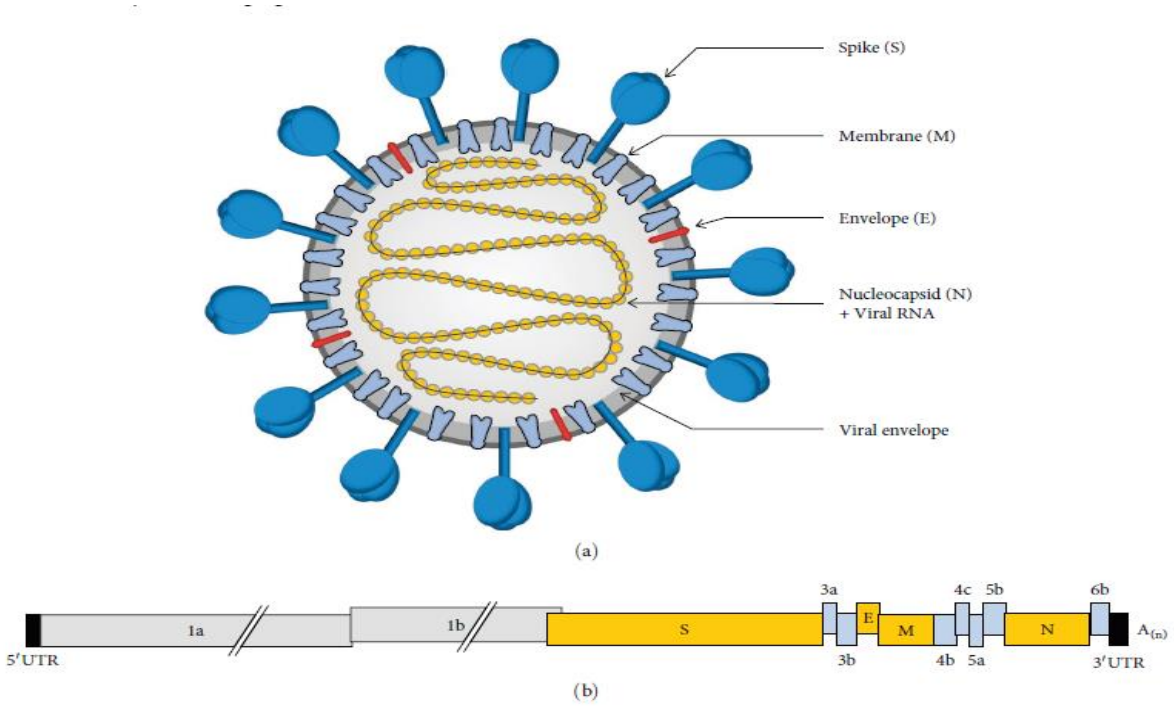


Figure 1: Schematic illustration of the infectious bronchitis virus.

(a) Architecture of the virus and (b) genomic structure of the virus. Gray shading is used to show the replicase gene that consists of open reading frames 1a and 1b, while orange shading is used to show the open reading frames of the structural proteins S, E, M, and N. The open reading frames of the accessory proteins 3a, 3b, 4b, 4c, 5a, 5b, and 6b are shown in blue. Black shading shows the 5′ and 3′ UTRs, while A (n) shows the poly (A) tail.

2.2. Clinical Signs of Infectious Bronchitis

The incubation period ranges from 18 to 36 hours after infection, and the viruses mostly replicate in the upper respiratory tract. The oviduct, kidneys, and caecal tonsils are often infected after a short period of viremia, which is when the virus multiplies and spreads within the host. The clinical presentation can vary depending on the chicken age, nutritional and immunological status, and virus genotype. Infected chickens show a wide range of symptoms, including respiratory symptoms like gasping, rales, snacking, coughing, sneezing, nasal discharge, and watery eyes, as well as reproductive symptoms like loss of egg production, egg shell deformities, and the appearance of false layers. Water and feed consumption may also

decrease. Chickens that haven't been vaccinated may show more signs of clinical illness. Nephropathogenic strains can significantly disrupt kidney function. IBV can cause secondary bacterial infections, which raise death rates (Falchieri *et al*., 2024; Ishag *et al*., 2025).

2.3. Pathogenesis of Infectious Bronchitis Virus

The spike protein of IBV is important for both pathogenicity and the range of host cells, just like other coronaviruses. Other structural and non-structural proteins, such as accessory proteins 3a and 3b, have also been associated with infectious outcomes. Viruses altered to lack these proteins exhibited diminished pathogenicity, with the absence of 3b exerting a more significant influence (Falchieri *et al*., 2024). It has also been shown that some non-structural proteins, such as nsp2, nsp3, and nsp15, lower the host's natural immune response. To stop the adaptive immune response, nsp7 and nsp16 can also stop the antigen from being presented. The other nsp13, nsp14, and nsp16 help make the viral mRNA cap, which makes it harder for the innate immune system to recognize it (Peng *et al*., 2022).

2.4. Transmission Dynamics of Infectious Bronchitis

The IBV has an incubation period (IP) of just 18 to 36 hours. It is very contagious because it can be spread by touching, inhalation, or eating virus particles that are infectious. It can also spread indirectly through aerosol droplets or feces, as well as through contact with objects that have the virus on them, like clothes, shoes, tools, and so on. Aerosol transmission is a major way that the virus spreads because there are a lot of viruses in the respiratory system during the first three to five days after infection. The viral load in the respiratory tract quickly drops below the detection threshold within the second week post-infection (PI). Also, uric acid from the kidneys and feces could spread the infection. Semen that has been infected can also spread the disease. Long-term excretion is probable during the chronic phase (Rana *et al*., 2021; Khan *et al*., 2023).

2.5. Laboratory Diagnostic Methods of Infectious Bronchitis Virus

2.5.1. Serological techniques

Antibody-based methods are used to check the immune response that was made or to find out about past IBV circulation. Hemagglutination inhibition (HI), viral neutralization (VN), enzyme- linked immunosorbent assay (ELISA), and agar gel precipitation (AGP) are some of the serological tests that can be used (Legnardi *et al*., 2020). The ELISA method is a sensitive serological test that gives results faster and with higher antibody titers than other tests. It is frequently employed to distinguish flocks based on elevated antibody titers. Numerous ELISA methodologies for the identification of IBV antibodies have been established and are extensively utilized. ELISA is a widely used method for IBV serological profiling because it is easy to use, fast, sensitive, and can be used to test a lot of samples at once (Shiferaw *et al*., 2022). Serological tests are less effective and yield inconclusive results in identifying novel or emerging IBV isolates because of the proliferation of various IBV serotypes and their lack of cross-reactivity (Bande *et al*., 2016).

2.5.2. Viral isolation

Since IBV levels are highest one week after infection, possibly even before the symptoms appear, samples should be taken as soon as IB-compatible signs are first seen while trying to isolate field viruses. The kidneys or the oviduct are good places to take samples, especially if there are lesions. However, tracheal tissues or swabs are the best places to take samples, especially during the acute period. Cloacal swabs and caecal tonsils can also be utilized for isolation; however, the virus recovery rate seems to be diminished (Jackwood & de Wit, 2013).To check for IBV at the flock or farm level, one should collect a pooled sample from both sick and healthy chickens. To keep the virus alive, samples should be quickly sent to the lab and kept on ice in a safe place.

Tracheal organ cultures (TOCs) or the allantoic cavity of SPF chicken embryonated eggs have been considered more efficient methods compared to cell culture for viral infection. IBV causes urate stones in the mesonephros, resulting in growth retardation, curled embryos, and

embryonal deaths in embryonated eggs, while ciliostasis is induced by the virus in TOCs. Lesions in embryonated eggs usually appear on the third passage, while ciliostasis is evident in the TOC after the first passage.

2.5.3. Molecular technique

The nucleic acid of the study virus is RNA. Due to the relatively more stable nature of DNA, it becomes imperative to use reverse transcription techniques to convert the RNA of the virus to a single strand of complementary DNA (cDNA) (Sundgren, 2020). One technique used successfully for detecting viral infections in field specimens and amplification of allantoic fluids is RT-PCR. All strains of IBV can be tested using target primers for the 3'UTR and S glycoprotein (Sun and Liu, 2015; WOAH, 2018).

2.6. Limitations of Serotyping and Genomic Accuracy in IBV Evolution

Typically, nucleic acid-based or antibody-based methods can be used to classify IBV, and these methods correspond to the results for genotyping and serotyping. This antibody interferes with the generation of the signal, preventing the enzyme-linked immunosorbent assay (ELISA) from differentiating between various serotypes, particularly when the coated antigen consists of whole virions (Lin & Chen, 2017). Mutations in specific epitopes or partial cross-reactivity often complicate serological characterization. More extensive molecular epidemiology research should be conducted to enhance understanding (Tegegne *et al*., 2020). Bio-molecular assay is beneficial for genetically identifying the circulating IBV strains to select a suitable vaccine and mitigate the transmission of IBV outbreaks within a specific geographic region. Additionally, genome sequencing can significantly enhance our understanding of IBV variability and provide essential insights into virus evolution (Marandino *et al*., 2023).

2.7. Infectious Bronchitis Virus status

2.7.1. Global

Major infectious bronchitis virus serotypes, such as Mass-type, 4/91 (793B or CR88)-like, D274-like (D207, D212 or D1466, D3896), and D3128, QX-like, and Italy 02, are found all over the world. Some serotypes, like the QX-like IB virus, the Mass strain from the USA, the 4/91 (CR88) strain from the UK, and the H120 strain from the Netherlands, are variants that have an effect in certain areas but have the potential to spread to other countries (De Wit *et al*., 2011a; Jackwood *et al*., 2012).

2.7.2. Infectious bronchitis virus status in Ethiopia

The first case of IBV was detected in 2017 from a flock located in Bishoftu, Ethiopia (Hutton *et al*., 2017). The first IBV serotype to be identified was 793B. However, Tesfaye et al. (2019) later found other IBV serotypes such as M41, D-274, and Qx; re-emergence of serotype 793B was also recorded, and another study was performed in Ethiopia (Table 1).

Table 1: An overview of the serological and molecular study status of IBV in Ethiopia

Year of study	Study area	Method	prevalence	Strain	Reference
2016	Debrezeit	Serological and Molecular	94.5%	793/B	(Hutton et *al*., 2017)
2017-2018	Oromia region and SNNPRS	Serological	-68.75% in commercial and 74.88% in backyard	M41, D274, 793B, and Qx from the backyard	(Tesfaye *et al.*, 2019)
2019 – 2020	Central Ethiopia (Shewa)	Serological	chicken level-90.5% holding Level:94%	Not reported	(Habte *et al*., 2022)
2020	Jimma Zone, Southwest	Serological and molecular	6%	793/B (GI-13)	(Tegegne *et al*.,2020)
2020-2021	Northwest Ethiopia	Serological	23.96%	Not reported	(Birhan *et al*., 2021)
2021	Bishoftu and mojo	Molecularly	-	4/91 like strain (GI-13).	(Wayou *et al*., 2021).
2021	Bishoftu and Holeta town	Serological	97.46%	Not reported	(Shifera *et al.*, 2022)
2021-2022	Bishoftu	Molecularly	80%	Not reported	(Demeke *et al.*, 2023)
2023	Central Gondar, Northern Ethiopia	Serological and Molecular	92.19% 11.54%	Not Reported	(Mihret *et al*., 2023)
2021-2022	East Shewa, Central Ethiopia	Serological and Molecular	97.96% 33.3%	Mass and 793/B	(Hirbaye *et al.*, 2024)
2023-2024	Mekele and Bishoftu	Molecularly	12.2%	Massachusets	(Berhan *et al.*, 2025)

2.8. Etiology of Infectious Laryngotracheitis and Molecular Biology of Infectious Laryngotracheitis Virus

The infectious Laryngotracheitis virus, or Gallid herpesvirus 1 (GaHV-1), is what causes ILT. It is a member of the Iltovirus genus and the Alphaherpesvirinae subfamily of the Herpesviridae family. The ILT genome consists of a 150-155 kb linear double-stranded DNA that encodes a unique long (UL), Unique short (US), and two inverted repeat (IR) sequences (Figure 2). The fully assembled complete genome sequence of ILT consists of 148 kb nucleotides, exhibiting a G +C content of 48.2 % (Gowthaman *et al*., 2020).

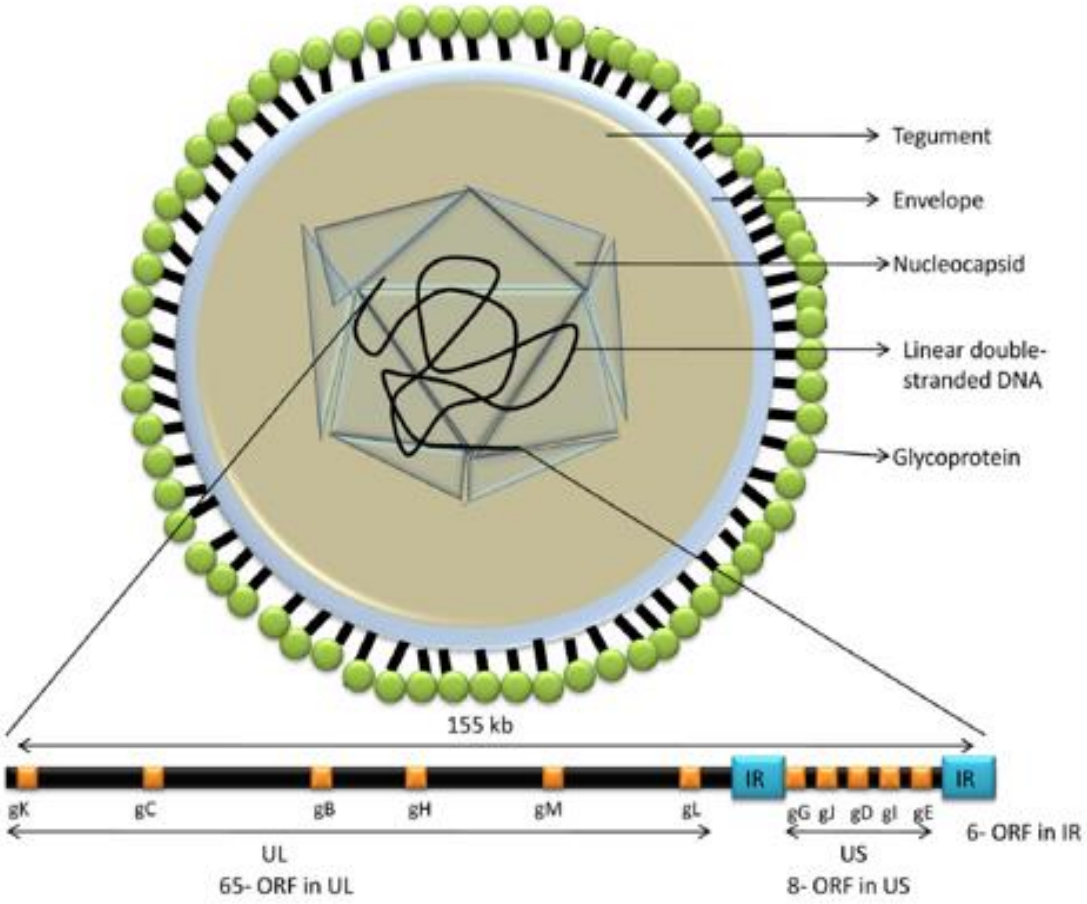


Figure 2: Structure of ILT virus (Gowthaman *et al*., 2020).

The ILTV virus particle interacts with cell surface receptors via the glycoprotein spikes present on the outer surface. Once bound, the viral envelope is fused with the host cell membrane; this results in the presence of the nucleocapsid inside the cell cytoplasm. After that, the nucleocapsid moves to the host cell nucleus; this marks the start of the replication process. Among approximately 70 proteins produced in the nucleus, most are viral structural proteins, while others are non-structural proteins that include enzymatic proteins required for the replication of viral DNA. After DNA replication, the genome is packaged to form nucleocapsids. Envelope formation takes place by budding off the nuclear membrane; mature virions are found in cytoplasmic vacuoles(Jonare, 2020).

2.9. Clinical Signs of Infectious Laryngotracheitis

Clinical symptoms of ILTV infection usually manifest six to twelve days following natural exposure to the virus. The symptoms of the disease manifest themselves in the form of hemorrhagic conjunctivitis, tearing, nasal discharges, and respiratory manifestations like rales, dyspnea, and expectoration of blood-streaked mucous discharge. Lesions characteristic of the disease include gross lesions in the upper respiratory tract and the sinuses, with morbidity, mortality, and reduction in egg production being high (Mo & Mo, 2025; Gowthaman et al., 2020), where mortality can be as high as 70% (Brown et al., 2020). Susceptibility includes all age groups in chickens, although prevalence is common between 4 and 18 months old. In peracute laryngotracheitis, mortality exceeds 50%, while in acute infectious laryngotracheitis, mortality rates are 10%-15%, even though the morbidity rates are comparable to peracute infectious laryngotracheitis. Chronic and mild laryngotracheitis have morbidity and mortality rates of 2%-5%. Egg production is invariably affected by the disease (Samour, 2015).

2.10. Virulence of Infectious Laryngotracheitis Virus

Virulence is the ability of a virus to cause disease in the host (Jonare, 2020). Herpesviruses have involved numerous immune evasion strategies and employ self-replicating mechanisms to survive inside their host. One way to do this is to get functional copies of host genes that code for cytokines like IL-6 (HHV-8), IL-10 (HHV-4, HHV-5), and IL-17 (SaHV-2). These

viral mimics, also called virokines, can either help the virus reproduce by making target cells grow faster or change the way cytokines send signals to cells to help the virus (Volkening *et al*., 2025). Most alpha herpesviruses, such as ILTV, have glycoprotein G (gG), which is a broad-range viral chemokine-binding protein (Coppo *et al*., 2018).

2.11. Transmission Dynamics of Infectious Laryngotracheitis

Chickens get infected with ILTV through the upper respiratory tract and eyes (Yegoraw *et al*., 2021). Another possible route of infection is ingestion, but this requires exposure of the nasal epithelium. Some of the causes of ILTV are contaminated dust, litter, beetles, drinking water, fomites, clinically sick chickens, and latently infected carriers (Brown *et al*., 2020). Upon infecting the upper respiratory tract of susceptible chickens, ILTV results in severe viral replication (Mo and Mo, 2025). The virus is frequently shed from the tracheal tissue and secretion for six to eight days post-infection. Following infection, the virus may be present at a very low level for as long as ten days (Yegoraw *et al*., 2021). During stress periods, for example, at the beginning of laying or rehoming, ILTV is reactivated and transmitted to susceptible hens (Hein *et al*., 2021).

2.12. Diagnostic Methods of Infectious Laryngotracheitis Virus

The laboratory diagnosis is essential for ILT, as similar clinical signs and lesions may be produced by other diseases such as infectious bronchitis, Newcastle disease, avian influenza, infectious coryza and mycoplasmosis. Various methods, including virus isolation and DNA detection, can be used to confirm ILT infection. A commercial ELISA for detection of antibodies against ILT is available, although ILT is isolated by primary cell culture and CAM injection of 9 to 12-day-old embryos. The CAM was inoculated with swabs or organ samples from the larynx, lung, conjunctiva, and trachea of chickens with clinical signs. The CK and CEL cell cultures were suitable for the isolation of ILT (Ou & Giambrone, 2012). Several molecular techniques have been described for the detection of ILTV DNA in clinical samples, but PCR has been the most useful. ILTV DNA was detected by nested PCR in formalin-fixed, paraffin- embedded tissues with or without syncytial cells and/or intranuclear inclusions. PCR

is more sensitive than virus isolation for clinical samples, particularly in the presence of other contaminating viruses such as adenoviruses (WOAH, 2021).

PCR assays involving the ICP4 locus are designed based on conserved regions within the subfamily of Alphaherpesvirinae (Creelan *et al*., 2006). The protein encoded by the gene acts as an initiator protein for the transcription of early and late genes (Mossad *et al*., 2022). Moreover, considering that the ICP protein is expressed before the replication of the viral genome in the host cells, its target will lead to reliable outcomes (Santander-Parra *et al*., 2022). Besides, since the differentiation between wild and vaccinated virus using the ICP4 locus is widely applied, it provides diagnostic advantages as well (Mossad *et al*., 2022).

2.13. Limitations of Serotyping and Genomic Accuracy in Infectious Laryngotracheitis Virus Evolution

Genomic pathogen detection and tracking methods identify disease and track its distribution by analyzing the genetic material of the pathogen. This strategy utilizes DNA sequencing techniques for analyzing multiple samples from pathogens and gaining information about their genomes quickly. Analysis of the genetic makeup of different strains allows one to detect the origin of the pathogen outbreak and track its sequence changes in time (Vashisht et al., 2023).

The establishment of control methods requires genomic surveillance of the circulating ILTV strain in a given geographic area (Choi *et al*., 2016). GaHV-1 strains are antigenically homogeneous, and differentiation of strains has been possible by analysis of differences in genetic make-up between vaccine strains and isolates associated with outbreaks. In countries where ILT outbreaks have occurred, a variety of genes and genomic areas have been used in the development of DNA-based diagnostic assays to distinguish vaccination strain from isolates associated with the outbreak. More recently, the whole genome sequences have permitted the identification of the origin of some of the isolates associated with outbreaks that are circulating in some chicken-producing countries (Menendez *et al*., 2015).

2.14. Status of Infectious Laryngotracheitis Virus in Ethiopia

The first case of ILT was recorded in the central and southern parts of Ethiopia following serological screening done in 2019. Prevalence rate was found to be significantly high in the case of backyard poultry production (34.4%) compared to commercial poultry production (13.3%) (Tesfaye et al., 2019). Another study found a high prevalence rate of 54.7% in the Ada'a District of Bishoftu city of Ethiopia (Roba, 2020). Nevertheless, molecular identification of the disease is rarely reported (Table 2).

Table 2: An overview of Ethiopian studies on infectious laryngotracheitis

Year of study	Study area	Production system	Type of laboratory test	Reported prevalence	References
2017-2018	Central and south Ethiopia	Commercial and backyard	Indirect ELISA	19.4% overall; 13.3% commercial & 34.4% backyard	(Tesfaye *et al.*, 2019)
2019	Ada'a district, Oromia	Backyard	Indirect ELISA	54.7% overall	(Roba, 2020)
2020–2021	Central Gondar, West Gojjam, and South Gondar	Mixed systems	Indirect ELISA	59.1% overall	(Birhan *et al*., 2022)
2022	Nekemte,	Small-scale production	Indirect ELISA	15.36% overall	(Balcha *et al*., 2023)
2021–2022	Robe Town, Southeastern Ethiopia	Commercial and Backyard	ELISA, Egg isolation & PCR	26.7% overall	(Abebe *et al*., 2024)
2021	East, West and North Shewa	Scavenging and intensive	Indirect ELISA	26.9 % chicken-level prevalence	(Habte *et al*., 2022)

3. MATERIALS AND METHODS

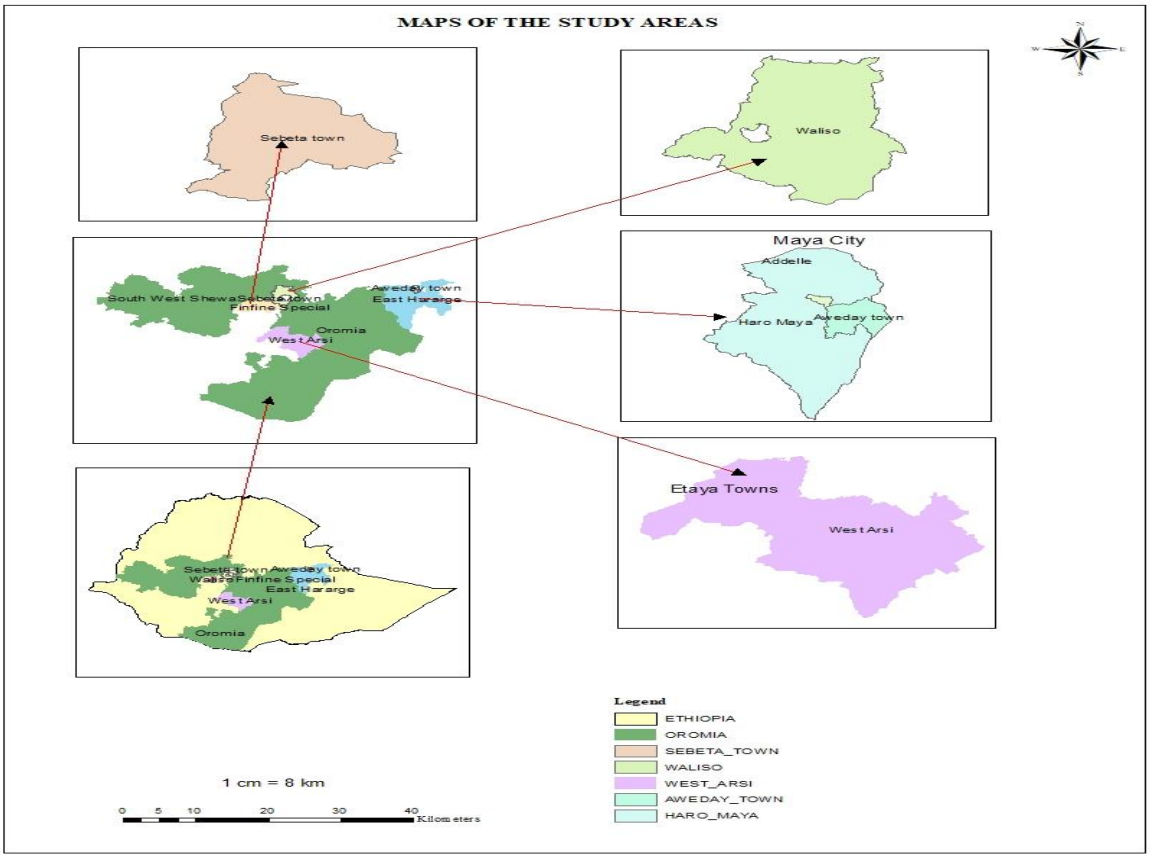
3.1. Description of Study Areas

The study was performed at four locations in Oromia Regional State, Ethiopia, namely Maya city, Waliso, Sebeta, which is commonly known as Sabata, and Eteya towns (Figure 1). Maya City is located about 510 km east of Addis Ababa in eastern Hararghe, on the main road to Harar. This location resulted from the consolidation of the sub-cities of Haramaya, Aweday, and Addele. The elevation of the study site ranges between 1,600 and 2,100 meters above sea level. A bimodal pattern of rainfall has been noticed in this area, where short rains occur between February and May, whereas long rains occur between June and September, with dry seasons between October and February. Annual rainfall varies between 118 and 866 mm with an average of 492 mm, whereas maximum and minimum temperatures are 17 and 9°C, respectively (Dahesa et al., 2025).

Sebeta town is situated 25 kilometers southwest of Addis Ababa. It is located in the tepid-humid mid-highland agro-ecological zone at an elevation of 2195 to 2300 m above sea level. The average annual rainfall is 955 mm, with July being the wettest month and November being the driest. The average temperature in Sebeta ranges from a minimum of 10°C to a maximum of 22°C. Farmers engage in mixed farming, which includes both crop cultivation and animal husbandry(Gemeda, 2025)

The Waliso district lies within the South West Shewa, 114 km away from Addis Ababa, and lies between altitudes of 1500 and 2900 m above sea level. The district's coordinates are 8°32′23.0″N and 37°58′16.3″E. It has a bimodal rainfall pattern with the first rains falling from March to April and the second rains falling from June to September. The average temperatures vary between 13.6°C and 25°C and have a mean temperature of 19.3°C. The total number of cattle, sheep, goats, horses, mules, donkeys and poultry of the district is 224,334, 39,543, 51,042, 7,625, 6,164, 16,320 and 147,679, respectively (Choramo *et al.*, 2024). Eteya town is situated 170 kilometers southeast of Addis Ababa and lies in an area of 937 square kilometers, with its coordinates being 8°08′N 39°14′E. It has an elevation between 1500 and 4170 meters

above sea level. This region experiences a biomodal rainfall pattern with the short rain falling between March and April, while the long rain falls between July and October, with temperature varying between 10 °C and 22.6°C (Yirga *et al*., 2018).



Source: The map was created using ArcMap 10.7.1

Figure 3: Map of the study areas

3.2. Study Population and Sample Size

The study was carried out on backyard chickens and commercial poultry farms that are apparently healthy and manifest signs of respiratory disease (swollen head syndrome (SHS), along with respiratory distress, coughing, sneezing, ocular and nasal discharge, difficulty breathing, and gasping. Although chicken, those decreased egg production, loss of water, and reduced feed intake. Chickens of various age groups (pullets, medium, adults), sex (male, female), breeds (Local, Crossbreeds, and exotic), management (backyard, commercial), and

non-vaccinated flocks were incorporated in the study based on farm owners' availability, willingness, and consent. Backyard flocking refers to a low- or no-input economic venture that uses few health care interventions (Rajkumar et al., 2021), and its bird derived products are dedicated mainly to home self consumption, and the number of birds in such a flock is usually small (5-50 or more chickens) (Chaiban et al., 2020). A purposeful sampling design was intentionally selected against random sampling due to the fact that identifying symptomatically affected flocks would provide greater chances of virus isolation and identification, and inclusion of clinically healthy birds was important for the accurate determination of seroprevalence to know post-exposure.

For molecular detection, 25 pool samples (n=125) were purposively collected from commercial poultry farms with symptomatic chickens, but only 12 pooled samples (n=60) were tested for molecular detection because of resource limitations. The sample size was decided based on the availability of the laboratory consumables to carry out molecular detection. For isolation and molecular detection, samples from five chickens were pooled into one. The sample size for seroprevalence was calculated using the Thrusfield formula, incorporating a 95 % confidence interval and a 5% desired absolute precision, considering the absence of prior research on Infectious Bronchitis Virus (IBV) and Infectious Laryngotracheitis Virus (ILTV) viruses in the study area, and assuming a 50% expected prevalence. Based on the absence of prior research on IB and ILT viruses in the study area, the following sample size was decided. Thus, the sample size was 384 chickens, as shown by the following formula (Thrusfield, 2018).

$$\text{n} = \frac{Z^2 \; x \; Pexp \; (1-Pexp)}{d^2}$$

$$\text{n} = \frac{(1.96)^2 \; x \; 0.5 \; (1-0.5)}{0.05^2}$$

Where, n = sample size, Z^2 = the value of z at a 95% confidence interval (1.96), Pexp = expected prevalence, and d=desired absolute precision (0.05).

3.3. Study Design and Sampling Methods

A cross sectional study design was conducted from November 2025 to May 2026 to molecularly detect, isolate, and determine the seroprevalence of the major poultry respiratory and immunosuppressive viral pathogens, including IBV and ILTV. The study was performed in selected areas of Ethiopia, namely Maya City, Waliso, Eteya, and Sebeta towns. These sites were purposely selected due to the limited number of previous studies conducted in the areas and their epidemiological significance in poultry production. Convenience sampling methods were employed, and flocks were selected primarily depending on accessibility and the willingness of the owner to permit sample collection. Chickens were randomly selected within each flock for blood sample collection, while for swab sampling, chickens showing clinical signs were purposefully chosen. The sampling procedure was standardized as much as possible.

3.4. Sample Collection and Transportation

3.4.1. Swap sample collection and preparation.

To lower the cost of the testing while still meeting the statistical sampling requirements for finding infection, the WOAH and others point out that up to eleven swabs from chickens or turkeys can be pooled without losing the RT PCR assay's sensitivity (Pieterse et al., 2022). Accordingly, samples taken from each individual were pooled together in sets of five based on flock sizes per farm; for farms that had fewer than 500 birds, only one pool was taken, whereas for those that had more than 500 birds, two pools were formed.

Chickens showing symptoms of respiratory distress, such as coughing, sneezing, dyspnea, and gasping (Appendix 1), oculonasal discharge, and a decrease in egg production were selected for sampling. Sterile cotton swabs were inserted into the mouth of each chicken and gently swabbed its oropharynx (Appendix 2). The samples were put directly into the sterile labeled universal bottles containing a 3 ml virus transport media with 10% antibiotic added. They put on ice box and transported them to Haramaya University molecular laboratory, where they

kept them at −80◦C. Then, they were put in an ice box and taken to the virology laboratory at NVI and stored in the same manner.

3.4.2. Blood sample collection and serum preparation

Antibody of maternal was expected to develop within the initial three weeks of life; therefore, chickens less than three weeks old were not included in the study. Chickens, which are apparently healthy, local, cross, and exotic breeds, both sexes, unvaccinated, commercial and backyard production systems are included. 384 blood samples (2–3 mL) were obtained using syringes with 3mL capacity and 22-gauge needles from the wing (Appendix 2).

To separate the serum from the blood clot, the blood sample was left to clot in the syringe overnight at room temperature in a slant position. After that, the sera were transferred into sterile 1.8 ml cryovial tubes and sent to the National Veterinary Institute (NVI) in an ice box. They were kept there at −20°C until they could be tested for anti- ILT and IBV. The data recording sheet had all the right information about each chicken, such as its age, breed, sex, and type of farm (Grankvist *et al*., 2019).

3.5. Laboratory Investigation

At NVI, techniques for Seroprevalence analysis, virus isolation, and molecular detection were carried out.

3.5.1. Serological analysis of IBV

An indirect ELISA (enzyme-linked immunosorbent assay) test method was employed in the serology laboratory, and the antibody response against IBV was determined. This test made use of a commercially available ELISA kit (ID.vet, ID Screen® infectious bronchitis virus antibodies against IBV in chicken serum or plasma; Louis Pasteur-Grabels, France). The entire process of testing was done according to the manufacturer's guidelines Appendix 3, making use of pre-coated 96-well flat-bottom ELISA plates with all necessary reagents provided by the kit. Before performing the test, all the samples and reagents were taken out of the

refrigerator to be allowed to reach room temperature for 30 minutes, and the samples were diluted in the ratio of 1:500 with the sample diluent buffer. Subsequently, 100 µl of the diluted samples was added into the microplate coated with purified IBV recombinant protein, and the plates were covered with sealants and incubated at 21 ^{0}C for 30 minutes. After 30 minutes' incubation, empty the well and wash the well 3 times with wash solution, and an anti-chicken horseradish peroxidase (HRP) conjugate was added to the wells and incubated at room temperature as mentioned above. The wells were emptied and washed 3 times again with wash solution, and 100 µl of a solution of tetramethylbenzidine (TMB) substrate/chromophore (IDEXX, Switzerland) was then added and incubated at 21 ^{0}C for 15 minutes in a dark place, resulting in the development of a blue color (Appendix 4). The reaction was stopped with 0.5 M sulfuric acid (IDEXX, Switzerland).

The reaction on the microtiter plate was quantified by measuring the color intensity photometrically at a wavelength of 450 nm using an ELISA reader. The result of the test was accepted as valid if the positive control's mean optical density value was greater than 0.250 and the ratio of the positive and negative control's mean values was greater than 3. The results were assessed using the ELISA sample to positive(S/P) for each serum. Serum samples were considered positive if their S/P value was greater than 0.2. The following formula was used to determine test positivity or negativity.

$$\frac{\text{S}}{\text{P}}\text{ ratio} = \frac{\text{ODsample}-\text{ODNC}}{ODPC-ODNC}$$

Where S/P stands for sample-to-positive ratio, ODS for sample optical density, ODNC for optical density of negative controls, and ODPC for optical density of positive control.

3.5.2. Serological analysis of Infectious Laryngotracheitis Virus

The detection of the antibodies against ILTV in chicken sera was performed using the indirect ELISA test kit ID Screen® ILT Indirect (310 rue Louis Pasteur, Grabels, France) according to the protocol provided by the manufacturer. The final concentration of sera tested was 1:500 in dilution buffer 14 by the initial dilution to 1:50 and further dilution to 1:10 in the ELISA plate.

For the initial step in the detection process, 245 µl of dilution buffer 14 was dispensed into the pre-dilution plate wells except for the control wells (A1, B1, C1, and D1). Following this step, 5 µl of the serum sample was dispensed into the buffer. In another ELISA microplate, 90 µl of the dilution buffer 14 was distributed in the analysis wells, and subsequently, 10 µl of the pre-diluted sera was added. As part of the control check, 100 µl of the negative control was put into A1 and B1, and 100 µl of the positive control was put into C1 and D1, respectively. After that, the plates were covered and incubated at 21°C for 60 minutes.

Subsequently, the wells were emptied of their content and then washed thrice with 300 µl of 1x wash solution. During washing, the plate was not permitted to dry. Following washing and drying of the wells, 100 µl of conjugate 1x was added to each well. This had been made by diluting 10x conjugate into 1:10 Dilution Buffer solution. The wells were then covered and incubated for one hour at a room temperature of 21°C. Following the drainage and washings of the wells with 1x wash solution thrice, 100µl of substrate solution was added to each well. This process was repeated in a dark incubator at a room temperature of 21°C for 15 minutes.

The addition of 100 µl of stop solution to each well eventually brought an end to the reaction process. The micro-titer ELISA plate was then inserted into the ELISA reader, where the amount of color intensity resulting from the ELISA test was determined photometrically at 450 nm. The values of the OD sample and OD positive control (PC) were established. The positive control optical density (ODPC) showed a significant level of antibody to the ILT antigen in chicken serum.Validity of the test was determined by the fact that the average optical density (OD) reading of the positive control was greater than 0.250 while the ratio of the positive and negative controls was more than 3. The S/P ratio was calculated according to the formula below, as recommended by the manufacturer of the test kit.

$$\frac{\text{S}}{\text{P}}\ \text{ratio} = \frac{\text{ODsample} - \text{ODNC}}{ODPC - ODNC}$$

where “S/P” stands for sample-to-positive ratio, "ODS" for optical density of a given sample, "ODNC" for optical density of the negative controls, and "ODPC" for optical density of the

positive controls. The ILT antibody status was said to be positive if the $\frac{S}{P}$ value was greater than 0.3 and negative if it was less than 0.3.

3.5.3. Molecular detection of IBV from direct field samples.

Because of the 3'UTR region's stability and minimal genomic variability in comparison to other regions of the viral genome, it was selected as the target site for the RT-PCR diagnosis method (Hewson *et al*., 2009).

Infectious Bronchitis Virus (IBV) Extraction: Following the manufacturer's instructions (Appendix 5), the QIAamp Viral RNA Mini Kit (Qiagen, Germany) was used to extract viral RNA from the 12 swab samples.

Conventional polymerase chain reaction for 3'UTR amplification: The Invitrogen Superscript™ III Platinum™ One Step RT PCR Kit (Thermo Fisher, Waltham, MA, USA) was used for RT PCR in accordance with the manufacturer's instructions. To detect IBV from the collected swab sample using one step RT- PCR, a pair of primers, All1F 5′-CAGCGCCAAAACAACAGCG 3' and Del1- R 5′ CATTTCCCTGGCGATAGAC-3', was used to amplify 433 bp of the conserved region of the 3'UTR. This primer identifies the IBV strain that targets the most hypervariable region with a conserved flanking region in the IBV genome (Hewson *et al.*, 2009). The final volume of master mix preparation was 25 µl (Table 3), which included the template. A thermocycler was used for conducting PCR, with polymerase activation at 50 °C for 30 minutes and an initial denaturation step at 95 °C for 15 minutes. Then, 35 cycles of 94 °C for 45 seconds for denaturation, 55 °C for 45 seconds for annealing, and 72 °C for 45 seconds for extension. The last step was an extension at 72°C for 5 min.

Table 3: Preparation of a One-step RT-PCR master mix for 3´UTR amplification

s/n	Type of reagent	For one reaction
1	RNase-free water	4 µl
2	5x RT-PCR Buffer	5 µl
3	5x Q-solution	5 µl
4	Primer-AIBV All1-F-Fow-5pm/µl 5´CAGCGCCAAAACAACAGCG-3´	2 µl
5	Primer AIBV- Del1 Rev 5pm/µl 5´CATTTCCCTGGCGATAGAC-3´	2 µl
6	10Mm dNTPs mix	1 µl
7	One-step RT-PCR Enzyme Mix	1 µl
8	Template	5 µl
7	Total volume	25

Agarose Gel Electrophoresis: 1.5% agarose gel was prepared by dissolving 1.5 agarose in 100 mL of 1x TAE buffer and heating the mixture until it was fully dissolved. The solution was then poured into a gel casting tray containing a comb and left at room temperature until it set. Once the gel had completely solidified, it was placed in an electrophoresis chamber containing 1x TAE buffer solution, and the comb was carefully removed. Next, 25 µL of each PCR product was combined with 5 µL of 1x pre-mixed loading dye and staining dye, and 10 µL of the resulting mixture was loaded into the appropriate wells. Positive and negative controls were included in addition to the sample. Then, 10 µL of molecular ladder with 100 bp was used as a marker for band size at both ends. The apparatus was securely positioned, and 120 V was applied for one hour. Once the power was switched off, the gel was transferred to the UV room, exposed to UV light, and then photographed with the Gel Documentation System (Lee et al., 2012). The expected band size for positive 3'UTR gene results was approximately 433 bp.

3.5.4. Molecular detection of Infectious Laryngotracheitis

DNA extraction was performed using the QIAGEN kit (QIAGEN® DNA mini columns kit, QIAGEN, Germany), following the manufacturer's instructions. 12 pooled Swab samples (n =

60) were used for DNA extraction. Samples were thawed for activation, and 200 µl of lysing buffer was added to each of the 12 processed cryovials separately. 200 µl of processed ILT sample was added to each cryovial containing 200 µl of lysing buffer and mixed. 20 µl of protease K was added to release contaminants, making the total volume 420 µl, and the suspension was mixed by pulse-vortexing for 15 seconds. After that incubated at 56°C for 10 minutes, then 200 µl of 96% ethanol was added and pulse-vortexed before being centrifuged.

After that, the mixture was then put in a centrifuge and centrifuged at 8000 rpm for 1 minute. Finally, 620 µl of the suspension was transferred to a QIAMPSpin column and centrifuged again at 8000 rpm for one minute. The liquid went through the column during centrifugation, but the DNA stayed on the membrane. After adding 500 µl of AW1, the mixture was centrifuged at 8000 rpm for 1 minute, then moved to a biosafety cabinet for suspension decanting. Then, 500 µl of buffer AW2 was added, and centrifugation occurred at 14,000 rpm for 3 minutes. Then, it was moved to the same empty vials after decanting the 500 µl of AW2 and spinning the column at 14,000 rpm for 1 minute to dry it off. The columns were put into new vials, and 50 µl of AE buffer was added to the membrane to release pure DNA. After a 5-minute incubation at room temperature, samples were centrifuged at 14,000 rpm for 1 minute to settle the DNA at the bottom of the vials. The DNA vials were ready for PCR processing after the columns were thrown away. They were kept at -20°C until the master mixed the following day.

Molecular diagnosis of ILTV was carried out using the 747 bp of ICP4 gene. The choice of this target region was based on the fact that it contains a nucleotide sequence conserved across members of the Alphaherpesvirinae subfamily (Creelan et al., 2006). In addition, because it is an immediate early gene that encodes a transcription factor (~175 kDa) expressed before viral genome replication, it serves as a reliable marker for detection (Sampath & DeLuca, 2008)

Conventional PCR amplification for the ICP4 gene of ILTV was conducted in a total reaction volume of 25 µl per sample. The PCR master mix was made to a total volume of 20 µl per reaction by adding reagents in successive order. In each individual reaction, the mixture consisted of 10 µl of IQ Supermix, 6 µl of Nuclease Free Water, 2 µl of the forward primer F2

(5'-CTTCAGACTCCAGCTCATCTG-3'), and 2 µl of the reverse primer 3R (5'-AATGAGCACGCAACCAGAAGTAA-3'). Master mix aliquots were transferred into individual PCR tubes followed by the addition of 5 µl of the extracted template DNA to reach a total reaction volume of 25 µl.

The PCR amplification process began with an initial denaturing step carried out at 95 °C for 4 minutes. 35 cycle were then conducted, whereby each cycle consisted of a denaturation step at 94°C for 1 minute, an annealing step at 60°C for 1 minute, and an extension step at 72°C for 90 seconds. The final extension step was done at 72°C for 10 minutes.

Gel Electrophoresis: The PCR products were separated on a 1.5% (w/v) agarose gel. To prepare the gel, 1.5 g of agarose was dissolved in 100 mL of 1x TAE buffer. The agarose solution was heated until fully dissolved, then poured into a tray fitted with an appropriate comb and left at room temperature to set. After the gel had completely solidified, it was placed in an electrophoresis chamber containing 1x TAE buffer, and the combs were carefully removed. Next, 25 µL of each PCR product was combined with 5 µL of 1x pre-mixed loading dye and staining dye, and 10 µL of the resulting mixture was loaded into the assigned wells. Positive and negative controls were included alongside the sample. A 100 bp molecular ladder (10 µL) was loaded into the first gel lane to serve as the size marker. Electrophoresis was performed at a constant voltage of 120 V for 1 hr. (Lee et al., 2012). After the run, the gel was examined under ultraviolet (UV) light, and the expected 747 bp bands were evaluated.

3.5.5. Infectious Bronchitis Virus isolation in an embryonated egg

Because of limited resources, two RT PCR-positive pooled samples were selected as a representative subset for virus isolation. Specific pathogen-free embryonated chicken eggs were obtained from the NVI of Ethiopia. The SPE eggs were incubated at 37°C and kept at 65% relative humidity. Temperature and humidity were checked daily until the day the samples were inoculated. The swab samples were centrifuged at 1500 rpm for 10 minutes to vortex and clarify them. Afterwards, the suspension was passed through a 0.45µm syringe filter (Millipore

USA). The resulting filtrate was kept at -80°C for later processing. Specific Pathogen Free (SPF) embryonated chicken eggs were used for the initial isolation of IBV (WOAH, 2018).

The embryonated eggs were disinfected with 70% ethanol, and a hole was made in a selected area of the egg, approximately two-thirds away from the embryo, under aseptic conditions. In brief, 0.2 ml of sample supernatant was inoculated into the allantoic cavity of 11-day-old embryos. The control egg was inoculated with PBS. The opening was sealed with melted candle wax, and the inoculated eggs were then incubated at 37°C. The eggs were candled each day for 5 days, and deaths occurring within the first 24 hours were regarded as nonspecific (Mechanical). Then, the allantoic fluids from all eggs were combined after harvest at 2–5 days post-infection (Appendix 6); this mixture was diluted 1/5 in antibiotic broth and used to inoculate another group of eggs, for a maximum of four passages. The infectious allantoic fluids were first cooled to 4°C, then collected, and finally kept at -20°C until RNA extraction was carried out. Because other respiratory viruses, such as avian adenovirus, can produce lesions similar to those caused by IBV, the virus was confirmed using a molecular detection method (WOAH, 2018).

3.5.6. Molecular confirmation of isolated egg for IBV

To establish conclusively that the lesions seen in the inoculated embryos resulted from IBV replication, allantoic fluid was obtained from eggs that showed embryonic alterations. As outlined in section 3.5.3, viral RNA was extracted from the recovered fluid, and all methods were done according to that and then analyzed by a second RT-PCR run under the same thermal cycling conditions, amplifying the same 433 bp region of the 3'UTR.

3.5.7. Isolation of Infectious Laryngotracheitis Virus in chicken embryo fibroblast cell culture (CEF)

Ten-day-old embryonated eggs were used to create the primary cell culture (Appendix 7). The growth media were discarded, and 0.5 ml of the field swab was used as inoculum and added to the monolayer CEF cell culture. Cells were incubated for 1 hr. at 37°C to allow virus attachment to cells and then supplied with maintenance media (2%) similar to control cell

culture. An inverted microscope is used every day to look for signs of viral cytopathic effect (CPE) in the cell culture (Appendix 8). The infected cell culture was frozen at -20°C, then thawed and propagated for two passages (Taha *et al*., 2017; Atta and Allawe, 2018).

3.6. Data Management and Analysis

Data obtained were entered into Excel 2019 software and analyzed using STATA version 17 software. The Pearson's chi-square ($\chi 2$) test was used to determine the association between the potential risk factor and viral revalence. logistic regression models were subsequently used to determine the direction and magnitude of these associations, with results expressed as Odds Ratios (OR) alongside their corresponding 95% Confidence Intervals (CI). For all statistical analyses, a P-value of less than 0.05 was considered statistically significant.

3.7. Ethical Approval and Considerations

The study was reviewed by the Animal Welfare and Research Ethical Review Committee at Addis Ababa University College of Veterinary Medicine and Agriculture, ensuring ethical considerations and adherence to public health and animal welfare principles. The committee approved the study, and if necessary, the humane sacrifice of severely ill chickens will follow established protocols (appendix 9). The significance of this research was evaluated from ethical perspectives, applicability, originality, and technical competence points of view. Finally, approval was granted with Reference number (VM/ERC/09/112/18/2026). 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.Seroprevalence of Infectious Bronchitis Virus and InfectiousLaryngotracheitis Virus

In this study, a total of 384 individual blood samples from chickens were tested for IBV and ILTV antibodies using the ELISA technique. The findings from the study revealed that there was a marked difference in the presence of the two viruses. The overall seroprevalence rate for IBV was 99.7% (383/384), showing that IBV infection was very prevalent among the sample population of the chickens used. On the other hand, the overall prevalence rate for ILTV was much lower, standing at 17.19% (66/384), most of which were from one geographical area only.

The sample taken from all three out of four sites indicated 100 % seroprevalence of antibody against infectious bronchitis virus (IBV). Hence, there was an occurrence of seroprevalence for IBV of 100% in Sebeta (n=97), Waliso (n=92), and Eteya (n=76). The level of exposure was slightly lower in Maya city (n=119) as well, but it was still a remarkably high rate of 99.2% (Table 6). However, in the case of ILTV, there was a highly varied geographical distribution of exposure to this virus. There was maximum local prevalence in Maya City, which reached 53.8% (64/119), compared to 1.1% (1/92) and 1.0% (1/97) in Waliso and Sebeta, respectively. No positive cases (0%) were detected among the 76 samples collected from Eteya. In comparison based on Eteya as a reference using logistic regression, there were statistically significantly higher odds of positivity in Maya City (OR=177.82; 95% CI: 10.77-2935.52; p = 0.000). On the other hand, the differences in the prevalence seen in Waliso (OR=2.51, p =0.575) and Sebeta (OR=2.38, p=0.597) towns were statistically insignificant (Table 4), thus implying no risk difference between the reference town and these two study towns.

Table 4: The Overall prevalence of IBV and ILTV based on the study sites

Location	Observation	Positive for IBV	Positive for ILTV	Prevalence for IBV (%)	Prevalence for ILTV (%)	P value for ILTV	OR ILTV	95% CI ILTV
Eteya	76	76	0	100	0	-	Reference	-
Sebeta	97	97	1	100	1	0.597	2.38	[0.10 - 9.21]
Waliso	92	92	1	100	1.1	0.575	2.51	[0.10 - 62.46]
Maya city	119	118	64	99.1	53.8	0.000	177.81	[10.77 - 2935.52]
Total	384	383	66	99.7	17.1%			

Note: CI – Confidence of Interval, OD - odds ratio

4.2. Risk factors for Infectious Laryngotracheitis Virus sero-prevalence

The production system structure variables were significantly associated with the presence of positive samples. The prevalence was higher among backyard production systems, where it was 34.4% (22/64), while in commercial production systems, it was only 13.75% (44/320). Univariate logistic regression analysis revealed that chickens kept in backyard production systems were more than three times more likely to have a positive test result compared with those in commercial production systems (OR=3.29, 95% CI [1.79, 6.02], p = 0.001) (Table 5)

The application of biosecurity measures at the farm level showed considerable efficacy against the pathogen. The prevalence of the pathogen was limited to 8.3% (20/241) in farms practicing regular farm cloth, while the prevalence was much higher, 32.2% (46/143), in farms that did not implement the practice. This practice was linked with an 81% decline in the odds of testing positive compared with no use of farm cloth (OR = 0.191; 95% CI = 0.11–0.34; p = 0.000). Likewise, the use of dedicated footwear by farm workers also emerged as a highly effective measure of biosecurity since it resulted in a prevalence rate of 7.3% (21/288) for farms using

dedicated footwear compared with a much higher prevalence rate of 46.9% (45/96) among non-users of dedicated footwear.

Table 5 : Total prevalence of ILTV in different production systems and different biosecurity levels

Factor	**Category**	**Nº of the sample**	**No. of sero-positive animals (%)**	**X^2**	**P value**	**OR**	**95% Conf. Interval**
Farm type	Commercial	320	44 (13.75 %)	15.93	-	Reference	-
	Backyard	64	22 (34.4%)	96	0.000	3.29	[1.79 - 6.02]
Farm cloth	No	143	46 (32.2%)	-	-	Reference	-
	Yes	241	20 (8.3%)	35.92	0.000	0.191	[0.11 - 0.34]
Dedicated Shoes	No	96	45 (46.9%)	-	-	-	Reference
	Yes	288	21 (7.3%)	79.26	0.000	0.089	[0.049-0.162]

4.3. Molecular detection of IBV and ILTV

Molecular analysis was carried out using 12 clinical samples collected from the field to determine the presence of Infectious Bronchitis Virus and Infectious Laryngotracheitis Virus (Table 6). To identify the presence of IBV, one-step RT-PCR was conducted by focusing on the highly conserved 3’ untranslated region (3’ UTR). The amplification reaction revealed a 433 bp product size indicative of IBV 3’ UTR (Figure 4). Out of the 12 pooled samples analyzed, 5(41.7%) were positive for IBV, while the rest of seven sample not show amplification. The same 12 infection-suspected clinical samples were used to extract the DNA in preparation for conventional PCR in order to amplify 747 bp of the ICP gene of the ILTV genome. Four out of 12 samples (33.3%) were found positive for ILT through successful PCR amplification Figure 5).

Table 6: Summary of molecular detection methods, genetic target, and positivity rates of IBV and ILTV

Target virus	methods	Target region/gene	Expected band size	Positive sample	Detection rate (%)
IBV	One-step RT-PCR	3'UTR	433 bp	5 / 12	41.7%
ILT	Conventional PCR	ICP gene	747 bp	4 / 12	33.3%

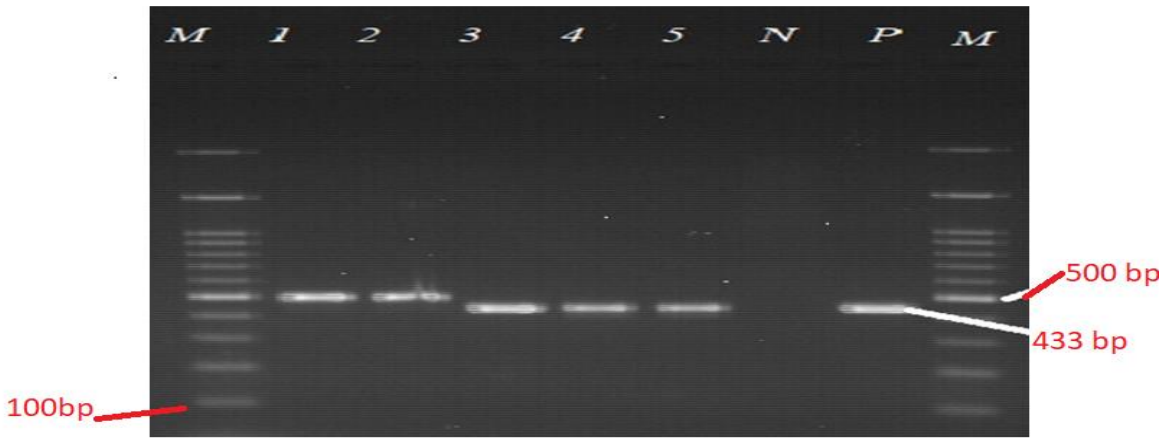


Figure 4: A conventional RT-PCR agarose gel showing the 433 bp amplicon.

The first and last lanes: 100bp molecular ladder (Biorabbit, Germany), lanes 1-5: tested samples that are positive for the 433-bp fragment, lane N: negative control, and lane P: positive control.

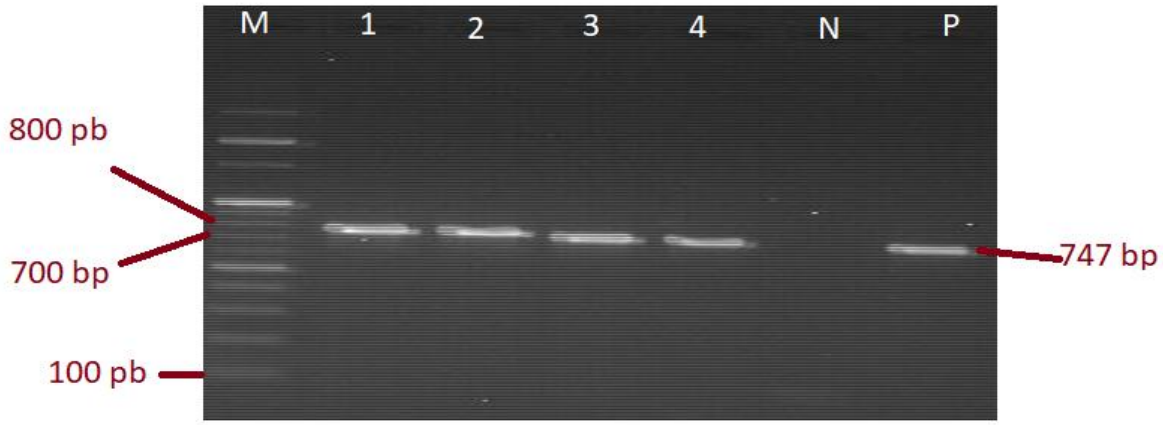


Figure 5: PCR amplification of a 747 bp fragment of the ICP4 gene from ILTV-infected field samples.

Lane M = 100-bp DNA ladder (Biorabbit, Germany), lanes 1-4 tested samples that are positive, lane N: negative control, lane P: positive control, and bp: base pair.

4.4. IBV isolation in embryonated eggs and molecular confirmation

Two of the five RT-PCR positive samples were used for virus isolation by inoculation in embryonated chicken eggs, and propagation was successful for up to four passages. Start from passage one; it showed obvious physical abnormalities such as embryonic stunting, curling, and feather dystrophy (Figure 6), while the control samples showed no change or mortality. The allantoic fluid harvested was repeatedly analyzed by RT-PCR to confirm the isolation was successful and to monitor virus replication. However, the confirmatory RT- PCR on allantoic fluid from later passages was negative for 3'UTR, indicating that detectable viral genome was lost during isolation, even though the virus was present in the original field sample.

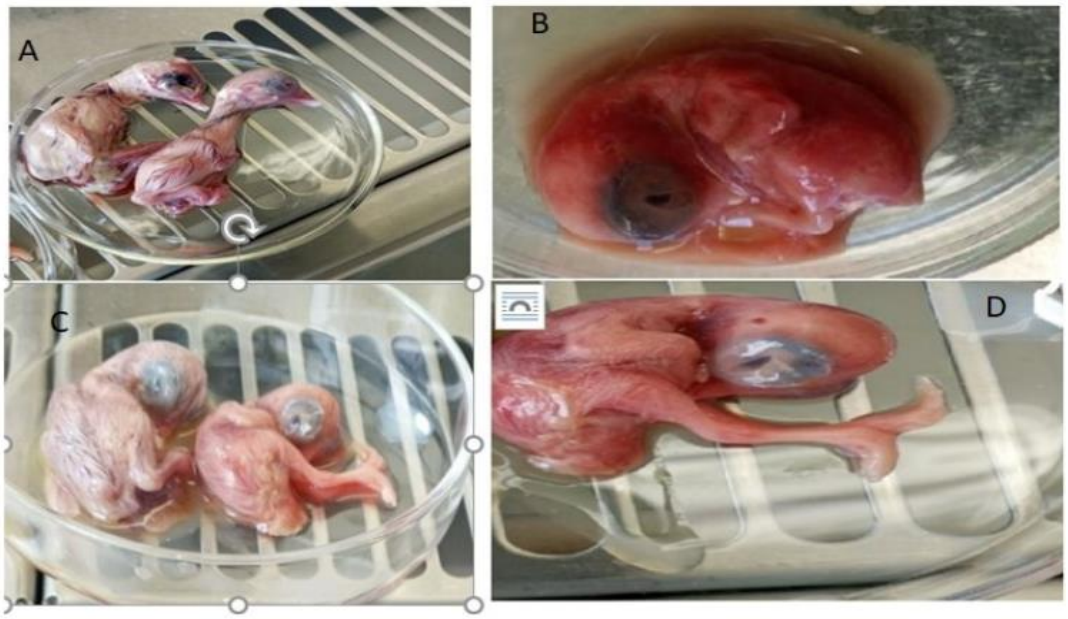


Figure 6: Isolation of IBV on embryonated egg:-

A: normal embryo; B, C, and D: curling, deformation, and stunted growth of the embryo after inoculation of IBV.

4.5. Infectious laryngotracheitis Virus propagation in Embryonic Fibroblast cell culture

The three positive pooled samples detected by PCR samples were filtered through a 0.45 µl minipore filter and then inoculated with 0.5 ml of it on CEF cell culture. Clear CPE with rounded, aggregated cells and separation from the flask surface was observed in three days post-inoculation using an inverted microscope at 40x magnification (Figure 7 B); the control CEF cell culture, shown in Figure 7 A, remained unchanged.

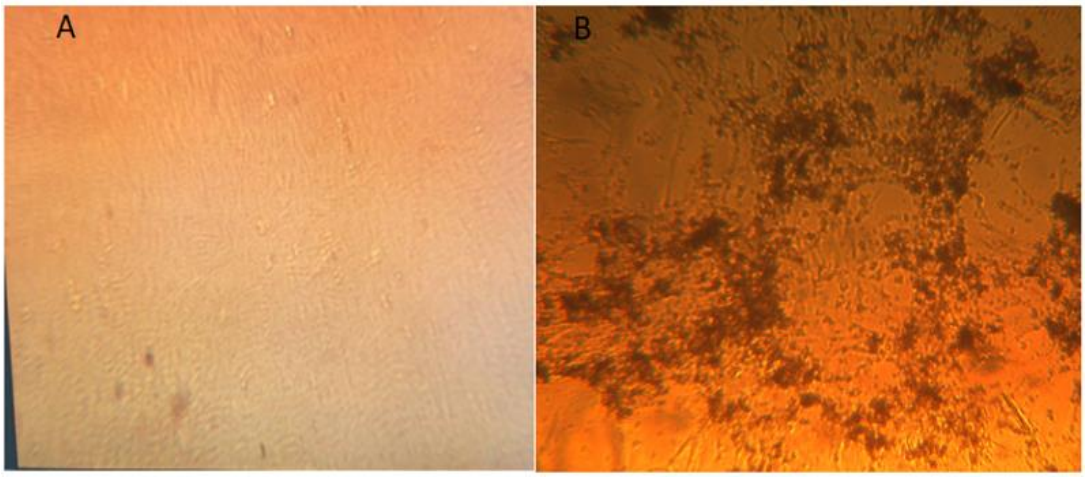


Figure 7: Infectious Laryngotracheitis virus isolation using CEF cell culture:-
(A) Control CEF cell culture, (B) CPE such as cell rounding, cell aggregation, and cell detachment in response to inoculation of field Infectious Laryngotracheitis virus.

5. DISCUSSION

In this study, the overall seroprevalence of Infectious Bronchitis Virus (IBV) in the backyard and commercial poultry flocks was an extremely high individual level seroprevalence (99.74%). Since the individual under investigation had never been vaccinated, this level of seroprevalence is indicative of field infection.

Similar results were obtained in the research done by Ayim-Akonor *et al.* (2018), where a seroprevalence rate of 100% in unvaccinated commercial laying flocks was documented in the Ga-East district of Accra, Ghana. Similar findings are indicated by the studies conducted by Shiferaw *et al.* (2022), where a seroprevalence rate of 97.46% (345/354) was reported in the areas of Bishoftu and Holeta in Oromia, Ethiopia. Slightly lower seroprevalence rates (94.5%) were indicated by the work done by Hutton *et al* (2017), where an institutional production system and a village production system were investigated in central Ethiopia. Further seroprevalence studies performed in southern Mozambique revealed that unvaccinated backyard flocks showed a cluster- based seroprevalence of up to 93.3% (Pinto *et al.*, 2022) and 91 % in central Ethiopia, reported by Habte *et al*. (2022).

Other studies have reported lower seroprevalence rates than this study. For instance, field surveillance by ELISA in unvaccinated flocks in poultry-dense areas of Pakistan showed an overall seropositivity of 84% (Kanwal *et al*., 2018). An overall seropositivity of 82.95% was reported in Nigeria (Ijoma *et al*., 2020), 70.6% in Sebeta Hawasa district Tesfaye *et al.*, 2019), 64.7% in the Ada'a district (Roba *et al*., 2021), 54.5% in a large scale serosurvey of unvaccinated backyard chickens in Iran (Shokri *et al*., 2018), and 23.96% in northwest Ethiopia (Birhan *et al*., 2021). Such wide variation is related to a substantial increase in viral circulation over time, as well as probable differences in sample sizes, agro- climatic conditions, and local poultry management systems.

The overall seroprevalence of Infectious Laryngotracheitis (ILT) detected in this study is 17.19% (66/384). This indicates a strong serological proof regarding the widespread

prevalence of Gallid Herpesvirus 1 in the studied areas of Oromia, thus filling an important gap in the local disease map. In comparison to other studies on Ethiopia's poultry population, this study falls within the middle baseline group, which matches closely with other findings where a prevalence rate of 17.33% was observed in Chittagong district of Bangladesh (Uddin *et al*., 2014). Another 19.4% was reported from the Amhara region of Ethiopia (Adam *et al*., 2025) and a 19.4% prevalence rate by Tesfaye *et al*. (2019) in commercial and backyard chickens from Central and South Ethiopia. Also, it agrees with the results obtained by Balcha *et al.* (2023) from a small-scale chicken production system of East Wollega of Oromia with 15.36% overall ILT seroprevalence.

On the Other hand, the finding of this study is lower than those of Jahan *et al*. (2012) and Rahman *et al*. (2018), who have found prevalence rates of 92.28% and 81.47%, respectively, in Bangladesh. Abebe *et al*. (2024) also observed a higher seroprevalence rate of 26.7% in backyard and small commercial chicken farms in Robe Town, southeastern Ethiopia. Likewise, in Ada'a district, Oromia region, Roba *et al.* (2020) reported the first Official documentation of ILT in backyard chickens, with a significant seroprevalence rate of 54.7%, and even higher cluster seroprevalence rates of up to 83.3%. Furthermore, the current results are lower compared to the regional seroepidemiological study carried out in the Northern part of Ethiopia by Birhan *et al.* (2022), which reported 59.1% (454/768) of the seroprevalence in chickens.

However, the overall prevalence recorded in this study (17.19%) is higher than that seen in Bangladesh 0.4 %, by Bhuiyan *et al*. (2019), Finland (12%), by Pohjola *et al*. (2019), and Iran 13 % in broiler by Ghalyanchi Langeroudi *et al* (2020). These variations may also be linked to variations in farm management and biosecurity, in addition to climate.

The prevalence rate of ILTV was significantly higher for the backyard production system (34.4%) compared to the commercial production system (13.75%). Backyard chicken farming mainly uses extensive, smallholder methods, and chickens are free-range. This makes it hard to maintain biosecurity. Because the chickens aren't controlled at home, they have more contact with wild birds. It's easier for diseases to spread, too, due to the mixing of different age groups. Additionally, these small-scale operations often skip veterinarians' care, like vaccinations, and

don't always keep things clean. As a result, there's more disease on these small farms. Because of these factors, the pathogen prevalence rate is higher in these backyard production systems. This is similar to the findings of Tesfaye *et al*. (2024), where the same 34.4% prevalence was found for backyard chickens in central Ethiopia, while the commercial farm system showed lower rates of 13.3%. Furthermore, these results correspond with Haesendonck *et al.* (2014) in Belgium, where a 30 % prevalence of the disease was noted. This result conforms to the global trends, according to which the pathogen prevalence rate is always higher for extensive or smallholder production systems than for intensive commercial farming operations Gentile *et al.,* 2024).

In this study, the protective effect of farm-level biosecurity practices against Infectious Laryngotracheitis virus was significant. The ILT prevalence was 8.3 % (20/241) on farms with routine farm cloth protocols compared to 32.2 % (46/143) on farms without these biosecurity measures. Additionally, adopting specific footwear for farm workers on the farm was shown to be another important operational practice for ILT prevention. Its adoption resulted in an incidence of 7.3% (21/288), but when this practice was completely absent, the incidence increased to 46.9% (45/96. The result obtained above is aligned with the previous risk factor assessments conducted on ILTV among livestock, where the researcher found that the absence of protective shoes and clothing significantly increases the chance of a positive ILT antibody test (Birhan *et al*., 2022; Gowthaman *et al*., 2020). This result is high in farm uses of farm clothing and shoes because the virus can enter through binding to the cloth and shoes.

In this study, the identification of IBV using molecular techniques showed that 41.7% were positive for IBV using the RT-PCR technique. Since there was no vaccination against the IBV in this particular case, it indicated that the infection was acquired via field contact. This result is aligned with results documented by Seger *et al.* (2016) in Iraq (40.62%) and Ayim-Akonor *et al.* (2018) in Ghana (40%). However, higher reports of molecular detection were reported, such as 100% by Al-Jallad et al. (2020) in Syria and 80% by Demeke *et al.* (2023) in Ethiopia. The molecularly positive results, which were lower than those of the current study, were published by (Gompo *et al*., 2019) 1% in Nepal, by Hutton, *et al*. (2017) 6% and by Tegegne *et al*. (2020) 6% in Ethiopia, by Domanska-Blicharz *et al.* (2020) 11.5% in Poland, 11.54 %

by Mihret *et al.* (2023),12.24% by Berhanu *et al*.(2025) in Ethiopia, 31.6% by Alsultan *et al.*, (2019) in Al-Hasa, 33.3 % by Hirphaye *et al*.(2024) and 39% by Tekelemariam *et al*.(2022) in Ethiopia. The variation of 41.7% in the current study may be due to the varying levels of implementation of biosecurity measures in the various farms.

The molecular analysis identified that 4 out of 12 swab samples (33.3%) were positive for ILTV. The presence of this specific 747 bp DNA fragment gives strong molecular proof for the circulation of GaHV-1 in the target chicken species. The conventional PCR method is one of the basic principles in diagnosing viruses in chickens, where targeting conserved regions of the virus genome ensures high analytical specificity of the test (Aydın *et al*., 2025).

The detection rate of 33.3 % obtained in this study adds to the growing evidence that ILTV is an active player in the complex of respiratory disease of Ethiopian poultry. In Ethiopia, the current study is comparable to, but slightly higher than, other recently published studies conducted using molecular analysis, emphasizing an increasing disease burden. Abebe *et al.* (2024) detected ILTV DNA in 4 of 15 samples (26.7%) by PCR amplification of a 688 bp region of the ICP gene in the Oromia Regional State of southeastern Ethiopia. Adam (2025) reported a positive rate of 14.8 % (4 out of 27) of oropharyngeal swab samples from the Central Gondar Zone targeting the same 688 bp region of the ICP4 gene. Tekelemariam *et al*. (2022) have reported that the molecular positivity rates of ILTV were found to be low, at 11%, among symptomatic chickens sampled from the live chicken markets of Addis Ababa, Ethiopia.

Globally, the results of this research correspond to those found endemically in the other countries that have emerging or developing poultry industries. For comparison, according to Ardiçli *et al*. (2022), the prevalence of the infection in Turkey was found to be 11.83 % based on a PCR test for the presence of 747 bp of the ICP4 gene. In another study conducted by Saasa *et al*. (2026) in Zambia, the pathogen was identified in 2 % (1 of 50) apparently healthy chickens. The high rates of positivity observed in this study (33.3%) compared to other studies above might be associated with differences in biosecurity measures, flock density, and the selection criteria of diseased birds used.

The two samples that were positive for IBV by molecular detection were used to carry out the virus isolation. The isolation was performed by using five Specific Pathogen Free (SPF) embryonated chicken eggs per sample, and embryos were observed daily for seven days after inoculation via the allantoic route. Mortality occurring within the first 24 hours after inoculation, due to mechanical injury, was discarded to prevent false positive mortality (Hirbaye *et al*., 2024). However, in this study, there was some degree of discordant results seen in a portion of the samples tested, with two positive molecular tests becoming negative after the second isolation process. Mismatch sensitivity has been widely reported in avian diagnostic virology and could be influenced by several main causes.

First, considering the instability of the virion form of the IBV, any changes in the environment or transport/storage conditions of the sample could lead to the degradation of the infectivity of the virus while the nucleic acid is preserved and remains detectable by the molecular test (De Wit, 2000). According to Compton (2020), PCR tests specifically detect fragments of nucleic acids and not live pathogens. In case in point, the first positive test conducted in the laboratory indicated the presence of fragments of the genome that do not necessarily have the biological activity necessary to infect host cells. Such discrepancies are common practice in field surveillance; Jones *et al*. (2011) described a case when out of 176 samples containing fragments of the IBV's genome detected by PCR analysis using the 3' UTR method, only three samples were positive in relation to the isolated live virus. Secondly, there can be samples containing very low concentrations of the virus, which can be detected by the high analytical sensitivity of the molecular assay, while they will lack sufficient biological activity necessary for egg inoculation (Meir *et al.,* 2010).

Their inoculation in SPF embryonated eggs gave rise to characteristic lesions. In particular, the stunting, curling, and feather dystrophy were observed, which corroborate the results obtained by Ul Hayat *et al*. (2018), Berhanu *et al*. (2025), and Mihret *et al*. (2023). The explanation for the occurrence of such lesions following the isolation process, but then followed by a negative PCR finding through egg passage, might be attributed to viral interference. Given the presence of the other viruses in addition to IBV, competition among the viruses occurs for the utilization of common receptors. As the co-infecting virus grows faster, it interferes with the binding and

replication of IBV. Thus, in such a case, the co-infecting virus outcompetes IBV in the host cells, resulting in the death of the embryos and hence the characteristic lesion development, while at the same time IBV gets reduced in quantity below the threshold detectable by PCR (Aouini *et al*., 2018). As such, the lesions observed could have been caused by other secondary infections present in the primary sample used, rather than IBV infection (Villarreal, 2010).

Successful isolation into primary CEF cells has been achieved in three independent pooled field samples that test positive for ILTV. The morphologic features seen in inoculated CEF monolayers, which include the progressive disappearance of their typical fibroblast-like elongation, significant cell rounding, the appearance of dense, dark, and irregular granular aggregates, and finally, cell detachment from the flask surface, are very much similar to the cytopathic features known for alphaherpesvirus infection. Its align with the cell culture findings presented by Portz et al. (2018), showing the permissive nature of primary CEF culture for ILT infection, resulting in significant cell rounding and subsequent cell death within 24-96 hours after inoculation.

Additionally, this observation is also well-supported by Magouz (2018), whose research was successful in using primary monolayer cultures of chicken embryo fibroblast (CEF) for isolating wild-type Gallid herpesvirus 1 respiratory pools, observing characteristic cytopathic changes in the period of 3-5 days following inoculation. It can be stated that the choice of this cell culture in the current study is very relevant in avian virology. According to Hughes & Jones (1988), primary cells isolated from chickens' kidneys or livers are characterized by a faster manifestation of cytopathic effects compared to fibroblasts at early passages. Still, the current research proves that primary CEF cells provide a very suitable system for visualizing the effect of viral infection. Nevertheless, in view of limited resources at hand, molecular confirmation of ILTV isolates could not be conducted repeatedly after isolation.

Limitations of the study: The results derived from this study provide important information, but there are some limitations associated with it. The purposive sampling technique used in the study ensured that the selected areas had minimal studies conducted in the past and emphasized chickens that exhibited clinical symptoms; although the method helped establish the virus's existence, even though this is an appropriate technique, it still created bias in the sampling and thus hindered its generalization to the asymptomatic chicken populations. There has also been limited availability of funds, which did not permit a sufficient molecular sample size; these resource limitations made downstream testing impossible, preventing genetic sequencing and the identification of a specific strain. This study did not address many backyard poultry production systems in the study areas due to time constraints.

6. CONCLUSION AND RECOMMENDATIONS

This study provides updated evidence of the exposure and current circulation of both the infectious bronchitis virus (IBV) and infectious Laryngotracheitis virus (ILTV) at the study sites. Testing performed by ELISA revealed extremely high levels of the IBV infection almost ubiquitously (99.74%) in comparison to a lower percentage of ILTV that was geographically varied (17.19%) until reaching a significantly higher level in May city (OR = 177.82, p = 0.000). In the risk factor analysis, the production system in the backyard was highly vulnerable to ILTV (OR = 3.29, p = 0.000), while wearing farm clothing (OR = 0.191, p = 0.000) and shoes (OR = 0.089, p = 0.000) provided significant protection against infection. Additionally, molecular tests performed on the samples obtained from the field Maya City showed that there was circulation of IBV (41.7%) and the ILTV (33.3%). Virus culture was conducted through the use of embryonated chicken eggs and chicken embryo fibroblast cell culture for IBV and ILT, respectively. Generally, the seroprevalence findings in multiple locations contribute to setting a baseline in the study area, yet the restriction of molecular testing and virus isolations to one geographical area serves to underline the urgent necessity of increasing sample numbers to conduct nationwide molecular characterization. Following the above-mentioned conclusions, the following recommendations can be made:

- To determine the real prevalence of the virus, it is recommended to expand surveillance to include asymptomatic chickens by random sampling of symptomatic and asymptomatic populations.
- Future research could benefit greatly from focusing on backyard systems, where risk and prevalence of ILT is substantially higher.
- In the future, it is necessary to emphasize the sequencing of viral genes to determine the exact strains in the country.
- Effective control of viruses requires the development of strain-specific vaccines that are closely matched to local field variants.

7. REFERENCES

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

Abebe, S., Ferrara, G., Getachew, B., Hirpa, E. and Moje, N., 2024. Serological and molecular investigation of infectious laryngotracheitis virus in chickens from Robe Town, Southeastern Ethiopia. *Animals*, ***14***(22), p.3227.

Abozeid, H.H., 2023. Global emergence of infectious bronchitis virus variants: evolution, immunity, and vaccination challenges. *Transboundary and Emerging Diseases*, ***2023***(1), p.1144924.

Adam, O., Oladele, O.A., Yimam, T.M., Getachew, B., Deresse, G., Birhanu, K., Legesse, A., Tefera, T.A. and Bitew, M., 2025. Serological and molecular detection of infectious laryngotracheitis virus in chickens in Central Gondar Zone, Ethiopia. *Frontiers in Veterinary Science*, *12*, p.1517373.

Adzhar, A., Gough, R.E., Haydon, D., Shaw, K., Britton, P. and Cavanagh, D., 1997. Molecular analysis of the 793/B serotype of infectious bronchitis virus in Great Britain. *Avian pathology*, ***26***(3), pp.625-640.

Al-Jallad, T., Kassouha, M., Salhab, M., Alomar, A., Al-Masalma, M. and Abdelaziz, F., 2020. Molecular characterization of isolated infectious bronchitis viruses from affected vaccinated broiler flocks in Syria. *BMC veterinary research*, ***16***(1), p.449.

Alp Onen, E. and Ozgur, Y., 2017. Investigation of Infectious bronchitis virus strains by real time RT-PCR and S1 gene sequencing in broilers. *Journal of Veterinary Science & Medical Diagnosis*, ***6***(5).

Alsultan, M.A., Alhammadi, M.A. and Hemida, M.G., 2019. Infectious bronchitis virus from chickens in Al-Hasa, Saudi Arabia 2015-2016. *Veterinary World*, ***12***(3), p.424.

Ardiçli, O., Demirbilek, S.K., Çöven, F. and Carli, K.T., 2022. A surveillance for avian coronavirus infectious bronchitis virus, infectious laryngotracheitis virus, avian metapneumovirus, and avian reovirus in poultry flocks with respiratory signs in Türkiye. *Turkish Journal of Veterinary & Animal Sciences*, ***46***(5), pp.687-697.

Armesto, M., Cavanagh, D. and Britton, P., 2009.The replicase gene of avian coronavirus infectious bronchitis virus is a determinant of pathogenicity. *PLoS One*, ***4***(10), .e7384.

Aouini, R., Laamiri, N. and Ghram, A., 2018. Viral interference between low pathogenic avian influenza H9N2 and avian infectious bronchitis viruses in vitro and in ovo. *Journal of virological methods*, *259*, pp.92-99.

Awad, F., Chhabra, R., Baylis, M. and Ganapathy, K., 2014. An overview of infectious bronchitis virus in chickens. *World's poultry science journal*, ***70***(2), pp.375-384.

Ayim-Akonor, M., Owusu-Ntumy, D.D., Ohene-Asa, H.E., Oduro-Abrokwa, A., Hammond, P., Appenteng, M. and Annan, D., 2018. Serological and Molecular Surveillance of Infectious Bronchitis Virus Infection in Free-Range Chickens and Guinea Fowls in the Ga-East District of Ghana. *Journal of Veterinary Medicine*, ***2018***(1), p.4949580.

Aydin, O., Bayraktar, E., Tali, H.E., Ozkan, I.E., Yilmaz, A., Umar, S., Bamac, O.E., Turan, N., Konuk, C., Sadeyen, J.R. and Chang, P., 2025. Phylogeny of multiple genomic regions of infectious laryngotracheitis virus in Turkish poultry flocks. *Poultry Science*, ***104***(5), p.104957.

Aziz, T., 2020. Avian infectious bronchitis virus: infection, evolution, and immunity https://www. thepoultrysite.com/articles/avian-infectious-bronchitis-virus-infection-evolution-and-immunity. *Erişim*, *6*, p.2020.

Balcha, T.K., Bulcha, B. and Etefa, M., 2023.Seroprevalence and Associated Risk Factors of Infectious Laryngotrachitis in Small Scale Chicken Production in Nekemte Town and Selectted Districts of East Wollega Zone, Oromia, Ethiopia.

Bali, K., Kaszab, E., Marton, S., Hamdiou, S.H., Bentaleb, R.K., Kiss, I., Palya, V. and 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*, ***12***(4), p.475.

Bande, F., Arshad, S.S., Omar, A.R., Bejo, M.H., Abubakar, M.S. and Abba, Y., 2016. Pathogenesis and diagnostic approaches of avian infectious bronchitis. *Advances in virology*, ***2016***(1), p.4621659.

Baumberger, C., Di Pillo, F., Tapia, D., Coloma, C., Orozco, K., Galdames, P., Oyarzun, C., Gárate, D., Torreblanca, C., Ruiz, S. and Jimenez-Bluhm, P., 2025. Prevalence and

Seroprevalence of Infectious Bronchitis Virus and Infectious Laryngotracheitis Virus in Backyard Poultry in Central Chile. *Animals*, ***15***(16), p.2364.

Berhanu, N., Hirpa, E., Mohammed, H., Legesse, A., Deresse, G., Assefa, E., Tesgera, T., Akalu, M., Abayneh, T., Bayissa, B. and Tesfaw, L., (2025): Isolation, molecular identification, and phylogenetic analysis of infectious bronchitis virus from commercial chicken farms in Mekele and Bishoftu, Ethiopia, 2023–2024. *Virology Journal*, ***22***(1), pp.1-15.

Bhuiyan, Z.A., Ali, M.Z., Moula, M.M., Bary, M.A., Arefin, N., Giasuddin, M. and Khan, Z.U.M., 2019. Seroprevalence of major avian respiratory diseases in broiler and sonali chicken in selected areas of Bangladesh. *Journal of advanced veterinary and animal research*, ***6***(4), p.561.

Bhuiyan, M.S.A., Amin, Z., Rodrigues, K.F., Saallah, S., Shaarani, S.M., Sarker, S. and Siddiquee, S., 2021. Infectious bronchitis virus (gammacoronavirus) in poultry farming: vaccination, immune response and measures for mitigation. *Veterinary sciences*, ***8***(11), p.273.

Bhuiyan, M.S.A., Sarker, S., Amin, Z., Rodrigues, K.F., Saallah, S., Shaarani, S.M. and Siddiquee, S., 2023. Infectious Bronchitis Virus (Gammacoronavirus) in poultry: genomic architecture, post translational modifications, and structural motifs. *Poultry*, ***2***(3), pp.363-382.

Birhan, M., Temesgen, M., Shite, A., Berhane, N., Bitew, M., Gelaye, E., Abayneh, T. and Getachew, B., 2021. Seroprevalence and associated risk factors of infectious bronchitis virus in chicken in Northwest Ethiopia. *The Scientific World Journal*, ***2021***(1), p.4553890.

Birhan, M., Syoum, A., Ibrahim, S.M., Fentahun, T., Mohammed, A., Berhane, N., Bitew, M., Gelaye, E., Atanaw, M.B., Getachew, B. and Dessalegn, B., 2022. Serological evidence of infectious laryngotracheitis infection and associated risk factors in chickens in Northwestern Ethiopia. *The Scientific World Journal*, ***2022***(1), p.6096981.

Borić, A.H., Nicević, M., Šabić, E., Terzić, I., Koro-Spahić, A., Šupić, J., Kapo, N., Prašović, S., Kustura, A., Ćutuk, R. and Softić, A., 2024. INFECTIOUS BRONCHITIS-IMPACT ON EGG PRODUCTION. *Journal of Hygienic Engineering & Design*, *47*.

Brown, L., Premaratna, D., Segal, Y. and Beddoe, T., 2020. Air sampling for detection of infectious laryngotracheitis (ILT) in commercial poultry flocks. *BMC research notes*, ***13***(1), p.556.

Cavanagh, D., 2005. Coronaviruses in poultry and other birds. *Avian pathology*, *34*(6), pp.439-448.

Chaiban, C., Robinson, T.P., Fèvre, E.M., Ogola, J., Akoko, J., Gilbert, M. and Vanwambeke, S.O., 2020. Early intensification of backyard poultry systems in the tropics: a case study. *Animal*, ***14***(11), pp.2387-2396.

Chen, L., Xiang, B., Hong, Y., Li, Q., Du, H., Lin, Q., Liao, M., Ren, T. and Xu, C., 2021.Phylogenetic analysis of infectious bronchitis virus circulating in southern China in 2016–2017 and evaluation of an attenuated strain as a vaccine candidate. *Archives of Virology*, ***166***(1), pp.73-81.

Choi, E.J., La, T.M., Choi, I.S., Song, C.S., Park, S.Y., Lee, J.B. and Lee, S.W., 2016. Genotyping of infectious laryngotracheitis virus using allelic variations from multiple genomic regions. *Avian Pathology*, ***45***(4), pp.443-449.

Choramo, A., Debelo, M., Tesfaye, A., Bedasa, C., Dima, C. and Guyassa, C., 2024. Serological detection of Newcastle disease virus in backyard poultry production system in Woliso District South West Shewa, Central Ethiopia. *Immunity, Inflammation and Disease*, ***12***(2), p.e1179.

Compton, S.R., 2020. PCR and RT-PCR in the diagnosis of laboratory animal infections and in health monitoring. *Journal of the American Association for Laboratory Animal Science*, ***59***(5), pp.458-468.

Cook, J.K., Jackwood, M. and Jones, R.C., 2012. The long view: 40 years of infectious bronchitis research. *Avian Pathology*, ***41***(3), pp.239-250.

Coppo, M.J., Devlin, J.M., Legione, A.R., Vaz, P.K., Lee, S.W., Quinteros, J.A., Gilkerson, J.R., Ficorilli, N., Reading, P.C., Noormohammadi, A.H. and Hartley, C.A., 2018. Infectious laryngotracheitis virus viral chemokine-binding protein glycoprotein G

alters transcription of key inflammatory mediators in vitro and in vivo. *Journal of Virology*, ***92***(1), pp.10-1128.

Creelan, J.L., Calvert, V.M., Graham, D.A. and McCullough, S.J., 2006. Rapid detection and characterization from field cases of infectious laryngotracheitis virus by real-time polymerase chain reaction and restriction fragment length polymorphism. *Avian pathology*, ***35***(02), pp.173-179.

Dahesa, G.D., Oche, T.W. and Kebede, I.A., 2025. Prevalence of mastitis and isolation of Escherichia coli from mastitic cows in Maya City, Eastern Hararghe Zone, Oromia, Ethiopia. *Discover Bacteria*, ***2***(1), p.12.

Demeke, H.D., Fufa, A.F., Ayana, H.W. and Abosse, J.S., 2023. Pathological and Molecular Characterization on Clinically Sick Chickens with Infectious Bronchitis Virus in Small Scale and Commercial Poultry Farms in Bishoftu Town, Ethiopia.

De Wit, J.J., 2000. Detection of infectious bronchitis virus. *Avian pathology*, *29*(2), pp.71-93.

De Wit, J.J., Cook, J.K. and Van der Heijden, H.M., 2011. Infectious bronchitis virus variants: a review of the history, current situation and control measures. *Avian pathology*, ***40***(3), pp.223-235.

Domanska-Blicharz, K., Sajewicz-Krukowska, J. and Lisowska, A., 2020. New PA/1220/98-like variant of infectious bronchitis virus in Poland. *Avian Pathology*, ***49***(4), pp.380-388.

Falchieri, M., Coward, V.J., Reid, S.M., Lewis, T. and Banyard, A.C., 2024. Infectious bronchitis virus: an overview of the "chicken coronavirus". *Journal of medical microbiology*, ***73***(5), p.001828.

Fehr, A.R. and Perlman, S., 2015. Coronaviruses: an overview of their replication and pathogenesis. *Coronaviruses: methods and protocols*, pp.1-23.

Feng, C., Liu, Y., Lyu, G., Shang, S., Xia, H., Zhang, J., Irwin, D.M., Wang, Z. and Zhang, S., 2022. Adaptive Evolution of the Fox Coronavirus Based on Genome-Wide Sequence Analysis. *BioMed Research International*, ***2022***(1), p.9627961.

Fereidouni, S.R., Globig, A., Starick, E. and Harder, T.C., 2012. Effect of swab matrix, storage time, and temperature on detection of avian influenza virus RNA in swab samples. *Avian diseases*, ***56***(4s1), pp.955-958.

Gemeda, D.G., Negese, Y.K., Lamecha, A.C., Hassen, B.M. and Nugusse, D.R., 2026. Assessment of Biosecurity Status in Commercial Chicken Farms Found in Sebeta Town, Shaggar City, Oromia Regional State, Ethiopia. *Veterinary Medicine and Science*, ***12***(1), p.e70782.

Gentile, N., Carrasquer, F., Marco-Fuertes, A. and Marin, C., 2024. Backyard poultry: exploring non-intensive production systems. *Poultry Science*, ***103***(2), p.103284.

GhalyanchiLangeroudi, A., Hosseini, H., Fallah, M.H., Aghaeean, L., Esmaeelzadeh Dizaji, R., Ziafati, Z., Modiri, A., Almasi, Y., BaGholamian, B., Ashouri, A. and Hajizamani, N., 2020. Serological survey of Infectious Laryngotracheitis in broiler flocks, Iran, 2018. *Iranian Journal of Virology*, ***14***(1), pp.1-5.

Gompo, T.R., Pokhrel, U., Shah, B.R. and Bhatta, D.D., 2019. Epidemiology of important poultry diseases in Nepal. *Nepalese Veterinary Journal*, *36*, pp.8-14.

Gowthaman, V., Kumar, S., Koul, M., Dave, U., Murthy, T.G.K., Munuswamy, P., Tiwari, R., Karthik, K., Dhama, K., Michalak, I. and Joshi, S.K., 2020. Infectious laryngotracheitis: Etiology, epidemiology, pathobiology, and advances in diagnosis and control–a comprehensive review. *Veterinary Quarterly*, ***40***(1), pp.140-161.

Grankvist, K., Gomez, R., Nybo, M., Lima-Oliveira, G. and Von Meyer, A., 2019. Preanalytical aspects on short and long term storage of serum and plasma. *Diagnosis*, ***6***(1), pp.51-56.

Gutierrez-Ruiz, E.J., Ramirez-Cruz, G.T., Camara Gamboa, E.I., Alexander, D.J. and Gough, R.E., 2000. A serological survey for avian infectious bronchitis virus and Newcastle disease virus antibodies in backyard (free-range) village chickens in Mexico. *Tropical Animal Health and Production*, ***32***(6), pp.381-390.

Habte, T., Gerber, P.F., Ibrahim, F., Groves, P.J. and Walkden-Brown, S.W., 2022. Seroprevalence of major respiratory diseases of chickens in central Ethiopia in different chicken production systems. *Poultry science*, ***101***(10), p.102065.

Haesendonck, R., Verlinden, M., Devos, G., Michiels, T., Butaye, P., Haesebrouck, F., Pasmans, F. and Martel, A., 2014. High seroprevalence of respiratory pathogens in hobby poultry. *Avian diseases*, ***58***(4), pp.623-627.

Hewson, K., Noormohammadi, A.H., Devlin, J.M., Mardani, K. and Ignjatovic, J., 2009. Rapid detection and non-subjective characterisation of infectious bronchitis virus isolates using high-resolution melt curve analysis and a mathematical model. *Archives of virology*, ***154***(4), pp.649-660.

Hein, R., Koopman, R., García, M., Armour, N., Dunn, J.R., Barbosa, T. and Martinez, A., 2021. Review of poultry recombinant vector vaccines. *Avian diseases*, *65*(3), pp.438-452.

Hirbaye, G., Tola, E.H., Moje, N. and Sori, T., 2024. Molecular and serological investigation of infectious bronchitis virus in the East Shewa, Central Ethiopia. *Veterinary Medicine: Research and Reports*, pp.81-90.

Hughes, C.S. and Jones, R.C., 1988. Comparison of cultural methods for primary isolation of infectious laryngotracheitis virus from field material. *Avian Pathology*, ***17***(2), pp.295-303.

Hutton, S., Bettridge, J., Christley, R., Habte, T. and Ganapathy, K., 2017. Detection of infectious bronchitis virus 793B, avian metapneumovirus, Mycoplasma gallisepticum and Mycoplasma synoviae in poultry in Ethiopia. *Tropical animal health and production*, ***49***(2), pp.317-322.

Ijoma, S., Shittu, I., Chinyere, C., Olawuyi, K.A., Gado, D.A., Nwagbo, I.O., Meseko, C.A. and Joannis, T.M., 2020. Sero-prevalence and serotypes of infectious bronchitis virus in free-range chicken in Plateau State, Nigeria.

Ike, A.C., Ononugbo, C.M., Obi, O.J., Onu, C.J., Olovo, C.V., Muo, S.O., Chukwu, O.S., Reward, E.E. and Omeke, O.P., 2021. Towards improved use of vaccination in the control of infectious bronchitis and Newcastle disease in poultry: understanding the immunological mechanisms. *Vaccines*, ***9***(1), p.20.

Ishag, Hassan Zackaria Ali, Abdelnasir Mohammed Adam Terab, Ebrahim Mohamad Abdalsalam Osman, El Tigani Ahmed El Tigani-Asil, Mohammed Saleh Albreiki, Oum Keltoum Bensalah, Asma Abdi Mohamed Shah, and Abdelmalik Ibrahim Khalafalla. "Clinical, pathological, and genotypic analysis of infectious bronchitis virus in broiler chickens in the Abu Dhabi Emirate, United Arab Emirates." *Frontiers in Veterinary Science* **11** (2025): 1474181.

Jackwood, M.W., Hall, D. and Handel, A., 2012. Molecular evolution and emergence of avian gammacoronaviruses. *Infection, Genetics and Evolution*, ***12***(6), pp.1305-1311.

Jackwood, M.W. and de Wit, S., 2013. Infectious bronchitis. *Diseases of poultry*, pp.139-159.

Jahan, M.S., Khan, M.F.R., Nazir, K.N.H., Amin, M.M. and Rahman, M.B., 2012. Sero-Surveillance of infectious laryngotracheitis in layer birds in Bangladesh. *Microbes and Health*, ***1***(2), pp.38-40.

Jones, R.M., Ellis, R.J., Cox, W.J., Errington, J., Fuller, C., Irvine, R.M. and Wakeley, P.R., 2011. Development and validation of RT-PCR tests for the detection and S1 genotyping of infectious bronchitis virus and other closely related gammacoronaviruses within clinical samples. *Transboundary and emerging diseases*, ***58***(5), pp.411-420.

Jonare, Liv. "Characterization of infectious laryngotracheitis virus from outbreaks in Swedish chicken hobby flocks." (2020).

Jones, R.C., 2010. Viral respiratory diseases (ILT, aMPV infections, IB): are they ever under control? *British poultry science*, ***51***(1), pp.1-11.

Kanwal, B., Channo, A.A., Kalhoro, N.H., Soomro, H., Korejo, N.A. and Tauseef, S., 2018. Prevalence and clinical pathology caused by infectious bronchitis virus in poultry birds at Sindh, Pakistan. *Journal of Veterinary Medicine and Animal Health*, ***10***(9), pp.231-236.

Khan, S.Z., Waqas, M. and Goyal, S.M., 2023. Infectious bronchitis: a challenge for the global poultry industry.

Kumar, S., Stecher, G., Li, M., Knyaz, C. and Tamura, K., 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. *Molecular biology and evolution*, ***35***(6), pp.1547-1549.

Lee, P.Y., Costumbrado, J., Hsu, C.Y. and Kim, Y.H., 2012. Agarose gel electrophoresis for the separation of DNA fragments. *Journal of visualized experiments: JoVE*, (62), p.3923.

Legnardi, M., Tucciarone, C.M., Franzo, G. and Cecchinato, M., 2020. Infectious bronchitis virus evolution, diagnosis and control. *Veterinary Sciences*, ***7***(2), p.79.

Lin, S.Y. and Chen, H.W., 2017. Infectious bronchitis virus variants: molecular analysis and pathogenicity investigation. *International journal of molecular sciences*, ***18***(10), p.2030.

Lin, S.Y. and Chen, H.W., 2017. Infectious bronchitis virus variants: molecular analysis and pathogenicity investigation. *International journal of molecular sciences*, ***18***(10), p.2030.

Magouz, A., Medhat, S., Abou Asa, S. and Desouky, A., 2018. Detection of infectious laryngotracheitis virus (Gallid herpesvirus-1) from clinically infected chickens in Egypt by different diagnostic methods. *Iranian journal of veterinary research*, ***19***(3), p.194.

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. and Pérez, R., 2023. Genome variability of infectious bronchitis virus in Mexico: high lineage diversity and recurrent recombination. *Viruses*, ***15***(7), p.1581.

Meir, R., Maharat, O., Farnushi, Y. and Simanov, L., 2010. Development of a real-time TaqMan® RT-PCR assay for the detection of infectious bronchitis virus in chickens, and comparison of RT-PCR and virus isolation. *Journal of virological methods*, ***163***(2), pp.190-194.

Menendez, K.R., García, M., Spatz, S. & Tablante, N.L. (2014). Molecular epidemiology of infectious laryngitis: a review. *Avian Pathology*, 43: 108–117.

Mihret, T., 2023. Seroprevalence, isolation and molecular detection of infectious bronchitis in backyard and commercial chickens in central Gondar zone, Northern Ethiopia. *Seroprevalence, Isolation and Molecular Detection of Infectious Bronchitis in Backyard and Commercial Chickens in Central Gondar Zone, Northern Ethiopia*.

Miłek, J. and Blicharz-Domańska, K., 2018. Coronaviruses in avian species–review with focus on epidemiology and diagnosis in wild birds. *Journal of veterinary research*, ***62***(3), p.249.

Mo, J. and Mo, J., 2025. Infectious Laryngotracheitis virus and avian metapneumovirus: a comprehensive review. *Pathogens*, ***14***(1), p.55.

Montassier, H.J., 2010. Molecular epidemiology and evolution of avian infectious bronchitis virus. *Brazilian Journal of Poultry Science*, *12*, pp.87-96.

Mossad, Z., Moussa, S.A., Saied, M., Fathy, M.M. and Zanaty, A.M., 2022. Molecular and genetic detection of infectious laryngeotrachitis disease virus in broiler farms after a disease outbreak in Egypt. *Virusdisease*, ***33***(4), pp.404-412.

Nguyen, V.G., Chung, H.C., Do, H.Q., Nguyen, T.T., Cao, T.B.P., Truong, H.T., Mai, T.N., Le, T.T., Nguyen, T.H., Le, T.L. and Huynh, T.M.L., 2021. Serological and molecular characterization of avian metapneumovirus in chickens in Northern Vietnam. *Veterinary Sciences*, ***8***(10), p.206.

Ou, S.C. and Giambrone, J.J., 2012. Infectious laryngotracheitis virus in chickens. *World journal of virology*, ***1***(5), p.142.

Peng, S., Wang, Y., Zhang, Y., Song, X., Zou, Y., Li, L., Zhao, X. and Yin, Z., 2022. Current knowledge on infectious bronchitis virus non-structural proteins: the bearer for achieving immune evasion function. *Frontiers in Veterinary Science*, *9*, p.820625.

Pereira, C.G., Saraiva, G.L., Vidigal, P.M.P., Fietto, J.L.R., Bressan, G.C., Moreira, M.A.S., de Almeida, M.R. and Júnior, A.S., 2016. Distribution of infectious bronchitis virus strains in different organs and evidence of vertical transmission in natural infection. *Archives of virology*, ***161***(12), pp.3355-3363.

Perez Contreras, A., van Der Meer, F., Checkley, S., Joseph, T., King, R., Ravi, M., Peters, D., Fonseca, K., Gagnon, C.A., Provost, C. and Ojkic, D., 2020. Analysis of whole-genome sequences of infectious laryngotracheitis virus isolates from poultry flocks in Canada: evidence of recombination. *Viruses*, ***12***(11), p.1302.

Pieterse, R., Strydom, C. and Abolnik, C., 2022. Effects of swab pool size and transport medium on the detection and isolation of avian influenza viruses in ostriches. *BMC Veterinary Research*, ***18***(1), p.48.

Pinto, S.C., Aleixo, J., Camela, K., Chilundo, A.G. and Bila, C.G., 2022. Seroprevalence of infectious bronchitis virus and avian reovirus in free backyard chickens. *Onderstepoort Journal of Veterinary Research*, ***89***(1), p.2042.

Pohjola, L., Tammiranta, N., Ek-Kommonen, C., Soveri, T., Hänninen, M.L., Fredriksson Ahomaa, M. and Huovilainen, A., 2017. A survey for selected avian viral pathogens in backyard chicken farms in Finland. *Avian Pathology*, ***46***(2), pp.166-172.

Portz, C., Júnior, A.B., Franco, A.C., Canal, C.W. and Reck, H., 2018. Comparision of different cell cultures for replication of infectious laryngotracheitis virus from chickens. *Acta Scientiae Veterinariae*, ***36***(2), p.101.

Rafique, S., 2018. Seroprevalence and molecular characterization of Infectious bronchitis virus variants from poultry in Pakistan. *Islamabad, Pakistan: Department of Microbiology, Quaid-i-Azam University).*

Rahman, M.M., Uddin, M.K., Hassan, M.Z., Rahman, M.M., Ali, M.Z., Rahman, M.L., Akter, M.R. and Rahman, M.M., 2018. Seroprevalence study of infectious laryngotracheitis virus antibody of commercial layer in Gazipur Districts of Bangladesh. *Asian Journal of Medical and Biological Research*, ***4***(1), pp.1-6.

Rajkumar, U., Rama Rao, S.V., Raju, M.V.L.N. and Chatterjee, R.N., 2021. Backyard poultry farming for sustained production and enhanced nutritional and livelihood security with special reference to India: a review. *Tropical Animal Health and Production*, ***53***(1), p.176.

Rana, C., Bhattarai, B., Magar, K.B.R. and Panth, Y., 2021. Avian infectious bronchitis and its management in Nepal: a review. *Journal of Agriculture and Natural Resources*, ***4***(2), pp.211-226.

Roba, Y.T., Tadesse, D., Assefa, Z. and Tesfaye, A., 2020. Seroprevalence of infectious laryngotracheitis disease in backyard chickens in villages of Ada'a district, Oromia, Ethiopia: first report. *Tropical animal health and production*, ***52***(6), pp.3109-3112.

Roba, Y.T., Wabi, B., Tsedale, T., Asamnew, T., Naol, M. and Fanos, T.W., 2021. Serological detection of antibodies against Gamma coronavirus infection in scavenging village chickens in Ada'a district, Ethiopia. *Biomedical Journal of Scientific & Technical Research*, ***33***(4), pp.26106-26110.

Ruano, M., El-Attrache, J. and Villegas, P., 2000. A rapid-plate hemagglutination assay for the detection of infectious bronchitis virus. *Avian diseases*, pp.99-104.

Saasa, N., Jesaya, O., Mwenda, R., Mukubesa, A.N., Chattah, C., Takada, A., Sawa, H., Siwila, J. and Kajihara, M., 2026. Molecular Detection and Characterization of Infectious Laryngotracheitis Virus (ILTV) Genotype VII-IX in Backyard Chickens in Lusaka and Chongwe Districts, Zambia.

Samour, J., 2015. *Avian medicine*. Elsevier Health Sciences.

Sampath, P. and DeLuca, N.A., 2008. Binding of ICP4, TATA-binding protein, and RNA polymerase II to herpes simplex virus type 1 immediate-early, early, and late promoters in virus-infected cells. *Journal of virology*, ***82***(5), pp.2339-2349.

Santander-Parra, S.H., Nuñez, L.F., Buim, M.R., Ferreira, C.S.A., Loncoman, C.A. and Ferreira, A.J.P., 2022. Detection and molecular characterization of infectious laryngotracheitis virus (ILTV) in chicken with respiratory signs in Brazil during 2015 and 2016. *Brazilian Journal of Microbiology*, ***53***(4), pp.2223-2232.

Seger, W., GhalyanchiLangeroudi, A., Karimi, V., Madadgar, O., Marandi, M.V. and Hashemzadeh, M., 2016. Genotyping of infectious bronchitis viruses from broiler farms in Iraq during 2014-2015. *Archives of virology*, ***161***(5), pp.1229-1237.

Shapiro, B.I., Gebru, G., Desta, S., Negassa, A., Negussie, K., Aboset, G. and Mechal, H., 2015. Ethiopia livestock master plan: Roadmaps for growth and transformation.

Shehata, A.A., Halami, M.Y., Sultan, H.H., Abd El-Razik, A.G. and Vahlenkamp, T.W., 2013. Chicken embryo origin-like strains are responsible for Infectious laryngotracheitis virus outbreaks in Egyptian cross-bred broiler chickens. *Virus genes*, ***46***(3), pp.423-430.

Sundgren, J., 2020. Development and validation of a novel quantitative PCR analysis method for HIV-1.

Shiferaw, J., Dego, T., Tefera, M. and Tamiru, Y., 2022. Seroprevalence of infectious bronchitis virus in broiler and layer farms of Central Ethiopia. *BioMed Research International*, ***2022***(1), p.8915400.

Shokri, S., Karimi, V., Langeroudi, A.G., Marandi, M.V., Hashamzadeh, M., Zabihipetroudi, T., Najafi, H. and Tehrani, F., 2018. Seroprevalence and genotyping of avian infectious bronchitis virus detected from Iranian unvaccinated backyard chickens. *Iranian Journal of Microbiology*, ***10***(1), p.65.

Sun, J. and Liu, S., 2016. An RT-PCR assay for detection of infectious bronchitis coronavirus serotypes. In *Animal Coronaviruses* (pp. 121-130). New York, NY: Springer New York.

Tai, Y.C., Hu, G.M. and Chen, C.M., 2025. Phylogenetic network of infectious bronchitis virus: exploring the impact of migratory birds on viral clustering, evolution, and recombination. *Veterinary Quarterly*, ***45***(1), pp.1-14.

Taha, Z. H., Allawe, A. B., & Kadhum, M. J. (2017). *Isolation and sequencing of field isolates of infectious laryngeotracheitis virus in Iraq*. *5*(5), 882–886.

Tegegne, D. Y. Deneke, T. Sori (2020). "Molecular epidemiology and genotyping of infectious bronchitis virus and avian metapneumovirus in backyard and commercial chickens in Jimma zone, southwestern Ethiopia," *Veterinary Sciences*, **7**(4), 187

Tekelemariam, T.H., Walkden-Brown, S., Atire, F.A., Tefera, D.A., Alemayehu, D.H. and Gerber, P.F., 2022. Detection of chicken respiratory pathogens in live markets of Addis Ababa, Ethiopia, and epidemiological implications. *Veterinary sciences*, *9*(9), p.503.

Tesfaye, A., Sahle, M., Sori, T., Kassa, T., Garoma, A., Koran, T., Dima, C., Guyassa, C., Hilu, H., Guta, S. and Tadesse, F., 2019. Infectious laryngotracheitis virus in commercial and backyard chicken production systems in central and South Ethiopia (first report) ILT in Ethiopian poultry production. *Journal of Applied Poultry Research*, ***28***(4), pp.1324-1329.

Tesfaye, A., Kassa, T., Mesfin, S., Garoma, A., Koran, T., Dima, C., Guyassa, C., Hailu, H. and 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), p.1001.

Thiry, E., Meurens, F., Muylkens, B., McVoy, M., Gogev, S., Thiry, J., Vanderplasschen, A., Epstein, A., Keil, G. and Schynts, F., 2005. Recombination in alphaherpesviruses. *Reviews in medical virology*, ***15***(2), pp.89-103

Thrusfield, M., 2018. *Veterinary epidemiology*. John Wiley & Sons.

Uddin, M.I., Sen, A.B., Islam, M.S., Shubhagata Das, S.D., Nasima Sultana, N.S., Ripa, R.N., Abul Kashem, A.K. and Kamaruddin, K.M., 2014. Seroepidemiology of infectious laryngotracheitis (ILT) in the commercial layer farms of Chittagong district, Bangladesh.

Ul Hayat, S., Gul, S. and Gul, S., 2018. 4. Isolation, identification and molecular characterization of virulent avian infectious bronchitis virus in Khyber Pakhtunkhwa, Pakistan. *Pure and Applied Biology (PAB)*, ***7***(2), pp.435-442.

Valastro, V., Holmes, E.C., Britton, P., Fusaro, A., Jackwood, M.W., Cattoli, G. and Monne, I., 2016. S1 gene-based phylogeny of infectious bronchitis virus: an attempt to harmonize virus classification. *Infection, Genetics and Evolution*, *39*, pp.349-364.

Vashisht, V., Vashisht, A., Mondal, A.K., Farmaha, J., Alptekin, A., Singh, H., Ahluwalia, P., Srinivas, A. and Kolhe, R., 2023. Genomics for emerging pathogen identification and monitoring: Prospects and obstacles. *BioMedInformatics*, ***3***(4), pp.1145-1177.

Villarreal, L.Y.B., 2010. Diagnosis of infectious bronchitis: an overview of concepts and tools. *Brazilian Journal of Poultry Science, **12***(2), pp.111-114.

Volkening, J.D., Spatz, S.J., García, M., Ross, T.A., Maekawa, D.A., Rosenthal, K.S., Zamora, A.C., Skipper, A., Blakey, J.R. and Paudel, R., 2025. A functional interleukin-4 homolog is encoded in the genome of infectious laryngotracheitis virus: unveiling a novel virulence factor. *PLoS pathogens*, ***21***(7), p.e1013219.

Wadas, I. and Domingues, I., 2025. Systematic review of phylogenetic analysis techniques for RNA viruses using bioinformatics. *International Journal of Molecular Sciences*, ***26***(5), p.2180.

Wayou, B.A., Kassa, G.M., Pasotto, D., Sori, T., Tucciarone, C.M. and Cecchinato, M., 2021. Molecular survey of viral poultry diseases with an indirect public health significance in central Ethiopia. *Animals*, *11*(12), p.3564.

Wickramasinghe, I.A., De Vries, R.P., Gröne, A., De Haan, C.A.M. and Verheije, M.H., 2011. Binding of avian coronavirus spike proteins to host factors reflects virus tropism and pathogenicity. *Journal of virology*, ***85***(17), pp.8903-8912.

WOAH (World Organisation for Animal Health). (2021). *Infectious laryngotracheitis (ILT)*. In *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (Chapter 3.3.5). Paris, France: WOAH.Available at: https://www.woah.org/en/what we do/standards/manual-of-diagnostic-tests-and-vaccines-for-terrestrial-animals/

World Organisation for Animal Health (WOAH). (2018). *Infectious bronchitis (chapter 2.03.02).*In WOAH Terrestrial Manual 2018. Retrieved from https://www.woah.org/fileadmin/Home/eng/Health_standards/tahm/2.03.02_AIB.pdf

Roba, Y.T., Tadesse, D., Assefa, Z. and Tesfaye, A., 2020. Seroprevalence of infectious laryngotracheitis disease in backyard chickens in villages of Ada'a district, Oromia, Ethiopia: first report. *Tropical animal health and production*, ***52***(6), pp.3109-3112

Roba, Y.T., Wabi, B., Tsedale, T., Asamnew, T., Naol, M. and Fanos, T.W., 2021. Serological detection of antibodies against Gamma coronavirus infection in scavenging village chickens in Ada'a district, Ethiopia. *Biomedical Journal of Scientific & Technical Research*, ***33***(4), pp.26106-26110.

Yirga Haile, Y.M., Tadesse, T. and Bekuma, A., 2018. Coprological Study on Lungworm Infection of Small Ruminants in and Around Eteya Town, West Arsi, Ethiopia. *American-Eurasian Journal of Scientific Research*, *13*(3), pp.47-52.

Yegoraw, A.A., Assen, A.M., Gerber, P.F. and Walkden-Brown, S.W., 2021. Transmission of infectious laryngotracheitis virus vaccine and field strains: the role of degree of contact and transmission by whole blood, plasma and poultry dust. *Veterinary research*, ***52***(1), p.91.

Zhao, Y., Kong, C., Cui, X., Cui, H., Shi, X., Zhang, X., Hu, S., Hao, L. and Wang, Y., 2013. Detection of infectious laryngotracheitis virus by real-time PCR in naturally and experimentally infected chickens. *PloS one*, ***8***(6), p.e67598.

Zhao, F., Han, Z., Zhang, T., Shao, Y., Kong, X., Ma, H. and Liu, S., 2014. Genomic characteristics and changes of avian infectious bronchitis virus strain CK/CH/LDL/97I after serial passages in chicken embryos. *Intervirology*, *57*(6), pp.319-330.

8. APPENDICES

Appendix 1: Clinical signs of Infectious Laryngotracheitis (ILTV) and IBV in chicken, exhibiting gasping and respiratory distress.

Appendix 2: a) Blood collection from the wing vein of a chicken and b) Oropharyngeal swab sample collection from a chicken.

Appendix 3: Protocol of indirect ELISA for IBV

1. 245 µL of dilution buffer 14 to all wells except the control wells A1, B1, C1, and D1.
2. In a pre-dilution plate set aside wells A_1, B_1, C1 and D_1 for the controls and add;

5 µL of each sample to be tested.

3. In the ELISA micro plate, add:

100 µL of the negative control to wells A1 and B1

100 µL of the positive control to wells C1 and D1

90 µL of dilution buffer 14 to as many wells as there are samples to be tested (not to control wells A1, B1, C1, and D1).

10 µL of the pre-diluted sample as prepared above.

4. Cover the plate and incubate 30min ± 3 at 21c° (5 °C).

5. Prepare the conjugate 1x by diluting the concentrated conjugate 10x to 1:10 in dilution buffer 3.

6. Empty the wells. Wash each well 3 times with at least 300 µL of the wash solution 1X. Avoid drying of the wells between washes.

7. Add 100ul of the conjugate 1X to each well.

8. Cover the plate and incubate 30min ± 3min at 21c° (5°C).

9. Empty the wells. Wash each well 3 times with at least 300 µL of the wash solution 1X. Avoid drying of the wells between washes.

10. Add 100 µL of the substrate solution to each well.

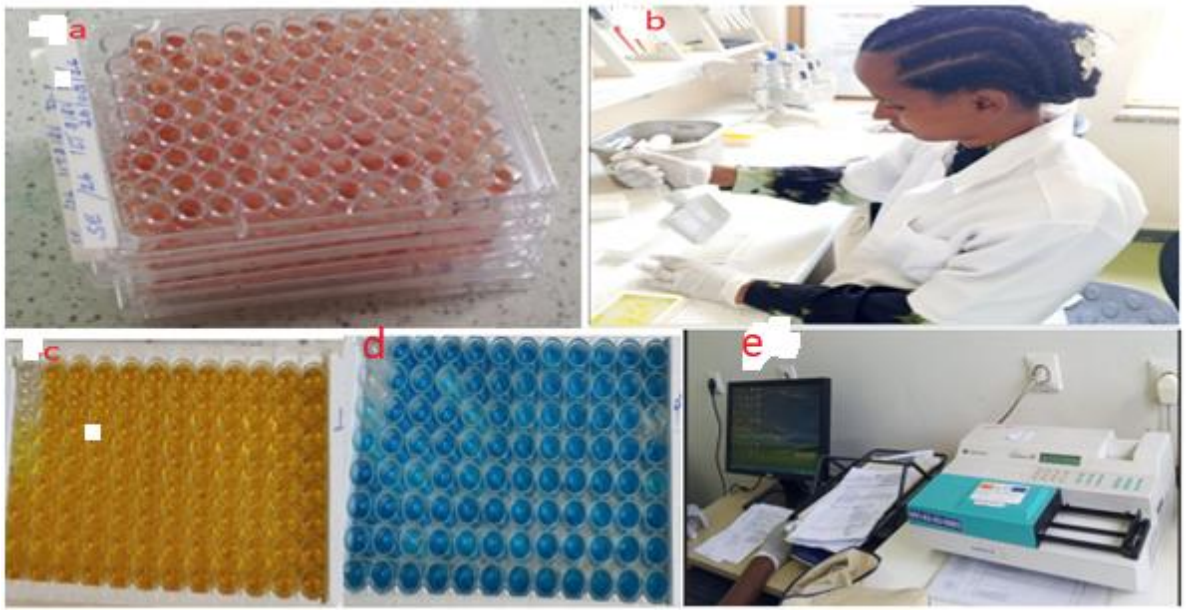
11. Cover the plate and incubate 15min ± 2min at 21c° (5°C) in the dark.

12. Add 100 µL of the stop solution to each well in the same order as in step no. 10 to stop the reaction.

13. Read and record the O.D. at 450nm.

Appendix 4: laboratory work during serological analysis:-

a) Plated samples into the well microplate , b) Introduction of reagents into the wells of the ELISA microplate; c) Formation of yellow color up on addition of the stop solution; d) Formation of blue color upon addition of substrate solution; e) Measurement and recording of optical density results using automated ELISA plate reader.



Appendix 5: 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 6: Laboratory work during isolation of virus:-

a) Incubation of the eggs, b) Embryo candling, c) inoculation of virus in to eggs, d) sealing of the eggs that have been inoculated with the viruses, and e) Allanntoic fluid harvest, and f) observation of embryo after virus inoculated.

Appendix 7: Primary cell culture preparation.

1. By using sterile technique, the embryo was removed 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 to 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, then 10% minimum essential media (MEM) was added.

8. Pour on tissue flask and incubate at 37°C until the cells are confluent.

Appendix 8: Laboratory works during the embryo cell culture preparation:-

5a Harvesting the embryo from an embryonated egg, 5b Trypsin treatment to dissociate cells, 5c Filtrating to obtain cell suspension, 5d Centrifugation of the cell suspension, 5e Inoculated cell culture flasks, 5f Observation for cytopathic effects (CPE).

Appendix 9: Ethical clearance

አዲስ አበባ ዩኒቨርሲቲ
የእንስሳት ሕክምናና
ግብርና ኮሌጅ
ቢሾፍቱ

ADDIS ABABA UNIVERSITY
College of Veterinary Medicine
and Agriculture
Bishoftu

Research Ethics Review Committee

Ethical clearance certificate

Certificate Ref. No: VM/ERC/09/112/18/2026

Name of Applicant: **Almaz Dawit Ragasa (BSc in Vet. Lab. Tech, MSc Student)**

Address: Department of Biomedical Sciences, College of Veterinary Medicine and Agriculture, Addis Ababa University

Title of the project: *Isolation and molecular detection of Infectious Bronchitis and Infectious Laryngotrachitis viruses in chickens in Maya city and seroprevalence in selected areas of Oromia, Ethiopia.*

Date of application: **November, 2025**

Nature of the project: **Field investigation**
Target animal species: **Domestic chickens**
Number of animals involved: **509**
Study area: **Selected areas of Oromia Region, Ethiopia**

Minutes No. and date of review: **VM/ERC/09/18/026, 26/03/2026**

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 Student Almaz Dawit Regasa.

Professor Getachew Terefe (DVM, PhD)
Chairman

Federal Democratic Republic of Ethiopia
Addis Ababa University
College of Veterinary Medicine & Agriculture

Signature

መልሱን በሚጽፉልን ጊዜ ባክዎን የኛን ደብዳቤ ቁጥር ይጥቀሱልን
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