Isolation And Molecular Identification of Infectious Laryngotracheitis Virus from active outbreak in Poultry: the Case of Mekelle Town and Lafto Wereda of Addis Ababa

Misganu Taso

A thesis submitted to the Department of Applied Biology

School of Applied Natural Sciences

Presented in partial fulfillment of the requirement for the degree of master's in biotechnology

Office of Graduate Studies

Adama Science and Technology University

October, 2024

Adama, Ethiopia

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Misganu Taso

Advisor: Hunduma Dinka (Professor.)

Co-Advisor: Yeneneh Tesfaye (Phd)

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DECLARATION

I hereby declare that this Master Thesis entitled "**Isolation And Molecular Identification of Infectious Laryngotracheitis Virus from active outbreak in Poultry: the Case of Mekelle Town and Lafto Wereda of Addis Ababa**" is my original work. That is, it has not been submitted for the award of any academic degree, diploma, or certificate from any other university. All Sources of materials that are used for this thesis have been duly acknowledged through citations.

_____ _____ _____

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RECOMMENDATION OF THE ADVISORS

We, the advisors of this thesis, hereby certify we have read the revised version of The thesis entitled **Isolation And Molecular Identification of Infectious Laryngotracheitis Virus from active outbreak in Poultry: the Case of Mekelle Town and Lafto Wereda of Addis Ababa** " prepared under my/our guidance by **Misganu Taso** submitted in partial fulfillment of the requirements for the degree of Masters of Science in Biotechnology.

Therefore, we recommend the submission of the revised version of the thesis to the department following the applicable procedures.

_____	_____	_____
Major Advisor	Signature	Date
_____	_____	_____
Co-advisor	Signature	Date

APPROVAL SHEET

We, the advisors of the thesis entitled "**Isolation And Molecular Identification of Infectious Laryngotracheitis Virus from active outbreak in Poultry: the Case of Mekelle Town and Lafto Wereda of Addis Ababa**" developed by **Misganu Taso** hereby certify that the recommendations and suggestions made by the board of examiners are appropriately incorporated into the final version of the thesis.

Major Advisor	Signature	Date
Co-advisor	Signature	Date

We, the undersigned, members of the Board of Examiners of the thesis by **Misganu Taso** have read and evaluated the thesis entitled "**Isolation And Molecular Identification of Infectious Laryngotracheitis Virus from active outbreak in Poultry: the Case of Mekelle Town and Lafto Wereda of Addis Ababa**" and examined the candidate during open defense. This is, therefore, to certify that the thesis is accepted for partial fulfillment of the requirement of the degree of Master of Science in Biotechnology.

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LIST OF ABBREVIATIONS

CAM	Chorioallantoic Membrane
CEO	Chicken Embryo Origin
CKC	Chicken Kidney cell
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediamine Tetraacetic Acid
FPV	Fowl Pox Virus
GaHV-1	Gallidalphaherpesvirus 1
HVT	Herpes virus Turkey
ICP	Infected-Cell Protein
ILT	Infectious laryngotracheitis
ILTV	Infectious laryngotracheitis Virus
MICE	Mortality Index for Chicken Embryos
NVI	National Veterinary Institute
ORF	Open Reading Frame
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
RNA	Ribonucleic acid
SPSS	Statically Package for Social Sciences
TAE	Tris-acetate-EDTA
TCO	Tissue Culture Origin
WOAH	World Organisation for Animal Health

ABSTRACT

Infectious laryngotracheitis (ILT) is a highly contagious upper respiratory tract disease caused by the Gallid herpesvirus 1 in chickens. In Ethiopia, there have been few scientific reports and molecular isolations of the virus regarding the status of ILT. Therefore this study aimed to isolate and identify the virus from outbreak areas using molecular techniques. Specimens were collected from chickens managed under intensive production systems that were suspected of having Infectious Laryngotracheitis (ILT) disease. A total of eighty-seven samples were collected, comprising 24 tissue samples and 63 tracheal swab samples from Mekele and Lafto Wereda in Addis Ababa, which were then pooled together. The Infectious Laryngotracheitis Virus was screened and isolated using a conventional polymerase chain reaction with a set of primers specifically designed to amplify a fragment of the ICP4 gene. The PCR products of the isolated DNA were analyzed through agarose gel electrophoresis. Consequently, one pooled sample from Mekele and three pooled samples from Lafto Wereda tested positive for ILTV among the eleven pooled samples from both study areas. The isolated virus was propagated via chorioallantoic membrane (CAM) inoculation in 9–11-day-old embryonated chicken eggs, as well as in chicken embryonic fibroblast (CEF), Vero, and DF-1 cell lines. Cytopathic effects (CPE) were observed following the inoculation of the virus in different cell types. Generally, in the study areas, the disease that posed a respiratory challenge and caused the outbreak in the chickens was the Infectious Laryngotracheitis Virus. To combat this challenge, all poultry farm owners in the study areas and the whole country should be aware of control measures for the disease.

Keywords: *Chorioallantoicmemberane, Infectious laryngo tracheitis, specimen, strain,*

1. INTRODUCTION

1.1 Background of the study

Poultry farming is one of the industries that is quickly growing and is crucial to the world's food security (Gowthaman *et al*., 2020). However, numerous diseases have become bottlenecks due to the effects of globalization, climate change, and the rapidly growing poultry population. One of the diseases that is an obstacle to poultry farming is infectious laryngotracheitis (Birhan *et al*., 2022).

Infectious laryngotracheitis (ILT), is a highly contagious upper respiratory tract illness that affects hens and is thought to be extremely dangerous for the health and welfare of poultry (Bagust *et al*. 2000). It was first described in the USA in 1925 and is of the commercially significant poultry illnesses that affect chickens. It is a severe, extremely contagious viral disease of poultry that mostly affects the upper respiratory system. Because of the ILT's severe economic effects on the poultry industry including morbidity, mortality, weight loss, and decreased egg production, the poultry industry is greatly affected by it (Tsiouris *et al*., 2021). ILT virus (ILTV) also known as Gallid herpes virus 1 (GaHV-1), is the cause of the disease.

The virulence of a given strain or isolate of ILTV determines how severe the clinical symptoms are, and fatality rates can range from 0% to more than 70% (Fuchs *et al*., 2007; Parra *et al*., 2016). Nasal discharge, conjunctivitis, and decreased egg production are signs of lesser forms of ILT, but severe forms include gasping, coughing, expectorating bloody mucus, and serious dyspnea (Sellers *et al*., 2004; Timurkaan *et al*., 2003). Pathomorphological changes are occasionally observed in the lungs as well as the conjunctiva, larynx, and trachea (Fuchs *et al*., 2007). Rojs et al. (2021) stated that, in the same way as other herpesviruses, ILTV can establish persistency in the trigeminal ganglia of the central nervous system after 7 days of acute infection. As a result, the virus is reactivated in stressful situations like the start of laying or the transfer and mixing of flocks. In Ethiopia, practically every rural family raises chickens as a valuable source of nourishment for the family and as a source of revenue (Birhan *et al*., 2022). Nevertheless, the industry continues to face obstacles related to bio-security and its significance for poultry farming, so it is likely to underestimate the potential role of those centers in

disseminating transmissible avian illness to a large number of backyard flocks and to those living in rural areas (Marga, 2023). In Ethiopia, few studies conducted that targeted the isolation and characterization of the virus by molecular tools. As a result, Birhan *et al*. (2022) suggested, that molecular confirmation and characterization of the virus from ILT cases should be considered from the disease outbreak occurrence areas of the country.

1.2 Statements of the Problem

Poultry farm is the second-most economically imperative livestock in Ethiopia. The chicken breeds participating in the grazing production system are mainly local breeds and hybrids between local and foreign breeds; less than 10% of chickens are imported. Closed intensive production systems are also common in Ethiopia, with small-scale intensive production being widely adopted in urban and peri-urban areas (Habte *et al.*, 2022). However, Birhan et al. (2021) stated that Poultry farms and farmers are still facing challenges such as diseases and predators, extension issues, the adaptation challenges of exotic chickens, and a lack of veterinary services problems in the poultry industry. Diseases that can threaten poultry health include several viruses that cause respiratory infections in poultry, like Newcastle disease, infectious bronchitis virus, infectious laryngotracheitis (ILT), pneumonia virus infection, and avian influenza (Tesfaye *et al*., 2019).

Even though, few studies have been conducted in Ethiopia that provide evidence of chicken exposure to the virus (Biran *et al*., 2022; Roba *et al*., 2020; Tesfaye *et al.*, 2019), so far there have been no proper study conducted that targeted for the isolation and characterization of the virus by molecular tools. Furthermore, Birhan *et al*. (2022) suggested, that molecular confirmation and characterization of the virus from ILT cases should be considered to use ILT vaccines.

1.3 Objectives of the study

1.3.1 General objective

The general objective of this research thesis is to isolate and identify Infectious Laryngotracheitis Virus in poultry from active outbreak areas by molecular tools

1.3.2 Specific objectives

- To detect and identify ILTV from active outbreaks in poultry by molecular tools
- To isolate the identified ILTV by using primary cells and Vero cell line

1.4 Significance of the study

In Ethiopia, chickens are frequently afflicted by infectious laryngotracheitis, a significant respiratory issue. So, the study had to employ specific techniques to identify and isolate the chickens suspected of having ILTV in the designated region. Accordingly, the study outcomes can contribute to confirming the presence of ILTV in the area under investigation. Furthermore, it is believed to hold valuable information on ways to prevent and regulate the condition (ILT), and it supports veterinarians in devising effective methods and campaigns to raise awareness and tackle ILT.

1.5 Research hypothesis

The research hypothesis is stated as:

H0: Infectious Laryngotracheitis virus is not present in the study area

HA: Infectious Laryngotracheitis virus is present in the study area

2. LITERATURE REVIEW

2.1 Etiology

2.1.1 The Virus

Infectious Laryngotracheitis is a disease that happens when a virus called infectious laryngotracheitis infects the body of the chicken. This virus is also known as Gallid herpesvirus 1 (GaHV-1) and belongs to a group of viruses called *Iltovirus*. These viruses are part of the *Alphaherpesvirinae* subfamily and *Herpesviridae* family (Gowthaman *et al*., 2020). ILT is prevalent around the world in regions with high levels of chicken production. Under electron microscopy, ILTV virions appear to be conventional herpes virions, with a DNA core inside an icosahedral capsid surrounded by a tegument layer and outer envelope glycoproteins (Thureen and Keeler 2006). The viral capsid is around 100 nm in diameter, while the total viral particle size ranges from 200 to 350 nm (Granzow *et al*., 2001). The ILTV genome has 80 open reading frames (ORFs), 65 of which are in the UL region, 9 in the US region, and 6 in the IR region (Lee *et al*., 2011; McGeoch *et al*., 2000; Thureen and Keeler, 2006). Among the 80 ORFs, sixty-three have homologies to the Herpes Simplex Virus-1 (HSV-1) genome in terms of position and structure of the deduced translation products. The envelope comprises glycoproteins gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, and gM, which are encoded by highly conserved ORFs UL27, UL44, US6, US8, US4, UL22, US7, US5, UL53, UL1, and UL10, respectively (Piccirillo *et al*., 2016).

The virus's native host is chickens, but it can also infect pheasants. In locations where there is a high bird density, it can also harm younger birds, however, it often affects chickens older than 20 days. In earlier times, photographs showcasing infected chicken embryo cells demonstrated the presence of nucleocapsids, which exhibited a structure resembling that of an icosahedron. These structures were similar to ones found in a virus called human herpes simplex virus Type 1. The hexagonal part of the virus is about 80-100 nanometers wide and the whole virus is surrounded by a lumpy envelope that is about 195-250 nanometers wide. Genetic material is made up of a long, double-stranded DNA molecule like that of other *Herpesviridae* family (Jones, 2021).

This virus belongs to a member of the family *Herpesviridae's* genus *Iltovirus* and subfamily *Alphaherpesvirinae* (Davison *et al*. 2009)). The genome of ILTV is linear and approximately 150 kb of double-stranded DNA and it contains G + C content of 48.16%

and a virus that is enveloped, non-segmented, and linear. Their viral particles are icosahedral in shape and include 162 elongated hollow capsomeres within hexagonal nucleocapsids that measure 80–100 nm in diameter (Gowthaman *et al*., 2020). The GaHV-1 genome has a density of 1.704 g/mL and a molecular weight of approximately 1 x 10^8, and it consists of a linear double-stranded molecule with a unique long region and a unique short region (Figure 1) surrounded by inverted repeat (Parra *et al*., 2016). In the early study as Menendez et al. (2014) stated the genes targeted for the differentiation of ILTV strains were gG, gB, ICP4, ORF A/B, and TK. However; currently a region of genes used for detection of virus by using PCR is infected cell protein (ICP4) (Kalin *et al*., 2020).

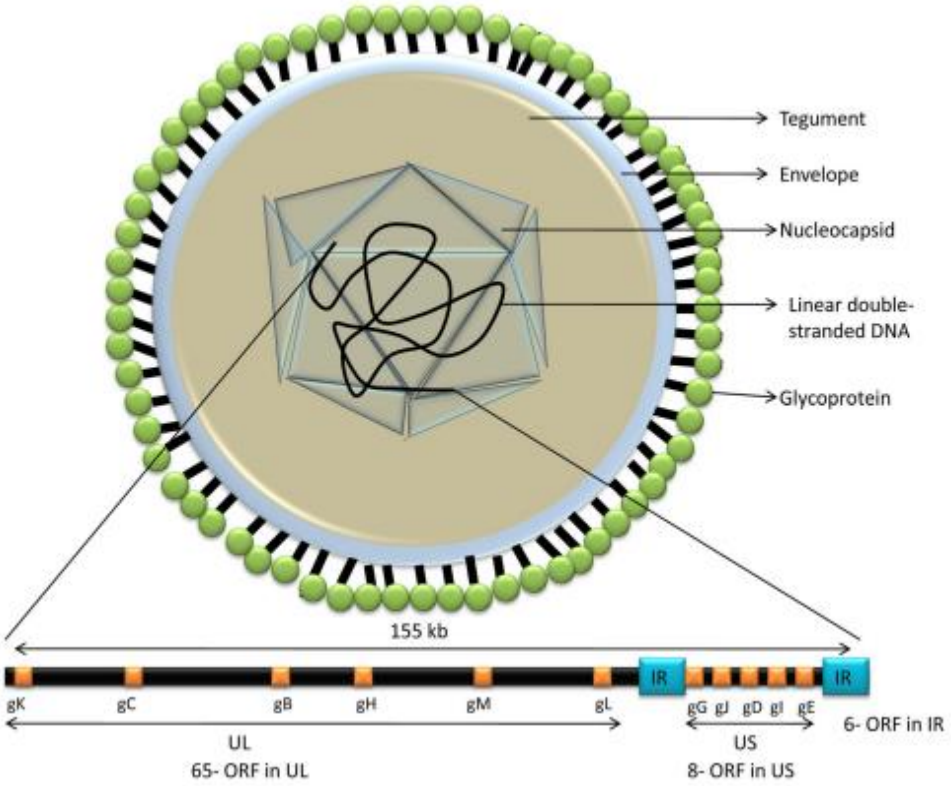

Figure 1: Structure of Infectious laryngotracheitis virus. Adopted from Gowthaman., et al. (2016).

2.1.2 Viral replication

ILTV replication occurs within the first week of infection (Bagust, 1986; Williams *et al*., 1992). It mostly affects the conjunctiva and tracheal mucosa, resulting in inflammation, serous or mucoid discharge, and respiratory discomfort (Coppo *et al*., 2013). ILTV's

interaction with the nasal cavity, conjunctival mucosa, and harderian glands is crucial for early virus multiplication and infection outcome (Beltrán *et al*., 2017). The respiratory system affects epithelial cells in the larynx and trachea, but not the sinuses, air sacs, or lung tissues (Hanson and Bagust 1991).

The replication of the virus occurs in the tracheal epithelium while poultry is exposed to the virus through nasal, oral, or conjunctiva as indicated in figure 2. It happens during the first week after infection, with Latency developing in the trigeminal ganglia and trachea, and then the virus can reactivate and spread at different sites (Tran *et al*., 2021). This virus also replicated in other cells like mucous membranes including air sacs, conjunctiva, lungs, and respiratory sinuses (Guy and Garcia, 2008).

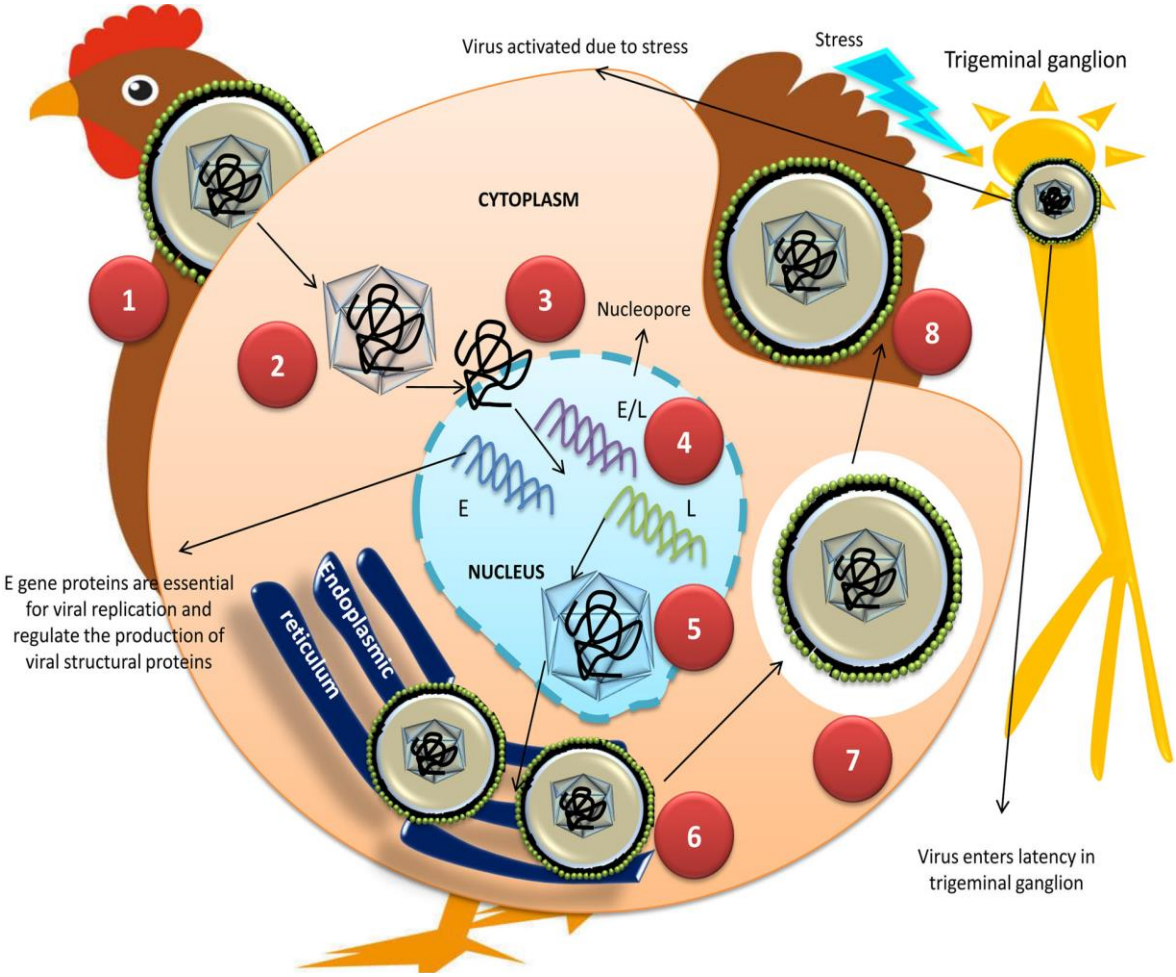

Figure 2: Replication of ILT virus. 1. Attachment. 2. Tegument and nucleocapsids are transferred into the cytoplasm. 3. Viral DNA released from the nucleocapsid enters the nucleus via nuclear pores. 4. During viral transcription and translation, three gene classes are expressed based on their expression levels:

early (E), early/late (E/L), and late (L). 5. Nucleocapsids containing DNA acquire an envelope while budding out of the inner lamellae of the nuclear membrane. 6. Virions enter the endoplasmic reticulum and form a second envelope before accumulating in cytoplasmic vacuoles. Exocytosis or cell lysis releases the virions from their vacuoles (7,8). Adapted from (Gowthaman *et al*., 2020).

2.1.3 Antigenicity of ILTV

As virus-neutralization and cross-protection studies done in the 1960s in the United States GaHV-1 vaccine strains were antigenically homogeneous. However, Cross-reactivity tests with heterologous antisera revealed modest antigenic differences among Australian field isolates from the 1970s (Garcia *et al*, 2013). The envelop glycoproteins of ILTV seem to be the potent immunogenic protein capable of stimulating humoral as well as cell-mediated immune responses in chickens. ILTV antigens are proteins like gB, gC, gD, gE, gG, gH, gI, gJ, gK, gL, and gM. They are important for viruses to enter cells and reproduce Among envelop glycoproteins, glycoprotein G (gG) is identified to facilitate virus entry cell-to-cell spread and functions as a broad-spectrum viral chemokine binding protein (Hidalgo, .2013).

2.1.4 Physico-chemical properties of ILTV

The enveloped nature of ILTV made the virus sensitive to an organic solvent such as chloroform, ether, and oxidizing agents like H2O2 (Gowthaman *et al*., 2020) but can survive for several months when stored in suitable diluents, such as glycerol (50%) and sterile skimmed milk at 4°C (Bagust *et al*., 2000). The sensitivity of ILTV to the temperature differs greatly between its strains. In respiratory exudates and chicken carcasses, the virus can remain infective for 10 days to 3 months at a temperature range of 13-23°C (Gowthaman *et al*., 2020).When stored at -20 °C to -60 °C, ILTV was viable for months to years (Ou and Giambrone, 2012). Chemical disinfectants derived from coal tar, formaldehyde, hypochlorite, and iodophor can effectively inactivate the virus (Guy and Garcia, 2008). Commercially available biofilm-reducing sanitizers are effective for removing residual vaccine virus from water lines and nipple drinkers after consecutive flock vaccination via drinking water (Ou *et al.*, 2011).

2.1.5 Pathogenesis

In the development of the disease, the virus mainly targets the cells in the trachea and epithelium of the larynx. It has been said that the virus is multiplying in the eyes, sinuses, lungs, and air sacs. Scientists have found that the virus can cause a lot of damage to these tissues. It can also make them bleed and harm the protective layer of cells. In experimental studies, tracheal tissues and secretions are present on days 6-8 of virus inoculation. The most common lesions like Bloody, mucopurulent, fibrinous exudate deposits and diphtheroid lesions were observed in the lumen of the trachea and larynx **(**Figure 2, A-D) (Yavuz *et al*., 2018)

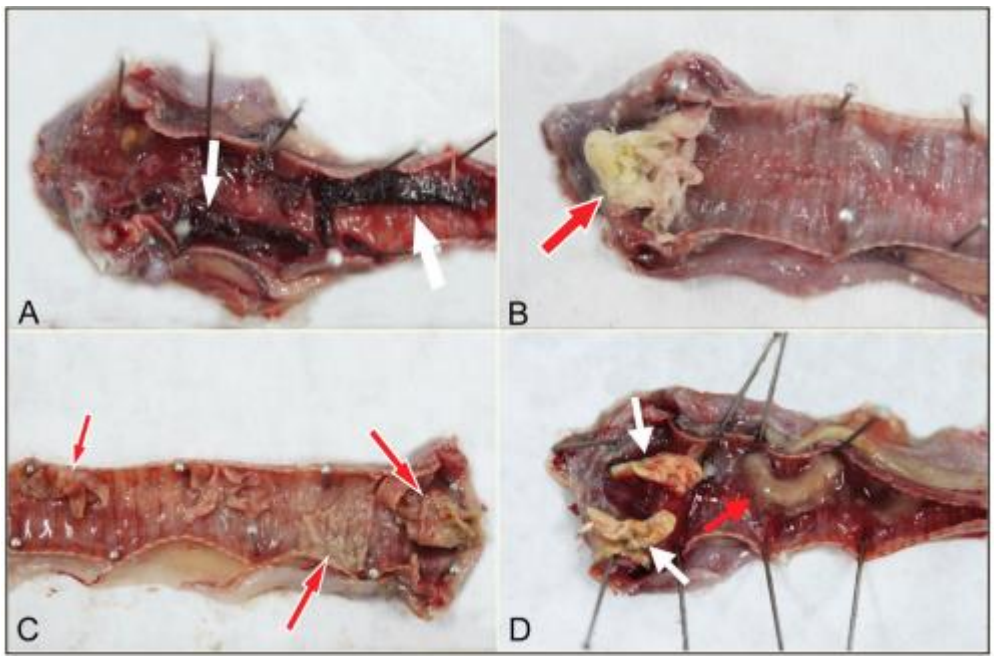

Figure 3: Tracheal lesion resulting from ILT diseases. Bloody(A), mucopurulent (B-D) Source: Yavuz *et al*. (2018).

2.2 Epidemiology of ILT

ILTV was first reported in the USA in 1925 and subsequently in Australia, Europe, and UK. It was regarded as the first viral disease of poultry for which the vaccine was administered via cloaca. As Linares et al. (1994), stated ILT continues to pose a significant threat and has adversely affected the global poultry industry since it was first identified. Birds of all ages, from eight days to four years old can contract ILTV infection, but those

over three weeks old are particularly vulnerable. Factors such as intensive poultry farming, the mixing of different bird species in the same area, and lapses in biosecurity frequently lead to ILT outbreaks worldwide. The rates of illness and death vary based on the virulence of the circulating ILTV strains (Oldoni *et al*.2009). Sporadic ILTV cases can arise in flocks that are not adequately vaccinated, either due to mistakes in vaccine administration or biosecurity breaches. In multi-aged layer farms, flocks that are not properly vaccinated may be exposed to ILTV when younger vaccinated birds are introduced (Hidalgo *et al* 2003). In densely populated poultry areas, both vaccine and field strains of ILTV can evolve to become more virulent due to the continuous presence of the virus, leading to outbreaks (Kotiw *et al*. 1995). Regions with high poultry density often suffer significant economic losses, with overall mortality rates reaching as high as 70% (Bagust *et al*. 2000).

At present, ILT is still a significant illness with reports from most nations in the world (Gowthaman *et al*., 2020). This disease is more prevalent in areas with high numbers of poultry and intensive poultry farming. Several causes have led to the global increase in ILT outbreaks, including the tendency towards denser chicken populations grown in shorter cycles, the raising of multiple kinds of poultry (commercial layers and broilers) in the same area, and inadequate biosecurity (Garcia *et al.* 2013). In general, unnoticed and occasional reactivations with active replication in the tracheal epithelium result in the shedding of the virus and the spread of infection to vulnerable birds (Bagust and Johnson 1995). Previous research has shown that the presence of long-term tracheal carriers among birds recovering from acute ILT infection significantly contributes to the establishment of latency (Gowthman *et al*, 2020). Additionally, backyard poultry flocks serve as a crucial source of infection for commercial poultry due to viral latency (Ojkic *et al.* 2006). Studies also indicate a high seroprevalence of ILTV (72%) in non-vaccinated flocks, highlighting the impact of backyard poultry on its epidemiology (Hernandez-Divers *et al*. 2008).

2.2.1 Current status of ILT in Ethiopia

In Ethiopia, Infectious Laryngotracheitis (ILT) has long been overlooked as a non-existent disease affecting poultry. However, a study conducted in the Hawasa region revealed the presence of the disease, marking the first report of Infectious Laryngotracheitis Virus (ILTV) in the Southern Nations, Nationalities, and Peoples Regional State (Mekibib et al., 2019). Despite the disease's economic impact and high transmissibility, there are few scientific studies addressing ILTV in Ethiopia. Furthermore, various studies conducted

across the country have confirmed the disease's presence in several regions, including Amhara (Birhan et al., 2022), Nekemt (Balcha et al., 2023), and around Bishoftu town and the Liban Cuqala district (Marga, 2023). Nevertheless, the clear clinical symptoms observed in the field, coupled with the government's increasing interest in commercializing poultry production, have created an urgent national need to identify the disease and develop effective intervention strategies (Birhan et al., 2022). As poultry production in Ethiopia continues to expand, inadequate biosecurity measures have contributed to a rise in various poultry viral diseases, including Infectious Laryngotracheitis. The overall prevalence of ILT was found to be 19.4%, with 13.3% in commercial poultry systems and 34.4% in backyard poultry systems. A significant difference in prevalence was observed between these two types of poultry production systems (Tesfaye et al., 2019).

2.2.2. *Host range of infectious laryngotracheitis disease*

Similar to other avian herpesviruses, ILTV has a very limited host range in both in-culture and in vivo settings. Other than domestic chickens, the only other natural hosts are pheasants. Moreover, the virus has been reported to infect turkeys in experiments and to be isolated from peafowl (Fuchs *et al*, 2007). ILTV has a small host range compared to other alphaherpesviruses. ILTV primarily infects chickens, although it has also been documented in peacocks, pheasants, turkeys, and guinea fowl (Bautista, 2003; Crawshaw and Boycott, 1982). Ducks are resistant to ILT infection but can serve as carriers (Yamada *et al*., 1980). Domestic and feral birds, including quail, guinea fowl, pigeons, starlings, sparrows, crows, and doves, have shown resistance to the disease (Beach, 1931; Brandly and Bushnell, 1934). Ou and Giambrone, (2012) declared all ages of chickens can contract the laryngotracheitis virus, however, those older than three weeks have a higher risk of infection.

2.2.3. *Transmission of infectious laryngotracheitis disease*

LTV enters the host through the eyes, respiratory system, and, to a lesser degree, the oral cavity.

Compared to contact with latently sick or carrier birds, direct bird-to-bird transmission is extremely common. It's crucial to direct transmission when mixing vaccinated and unvaccinated chickens. There is no proof of either vertical transmission or virus transmission via the eggshell (Oldoni *et al*. 2007).

As previously described by Cook *et al* (1992) an additional source of infection for naive birds is carrier birds who have survived earlier epidemics. Compared to birds who have recovered clinically or latent carriers, sick birds spread the disease more easily through oral secretions. The virus is typically transmitted into a flock through direct contact with respiratory exudates or indirect/mechanical transmission of contaminated items, such as litter, feed bags, feathers, vehicles, dust, footwear, clothing, and movement of people (Yegoraw *et al* 2021). Ou *et al* (2011) demonstrated that ILTV can survive in the biofilm of drinking water lines and spread to susceptible birds. Darkling beetles and mealworms are also sources of infection for birds, and the live virus has been demonstrated in darkling beetles even 42 days after the illness outbreak (Ou and Giambrone 2012).

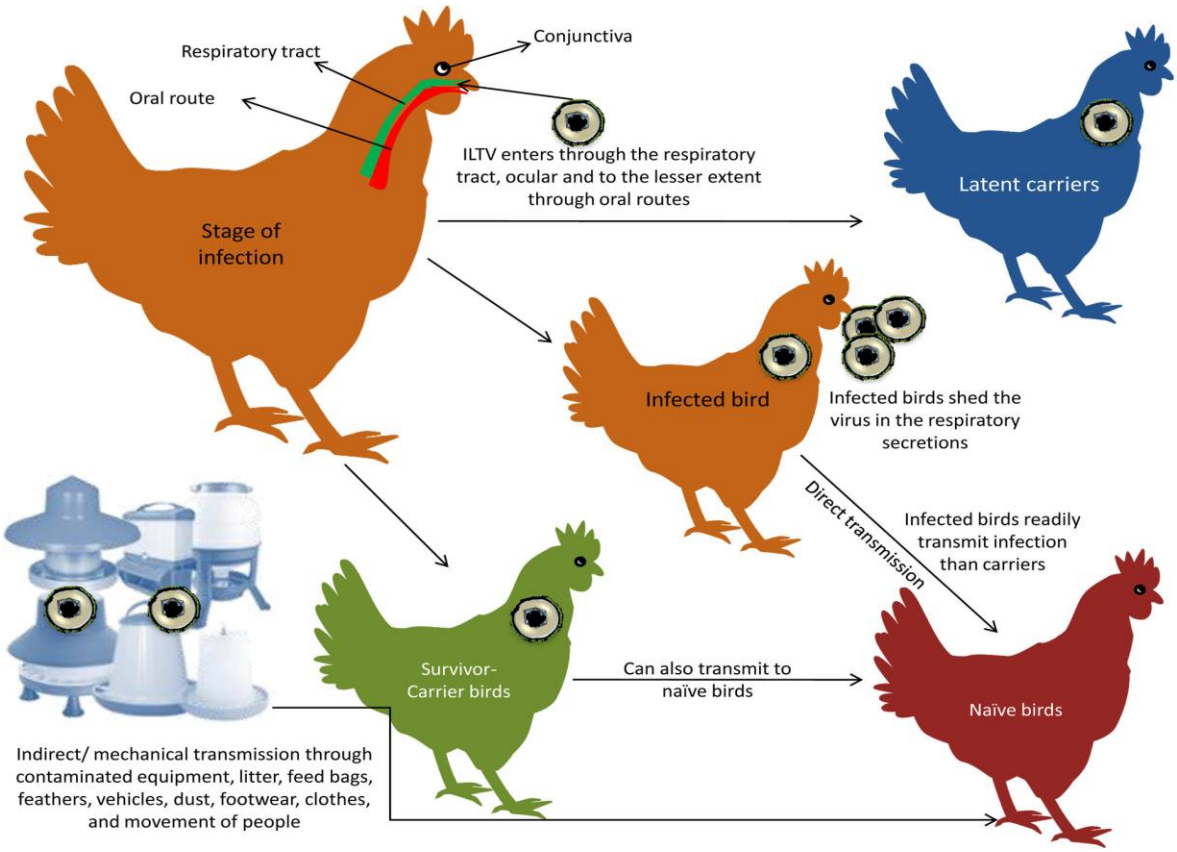

Figure 4: Transmission pattern of the ILT virus. Adopted from (Gowthaman *et al*., 2020).

2.3 Clinical signs of the ILTV

The disease has two forms, severe and mild forms. In the worst case, the main symptoms are difficulty breathing, coughing up blood, and moderate to severe eye inflammation. It can be severe, with a high chance of getting sick (90-100%) and even dying (up to 70%)

(Parra *et al*., 2016) (Fuchs *et al*., 2007). Birds may lengthen their necks to allow air to pass through the swollen and mucus-filled airway while wheezing loudly. Coughing and shaking of the head and neck may occur in affected birds trying to remove the tracheal secretions. As a result, blood is seen on the beaks and feathers of some birds. Reduced egg production and mortality differ based on the severity and pathogenicity of the strain (Gowthaman *et al.,* 2020). Large vacuoles having viruses in the infected cytoplasm and the creation of tubular structures are two other unusual characteristics frequently seen during ILTV propagation (Fuchs *et al*. 2007).

2.4 Diagnosis of the ILTV

For a preliminary diagnosis with pathogenic strains of ILT, necropsy results and clinical symptoms are sufficient. However, clinical signs may not be indicated in cases involving milder strains of ILT. The most common test for diagnosis of ILT is microscopic examination of the trachea (histopathology). In samples, containing the virus, multinucleated epithelial cells with eosinophilic intranuclear inclusion bodies can be detected (Sellers *et al.*, 2004).

Although traditional testing methods are cost-effective and widely utilized in laboratories, they have limitations such as inadequate detection capabilities, requiring extensive effort, and prolonged testing durations. Due to their high sensitivity, precision, speed, reproducibility, and simplicity, several molecular-based methods, including PCR, real-time PCR, nested PCR, restriction fragment length polymorphism (RFLP), and in situ hybridization, have been used to identify the ILTV (Zhao *et al*. 2013).

2.5 Virus isolation

The ILT virus can be found in samples taken from swabs, tissue, and fluids from the trachea, larynx, lungs, and conjunctiva. It is isolated and propagated by chorioallantoic membrane (CAM) inoculation in 9–11-day-old embryonated chicken eggs. In CAM infected with ILTV, opaque plaques can be seen as early as 48 hours after inoculation, and embryo mortality happens two to eight days later (Paji & Polaˇ, 2022)(Magouz *et al*., 2018; Wolfrum, 2020).

As the egg passage increases embryonic survival time increases thus leading effective replication of the virus to significant titers (Gowthaman *et al*., 2020). Viruses can also be

isolated by cell culture, including CEL and CK, but the most preferable and sensitive method for isolation is inoculation in the chorioallantoic membrane (CAM) of embryonated chicken eggs (Hitchner & White, 1958) and the CEL system. In cell cultures, large cells with multiple nuclei are seen after 24 hours. In both the laboratory and cell cultures, you need more than one passage to isolate a virus (Parra *et al*., 2016).

2.6 Sequencing of ILTV

ILTV genes are targeted for PCR followed by sequence analysis for identification of product amplicons for strain identification purposes, for example, ICP4 may be amplified by PCR using the primers (Chacón & Ferreira, 2009), and the PCR product can be purified using a disposable mini-column method and submitted for DNA sequencing. Different software programs are used for analysis including clusterW, which may be used for the analysis of the sequences and comparing them with the existing sequences in GenBank. It is important to analyze multiple genes in a sequence to identify the ILTV strains correctly. Whole genome sequencing has also been reported in several publications as a means of fully characterizing viral strains (WOHA,2021).

2.7 Prevention and Control

ILT still poses a significant threat to the chicken industry worldwide. Enhanced understanding of the virus, its transmission, and effective preventive measures can help prevent disease outbreaks (Gowthaman *et al*., 2020).

The Control of ILTV largely depends on vaccination and biosecurity measures (García & Zavala, 2019) and, the development of coordinated control programs in collaboration with veterinary professionals, government organizations, labs, and poultry farmers (Dufour-Zavala 2008). As (Oldoni & García, 2007) reported the vaccines commonly used against ILTV are recombinant ILTV vaccines with either herpes virus of turkeys (HVT) or fowlpox virus (FPV) as vectors, and live attenuated vaccines. Depending on the attenuation method attenuated vaccines can be grouped into chicken embryo origin (CEO) and tissue culture origin (TCO). Typically, attenuated vaccines are preferred over recombinant vaccines due to their ability to provide rapid and long-lasting protection while eliciting more robust immune reactions in the body. Attenuated vaccine viruses also suffer from reversion to virulence by multiple passages and cause bird-to-bird transmission within

flocks. This event happens more often in CEO vaccines because they are less weakened compared to TCO vaccines (Contreras *et al*., 2020).

To effectively combat and manage infectious laryngotracheitis (ILT), a cooperative effort between the government and industry is crucial, stopping viruses from spreading by getting vaccinated and other methods. It is crucial to quickly identify and remove chickens that are infected to stop the illness from spreading further (Wolfrum, 2020).

2.8 The economic importance of infectious laryngotracheitis disease.

The economic impact of ILT includes reduced egg production, weight loss, increased vulnerability to other respiratory infections, and high rates of mortality and morbidity (Couto *et al*, 2015). According to Garcia et al. (2013), investors might expect multimillion-dollar losses annually in the United States because of GaHV-induced mortality, lower egg production, vaccination costs, and vaccine responses that impair performance, especially in broilers.

2.9 Vaccination

In the absence of appropriate treatment, ILTV can be effectively controlled with good biosecurity procedures and vaccinations. Nonetheless, ILTV was the first important poultry disease with an effective vaccination (Gibbs, 1934). However, the disease remains a significant issue in poultry-rich areas (Chacón *et al*., 2015; Couto *et al*., 2015; Gowthaman *et al*., 2016; Yan *et al*., 2016). Modified live attenuated ILTV vaccines, such as CEO and TCO, have been used for decades. The first commercially available vaccinations were chicken embryo-origin live attenuated vaccines, launched in the 1950s and early 1960s (García and Zavala, 2019). According to Andreasen *et al*. (1989), CEO vaccines provide superior protection than TCO vaccines. Vaccines are used both to prevent and control virus spread during outbreaks (Bagust *et al*., 2000).

ILTV vaccine is given at 6-8 weeks of age, followed by a booster at 12-15 weeks for layers and breeders (Gingerich and Carver 2006). These vaccinations stimulate the immune system by infecting the trachea without causing illness. According to Neff *et al*. (2008), the best protective immunity occurs 15 to 20 weeks post-vaccination and can continue up to a year. Aston *et al*. (2019) found no evidence of interference between ILT and other

vaccinations if the immunization interval exceeds 2 weeks. ILTV immunization is not recommended for broilers due to economic concerns (Giambrone *et al*., 2008).

Vaccine administration routes are crucial for protecting against adverse responses. Eye drops are safer and more effective than other ways of application, such as drinking water or sprays. To produce sufficient protection, an ILTV vaccine must have a titer of more than 102 plaque-forming units/ml when administered by non-oral methods (Raggi and Lee, 1965). CEO and TCO vaccinations may cause vaccinal laryngotracheitis in the field due to reversal to the virulent form during bird-to-bird transmission (Chacón *et al*., 2015; Dufour-Zavala, 2008).

Vaccination can create latent carrier birds that spread infection to unvaccinated flocks (Bagust, 1986). Stress situations, such as lay, travel, and vaccination, can reawaken latent viruses and cause intermittent shedding of ILTV, spreading disease to susceptible birds (Guy *et al*., 1990; Hughes *et al*., 1991). The widespread use of live attenuated vaccinations has led to fresh outbreaks of ILT in many parts of the world. Experiments have shown that poor mass CEO immunization can lead to extended ILT infections (García, 2017). A study by Palino-Tapia *et al*. (2019) found that CEO vaccination provided superior protection for birds at 35 weeks of age than TCO or HVT-LT.

3. MATERIALS AND METHODS

3.1 Description of Study Area and Populations

The study was conducted in poultry disease outbreak areas where ILTV is suspected focusing on layer and commercial settings in Mekele, Lafto wereda of Addis Ababa, Ethiopia. Mekelle is the capital and commercial hub of the Tigray National Regional State in northern Ethiopia (Fig. 5. 1). Geographically, the town is positioned at a longitude of 39°33'E and a latitude of 13°32'N, extending into the central highlands of Ethiopia. The elevation of Mekelle ranges from 1,965 meters to 2,220 meters above sea level. The town is flanked by mountain ranges to the east and north. From a climatological perspective, the region is categorized as "Woina Dega" (temperate), characterized by an effective temperature range of 14°C to 20°C (Beyene, 2015). Addis Ababa is the capital of Ethiopia and functions as the diplomatic hub of the African continent. Geographically, it is situated at the center of the country, with coordinates ranging from 9°2′ N to 9°5′ N latitude and 38°45′ E longitude (Mekonnen *et al*, 2024).

The mean annual rainfall distribution is approximately 2,000 mm over the southwestern highlands and less than 300 mm over the south-eastern and north-eastern (Elzopy *et al*, 2021). Ethiopian poultry population (chicken) is estimated to be around 56.06 million, with indigenous (88.19%), hybrid (6.45%), and foreign breeds (5.36%). They are owned by every rural family, urban and semi-urban setting to provide protein and revenues as stated by Sime (2022).

Experimental studies were undertaken at the National Veterinary Institute, located in Bishoftu town. The town is situated 47 km southeast of the capital city, Addis Ababa at 9°N and 40° E. The place has two main periods of rainfall: a long one from June to September and a short one from February to April. The average yearly rainfall is between 450 mm and 1,000 mm, and the temperature usually ranges from 17°C to 30°C (Genet & Engdawork, 2020). Both sexes and greater than 8 weeks old exotic chickens reared in the area clinically suspected of ILT were used for virus isolation and characterization, and ILTV with typical signs of the diseases were sampled.

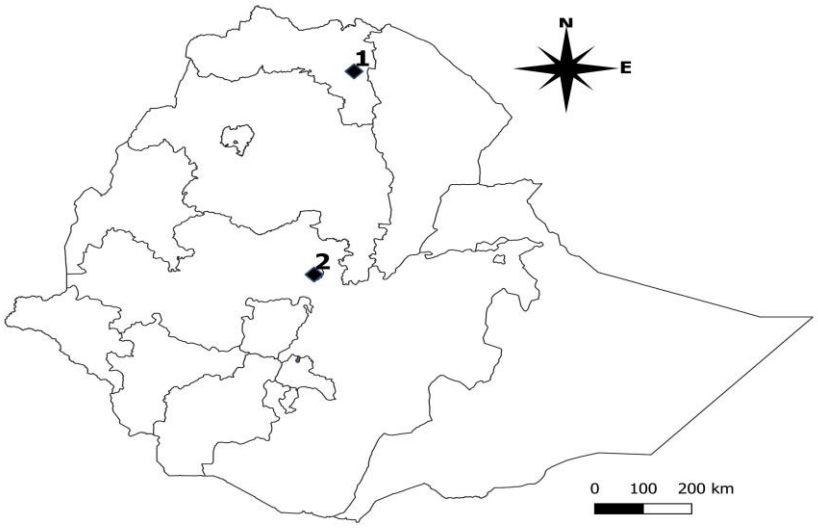

Figure 5:Map of the study area prepared by GIS software. Where 1: Study area one (Mekele) and 2: Study area two (Lafto)

3.2 Study design

Active outbreaks were investigated in a cross-sectional study designed together with poultry farm owners and veterinary professionals who are working in the district veterinary clinics. The samples were purposively collected based on the reports of ILT disease outbreaks to district Animal Health Service Departments and farm owners.

3.3 Sample collection

As Garcia et al (2013) describe clinical samples suitable for isolation can be swabs, tissue homogenates, or exudates from the trachea, larynx, lung, and conjunctiva. Hence swab samples from the trachea and trachea tissue from clinically apparent cases were collected for clinical data. Virus isolation and identification were conducted at the National Veterinary Institute (NVI).

Specimens were collected from ILT non-vaccinated chickens that are managed under intensive production systems in the Mekele, Tigray, and Lafto wereda of Addis Ababa. Accordingly, a total of 87 (eighty-seven) samples (tracheal swabs and tracheal tissue) which means 39 samples (12 tissue and 27 swabs) from Mekele and 48 samples (12 tissue and 36 swabs) were collected from Lafto wereda (Table 1).

Table 1: A summary of the samples collected by study area and sample types.

S.N	Study area	Farms	Type of sample	No of the collected sample
1.	Mekele	Farm1	swab	3
			Tissue	3
		Farm2	swab	2
			swab	2
			Tissue	4
		Farm3	Swab	3
			Swab	4
		Farm4	Swab	3
			Swab	5
		Farm5	Tissue	3
			Swab	5
		Farm6	Tissue	2
2.	Lafto sub-city	Farm1	Tissue	6
			Swab	7
		Farm2	Swab	6
			Swab	6
			Tissue	6
		Farm3	Swab	4
			swab	6
		Farm4	swab	7

The swabs were taken from chicken trachea that has dyspnea, expectoration of bloody mucus, conjunctivitis, sinusitis, swollen infra-orbital sinuses, and nasal discharge. The swabs were put into a cryovial tube containing a virus transport medium (VTM) supplemented with antibiotics and antifungal drugs. Additionally, from the bird, that is killed by barbiturate or other injection rather than by cervical dislocation, only the trachea tissue was collected in nutrient broth supplemented with antibiotics and antifungal drugs (WOHA, 2021). All collected samples were shipped to NVI in the icebox and kept at -80C^{o} until used for lab work.

3.4 Sample Preparation

Due to the shortage of reagents, chemicals, materials, and equipment used for sample processing and analysis, the collected samples were pooled together for further processing.

3.4.1 Tissue sample processing

The frozen tissue sample from the trachea was removed from the deep freezer, thawed at room temperature, and taken out of the labeled bottle in the Class II cabinet. The collected samples were combined and labeled as tissue samples from Mekele, marked with Mt which included 6 tissue samples. Similarly, samples from the Lafto wereda were grouped and labeled as Lt (which means tissue samples from Lafto), with each pool containing 6 tissue samples. Subsequently, 1 gram of tissue sample was meticulously extracted in a sterile manner.

To prevent contamination, the tracheal tissue sample was washed three times with a sterile phosphate-buffered saline solution containing penicillin-streptomycin (10,000U:10,000 μg), following the method described by Taha et al. (2017). The tissue sample was then sliced into small pieces using sterile tools and further crushed into smaller fragments with a mortar and pestle. A suspension of the tracheal sample was prepared in a sterile PBS solution supplemented with penicillin-streptomycin, transferred to a sterile centrifuge tube, and centrifuged at 3000 rpm for 10 minutes. The resulting supernatant was aseptically transferred to another sterile test tube, stored at -80°C (Annex 1), and utilized for virus isolation and molecular detection, as outlined by Marga (2023).

3.4.2 Swab sample processing

The Swab sample was taken out of -80°C then thawed and filtered in a biosafety cabinet with 0.22 microliter pore size filter pad to reduce bacterial and other contaminants. The sample collected from Mekele was labeled as ‘ms’ which contained 3 pooled swab samples. Each pooled sample contained 9 samples. In total, samples collected from Mekele were pooled into 5 samples (2 pooled tissue and 3 pooled swabs).

Similarly, the swab samples from Lafto Wereda were labeled as ‘ls’ and contained 4 pooled samples. Each pooled sample contained 9 swab samples. In general, the samples

collected from the Lafto wereda were pooled into 6 samples (2 pooled tissue and 4 pooled swabs).

Consequently, 11 pooled samples, comprising 4 tissue samples and 7 swab samples were processed from ILT-suspected chickens. Eventually, 2 ml of virus suspension was taken from each pooled sample and transferred into two different cryovials. One milliliter was used for the inoculation of embryonated eggs, and the other milliliter was sent to the molecular laboratory for the molecular characterization of the virus.

3.5 Virus Isolation in fertile eggs and cell culture

The prepared and harvested 10% tissue suspension supernatants and swab samples were used for the infectious laryngotracheitis virus isolation method (embryonated egg inoculation). The eggs utilized were sourced from the avian vaccine production department at the National Veterinary Institute (NVI). The specimens were inoculated onto the chorioallantoic membranes (CAMs) of 10-day-old specific-pathogen-free (SPF) chicken embryos (Annex 2) as described by Shehata et al. (2013) and Contreras et al. (2020).

A total of 66 eggs were used for this purpose. For each passage, 22 eggs were used, with 20 for virus inoculation and 2 for control. The eggs were then incubated at 37°C, 65% humidity, placed horizontally, and examined daily for 5 days, after which the allantoic fluids were harvested. Three blind passages were conducted in SPF eggs as previously outlined by Portz et al. (2008). Briefly, embryonated eggs were disinfected with 70% ethanol. A small hole was drilled in the air cell of the eggs, except for the control, and the eggs were placed horizontally by turning the egg roller off. Inoculation was performed using a 30-gauge insulin needle.

A volume of 0.2 mL of 10% tracheal tissue suspension/supernatant and tracheal swab were inoculated into each of five chorioallantoic membranes (CAMs) of 10-day-old embryonated specific pathogen-free (SPF) eggs. The hole at the inoculated site on the embryonated eggs was sealed with a drop of melted candle wax. The inoculated embryonated eggs were incubated at 37°C and candled daily for 5 days, and mortality was recorded. Any mortality within the first 24 hours post-inoculation was considered non-specific and discarded. The embryos dying within 48 hours, 72 hours, 96 hours, and 120 hours were chilled at 4°C for 24 hours, cleaned with 70% ethanol, and opened aseptically. The embryos were then examined for gross ILT lesions. Allantoic fluids from eggs with

dead embryos were harvested by grasping them with sterile forceps, sucking fluids with a micropipette, and adding them into the universal bottle.

The harvested allantoic fluids were mixed by vortexing, and 500 µL was added into sterile cryovial tubes for molecular analysis and stored at -20°C. On the other hand, Vero, DF-1, and CEF cells were cultured in 25 cm^2 tissue culture flasks at 37°C, starting with an initial concentration of 5.0 x 10^5 cells/mL in Eagle's Minimal Essential Medium. Monolayers were grown in 25 cm^2 tissue culture flasks until reaching 90% cell confluence. Subsequently, the cells were inoculated with 1 mL of each viral isolate. Adsorption was allowed for 40 minutes at 37°C, and the medium with 2% fetal calf serum was brought up to a total volume of 100 mL. The inoculated African green monkey kidney(Vero), chicken embryo fibroblast (DF-1), and primary chicken embryo fibroblast (CEF) cell lines were then incubated at 37°C and monitored daily for 96 hours to observe the development of viral cytopathic effect (CPE) using an inverted microscope. The affected cells were harvested, centrifuged at 3000 rpm for 10 minutes, and stored at -20°C. DNA extraction was performed following the protocol described by Portz et al. (2008).

3.5.1 DNA Extraction

DNA extraction from allantoic fluid which is harvested from inoculated chicken embryonated egg and virus inoculum from Vero, DF-1, and CEF cells was performed in the National Veterinary Institute (NVI) molecular biology laboratory with the QIAGEN™ DNA Mini Column Kit (QIAGEN, Germany) based on the manufacturer's instructions.

Harvested Allantoic fluid and virus inoculum were melted at room temperature, and mixed by vortexing for 15 seconds. 200 µl of the volume of the sample was utilized for the extraction of DNA as previously described by Dormitorio et al (2013). Briefly, 200 µl of the allantoic fluid was incubated with 20 µl of proteinase K, and 200 µl of lysis buffer (AE) at 56 °C for 10 min. Next to incubation, 200 µl of 96% ethanol was added and mixed by vortexing for 15 sec. The 620 µl mixtures were transferred to the QIAGEN spin column and centrifuged at 8000 rpm for 1 min and the QIAGEN spine columns were placed in a sterile collection tube. The mixtures were added with 500 µl AW1 buffer and centrifuged at 800 rpm for 1 min followed by 500 µl AW2 buffer and centrifuged at 14,000 rpm for 3 min. DNA was eluted with 30 µl of elution buffer (AE) provided in the kit and stayed at room temperature for 5 min centrifugation, then it was centrifuged for 1 min at 8000 rpm.

Finally, the extracted DNAs were stored at +4°C until the next test was performed (Annex 3).

3.5.2 Polymerase Chain Reaction (PCR) for Amplification of ILTV

Polymerase Chain Reaction was performed with a set of primers that specifically amplified the 688 bp fragment of the ICP4 gene. It was conducted in Bio-Rad 2729™ Thermal Cycler in a reaction volume of 25 µl, containing 5 µl of 10X Dream Taq™ buffer, 2 µl RNAs free water, 5 µl of each 2 mM of deoxynucleotide triphosphate, 5 µl of Dream Taq™ DNA polymerase, 2 µl of 5 pm/µl Primer-ILT- Forw- 5 pm/µl 5´-ACT-GAT-AG C-TTT-TCG-TAC-AGC-ACG3´ and 2 µl of Primer-ILT- Reve- 5 pm/µl 5´-CAT-CGG-GAC-ATT-CTC-CAG-GTA-GCA-3´ (Chacon and Ferreira, 2009), 5 µl template DNA. 5 µl of RNAs free water was used as a negative control (Annex 4).

Thermal cycling conditions were conducted as follows; one cycle of initial denaturation at 94°C for 3 min followed by 35 cycles of a three-step amplification protocol: Denaturation at 94 °C for 30 sec, annealing at 62 °C for 45 sec, and elongation at 72 °C for 1:50 sec each, and finally one cycle of elongation at 72 °C for 3 min then it was put at 4 °C until the machine off (Annex 5).

3.5.3 Agarose Gel Electrophoresis

PCR products of isolated DNA from embryonated eggs and harvested inoculum were analyzed by 1.5% (w/v). Agarose electrophoresis gel stained with 4 µl GelRed (alternative to traditional ethidium bromide (EtBr) stain for detecting nucleic acid) and 1X TAE buffer. Briefly, 5 µl of each PCR product was mixed with 5 µl loading buffer by pipetting, and 5 µl of 100 bp DNA molecular ladder was added into the first lane. In contrast, in the remaining wells, 10 µl of sample DNA with 2 µl of DNA loading dye (50 % glycerol, 6x TAE, 1 % bromophenol blue) was loaded by using micropipettes.

The gel-running tank was connected to the power supply and voltage was adjusted to 120 volts and run for 1:20 hours. Then the gel was observed under a UV trans-illuminator for target size bands and a Polaroid photograph by the Gel documentation system (Annex 6).

3.6 Data analysis

The collected data was entered and stored into Microsoft Office Excel spreadsheet 2010. Descriptive statistics was used to summarize data from field surveys.

3.7 Ethical Considerations

Ethical clearance was obtained from the National Veterinary Institute. After reviewing and discussing the project proposal, it was scientifically arid and ethically sound regarding relevance, originality, and technical competence. The experiment followed ethical guidelines for lab animal testing and had approval with reference number NVI/374 (Annex 7).

4. RESULTS

4.1 Clinical Findings during the Sample Collections and postmortem lesion on the trachea

While collecting samples, clinical symptoms such as increased egg production, nasal discharge, and blood exudate were observed in both outbreak regions of the study (Table 2).

Table 2: summary of clinical findings and mortality observed during sample collection

s.n	Area	Farm	Total number of chickens	No of cases	Clinical symptoms	No of death	Morbidity (%)	Mortality (%)	Fatality (%)
1	Mekele	F1	460	18	Gasping Decreased egg production	3	3.91	0.65	16.67
		F2	540	12	Nasal discharge Blood exudate on the trachea Gasping	2	2.22	0.37	16.67
		F3	800	24	Gasping	7	3	0.88	29.17
		F4	480	14	Infraorbital sinuses Gasping	3	2.92	0.63	21.43
		F5	850	20	Nasal discharge Infraorbital sinuses	3	2.35	0.35	15

		F6	650	34	Blood exudate on the trachea Gasping	1	5.23	0.15	2.94
2	Lafto wereda	F7	700	22	Infraorbital sinuses	5	3.14	0.71	22.73
		F8	620	17	Gasping Blood exudate on the trachea	4	2.74	0.65	23.53
		F9	350	21	Infraorbital sinuses	3	6	0.86	14.29
		F10	480	19	Decreased egg production Infraorbital sinuses	4	3.96	0.83	21.05

Furthermore, many chickens exhibited respiratory issues such as difficulty breathing, gasping, and coughing up bloody mucus. There was also swelling in the infraorbital sinuses and blood clots found within the trachea, as shown in Figure 6.

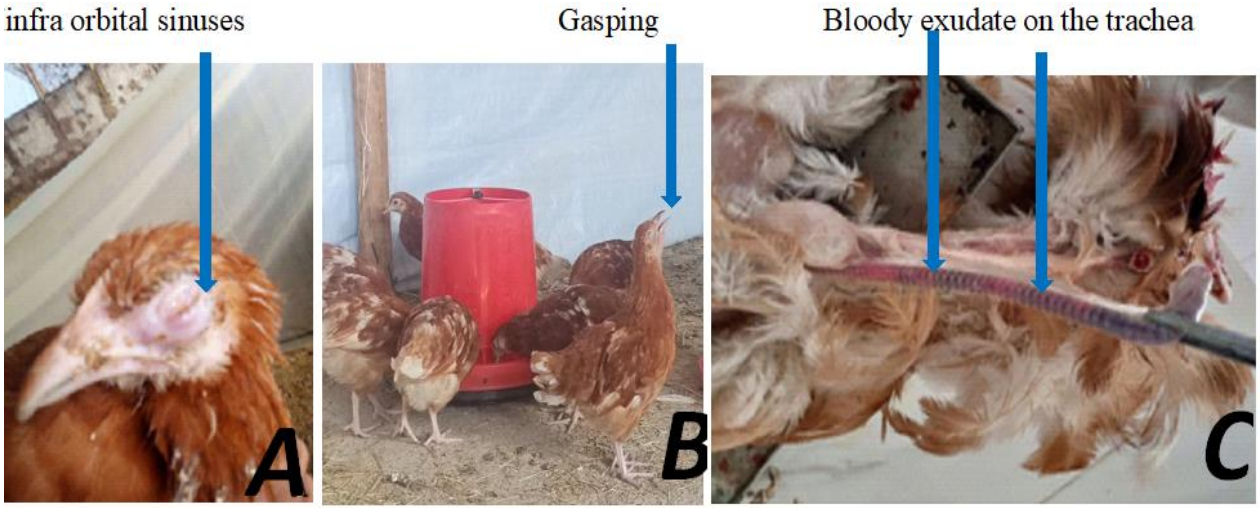

Figure 6:The representative pictures collected during sample collection and postmortem findings.Where (A) Infra orbital sinuses; (B) gasping; (C) bloody exudate on trachea during postmortem finding.

In a study involving the examination of 122 chickens in the Mekelle area, it was observed that 34 chickens (27.8%) exhibited infraorbital sinuses. Additionally, 102 chickens (83.6%) displayed signs of gasping, while 32 chickens (26.2%) presented with nasal discharge. Furthermore, 18 layers of chickens (14.7%) experienced a reduction in egg production, and 46 chickens (37.7%) exhibited bloody exudate on the trachea during postmortem examinations. In a separate inspection of 79 chickens in Lafto Wereda, 17 chickens (21.5%) were noted to be gasping, 62 chickens (78.4%) showed infraorbital sinuses, and 17 chickens (21.5%) had bloody exudate on the trachea.

4.2 ILTV screening by molecular tools (PCR)

A total of 87 samples were consolidated into 11 suspensions and subsequently submitted to the molecular laboratory for screening to determine the presence of Infectious Laryngotracheitis Virus (ILTV). Among these, four samples tested positive for ILTV, leading to the amplification of a 688 bp fragment (refer to Figure 7). The positive samples comprised one tissue specimen from L4, collected in Mekele, and three swab samples (L1, L2, L3) obtained from the Lafto wereda. The incidence of ILT was identified in 4 out of 11 samples, representing a prevalence rate of 36.3% among the tracheal tissue and tracheal swabs (see Table 3).

Table 3: Detection of the virus in the pooled samples by PCR

S.N	Area	Pooled sample			Positive			Negative		
		Tissue	Swab	Total	Tissue	Swab	Total	Tissue	Swab	Total
1	Mekelle	2	3	5	1	-	1	1	3	4
2	Lafto	2	4	6	-	3	3	2	1	3
	Total	**4**	**7**	**11**	**1**	**3**	**4**	**3**	**4**	**7**

The detection of the ICP4 gene was accomplished through polymerase chain reaction (PCR) using primers available in the molecular laboratory at the National Veterinary Institute (NVI), in accordance with the methodology outlined in Annex 3-5. Subsequently, these positive samples were characterized, and the virology laboratory proceeded with the inoculation of embryonated eggs as well as Vero, DF-1, and CEF cell cultures.

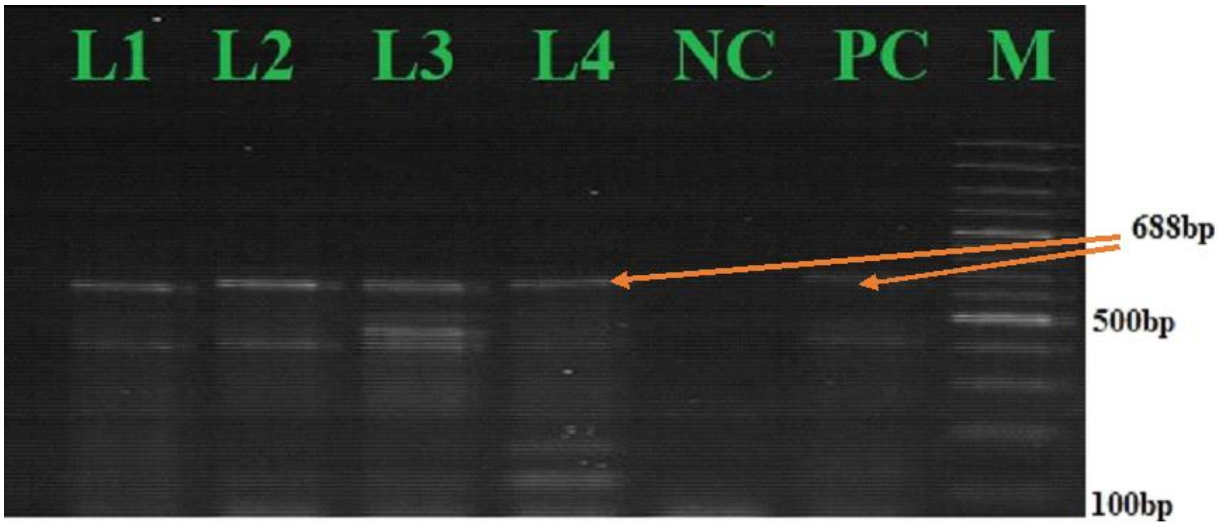

Figure 7:Conventional PCR Agarose gel showing amplification of ICP4 gene with 688 bp band size. Lanes L1-L4 positive samples, NC: Negative control, PC: positive control, and M: molecular ladder 100 bp.

4.3 Propagation of the ILTvirus on Embryonic Eggs

Positive samples identified through PCR were subsequently inoculated into 10-day-old specific pathogen-free (SPF) embryonated eggs. A total of 66 eggs were utilized, with 22 eggs allocated for each passage: 20 for virus inoculation and 2 serving as negative controls. The mortality of the embryos attributed to infectious laryngotracheitis virus (ILTV) was monitored within a timeframe of 48 to 96 hours post-inoculation, as detailed in Table 4.

Table 4: Mortality of Embryo at different times from three consecutive passages

Sample code	Inoculated amount(ml)	Passage number vs. mortality at different time											
		Passage1				Passage 2				Passage 3			
		24 hr	48 hr	72 hr	96 hr	24 hr	48 hr	72 hr	96 hr	24 hr	48 hr	72 hr	96 hr
Code1	0.2	0	1/5	1/5	0	0	1/5	1/5	1/5	0	3/5	1/5	0
Code2	0.2	0	0	2/5	0	0	2/5	1/5	0	0	2/5	2/5	0
Code3	0.2	0	1/5	1/5	0	0	1/5	2/5	0	0	3/5	1/5	0
Code4	0.2	0	1/5	0	1/5	0	1/5	2/5	2/5	0	1/5	3/5	0

After 2 days post-inoculation, the dead embryos were chilled at +4 ^{o}C for 24 hours. After 24 hours the dead embryonic eggs were opened and they contained a hemorrhage, thick and brown necrotic opaque chorioallantoic membrane (Figure 8 B)

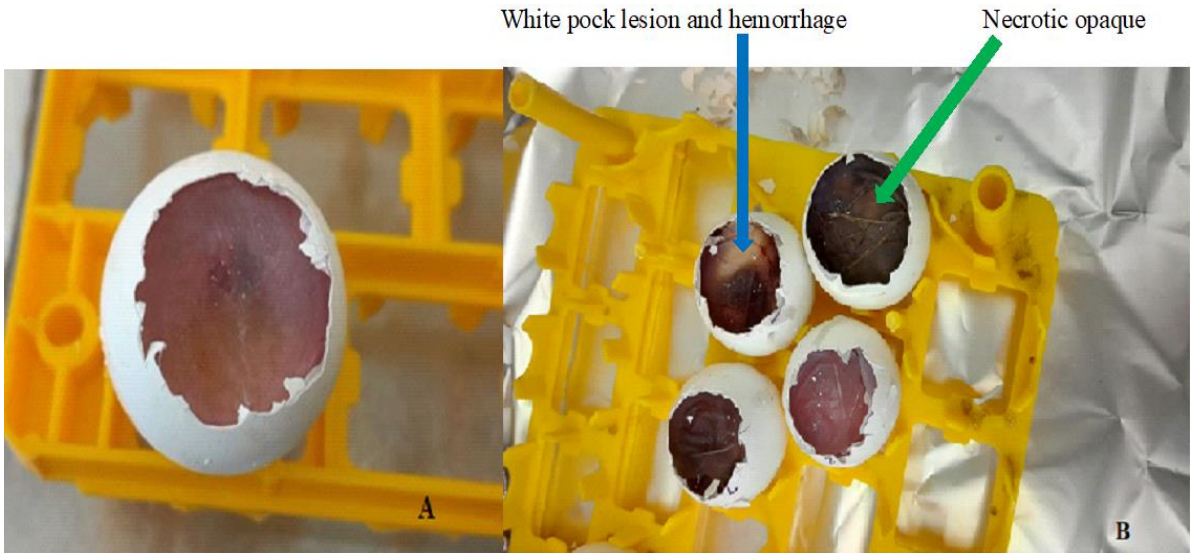

Figure 8: The representative image captured following the chilling and subsequent opening of the infected embryonated egg on day 4. In this image, (A) depicts the negative control, which consists of un-inoculated embryonated eggs, while (B) illustrates the ILTV-inoculated embryonated eggs. The latter exhibit white pock lesions and hemorrhaging, as indicated by the blue arrow, and necrotic opaque areas, as denoted by the green arrow.

Upon the examination of dead embryonated eggs, notable morphological alterations, such as congestion within the embryo, were visually documented (Figure 9 B)

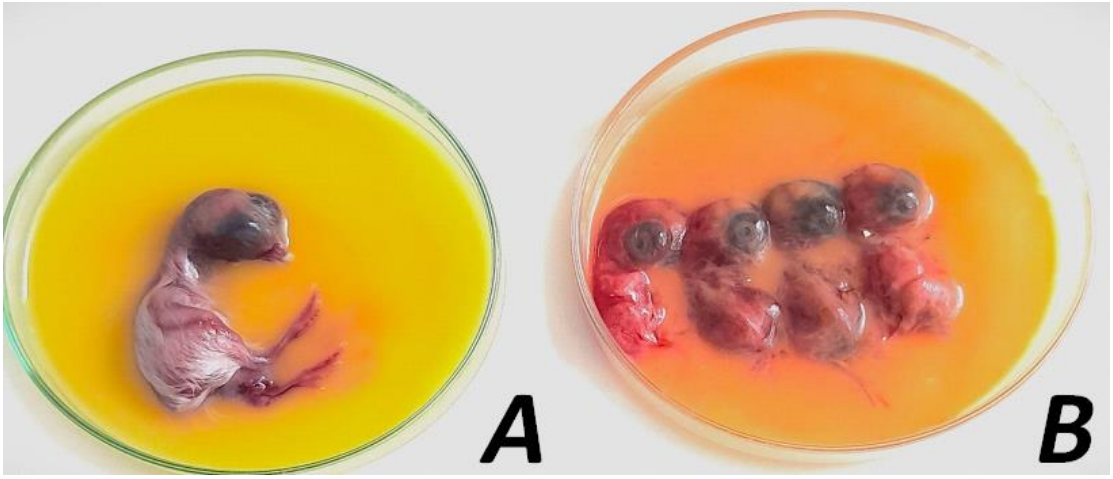

Figure 9: Representative picture which showed the morphology of negative control embryo (A) and ILTV inoculated Embryos (B).

4.4 Detection of the virus on the CEF, Vero, and DF-1 cells

The inoculated Infectious Laryngotracheitis Virus (ILTV) on primary cell cultures of Chicken Embryo Fibroblasts (CEF) exhibited cytopathic effects (CPE) within three days post-inoculation, Typical CPE includes cell rounding, detachment from the surface, syncytia (cell fusion), or cell lysis (bursting) were observed. These morphological changes help in detecting the viral presence as illustrated in figure10

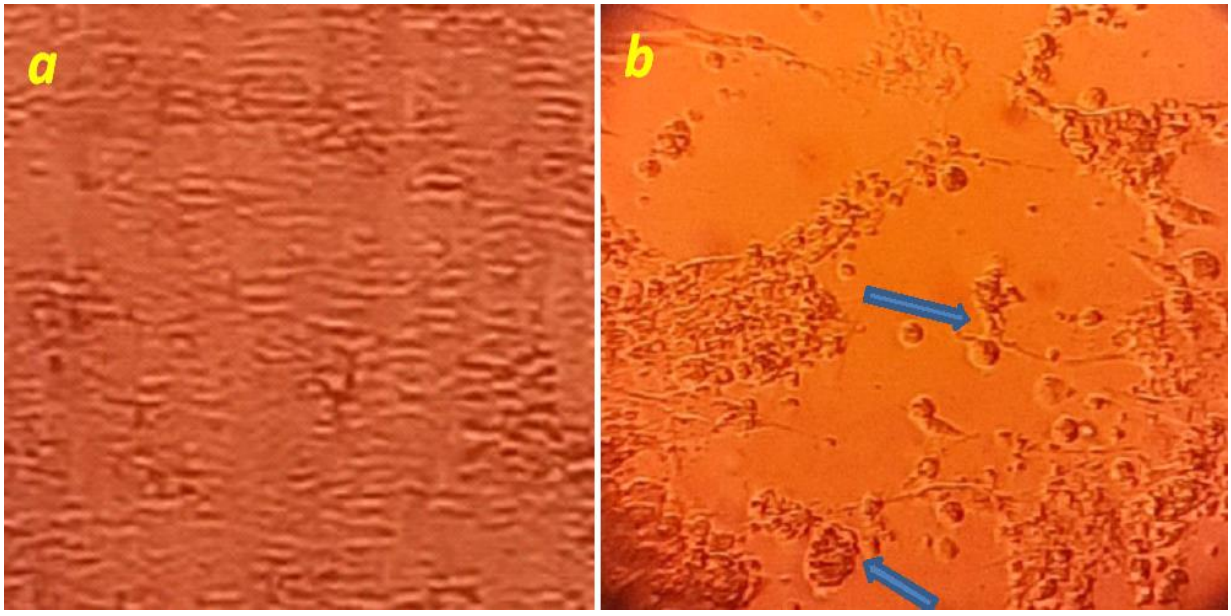

Figure 10: Representative picture taken from CEF primary cell.

In the absence of ILTV infection (a), the infection of CEF cells resulted in cellular swelling and the formation of multinucleated giant cells, also known as syncytia, as evidenced by the blue arrow in the cell culture image taken 24 hours post-infection (b)

The isolated virus was grown in the African green monkey kidney (Vero) cell line and produced cytopathic effects (CPE). Changes resulting from the inoculated virus were observed on the infected confluent Vero cells during 72 hours post-inoculation. The CPE appeared in the form of cell rounding on day 3 (Figure 11, B). Additionally, there were detachments of dead cells from the tissue flask on days 4 and 5 post-inoculation, as indicated in Figures 11C and 11D, respectively.

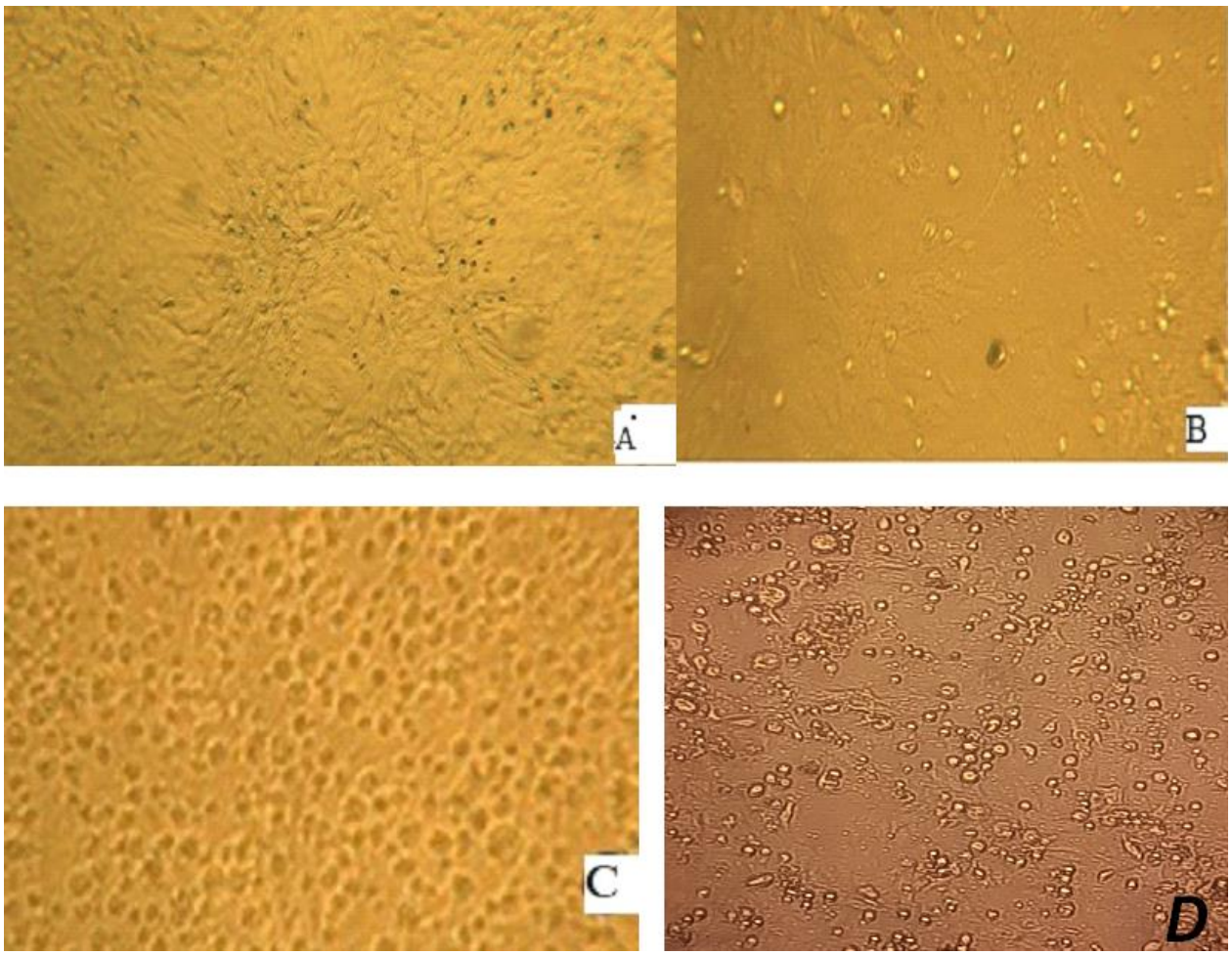

Figure 11: The representative pictures showing CPE established on Vero cells. (A) negative control; (B) cytopathic effect (CPE) at day 3 of post-infection; (C) CPE at day 4; and (D) CPE at day 5 established on Vero cells.

The virus was inoculated on the Duck embryo fibroblast (DF-1) cell lines to detect the virus destruction on the tissue culture flask and the CPE was established and characterized by fusion of cell nuclear as shown in Figure 12.

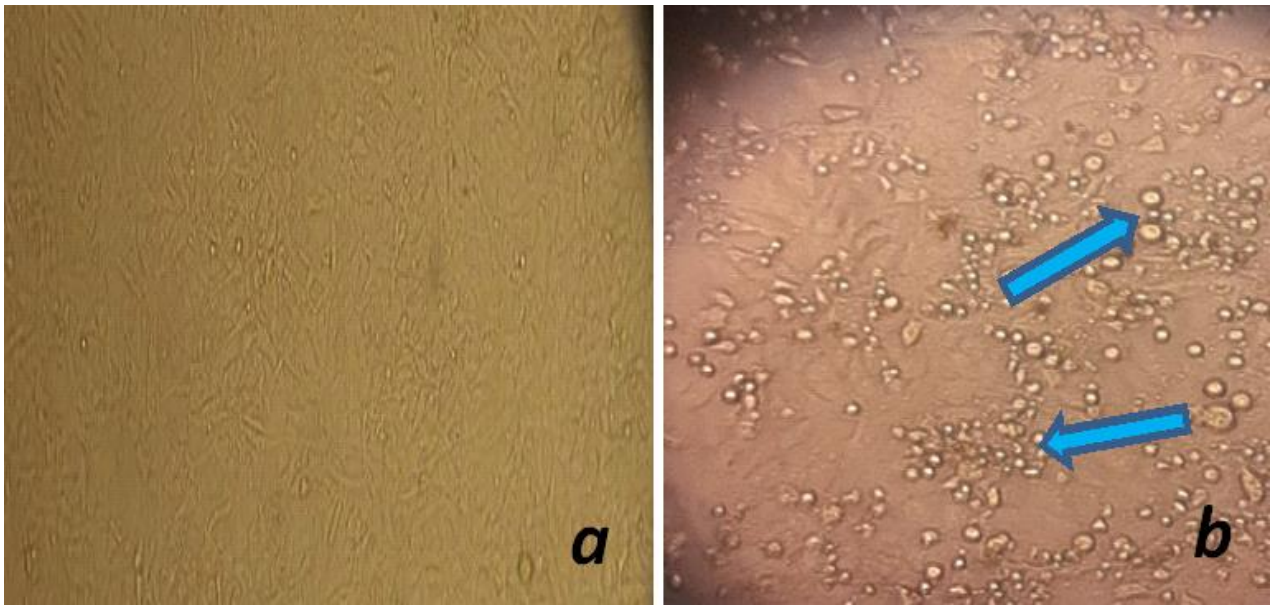

Figure 12: The representative pictures indicating CPE of infection on DF-1 cell. (a) was a negative control and (b) a cytopathic effect (CPE) indicated as round and aggregation of cells as showed in the blue arrow at 48 hours of post-infection on chicken embryo fibroblast (DF-1) cells.

5. DISCUSSION

The present study revealed that the cause of the outbreak was ILTV disease in the suspected area. It presents the isolation and characterization of 87 ILTV suspected samples collected from chickens between January 2023 and May 2024 from two outbreak areas located in the Mekelle and Lafto wereda of Addis Ababa, Ethiopia. Chickens are the primary natural hosts for ILTV, as stated by Yi et al. (2024). During sample collection in this study, two forms of clinical presentations were observed: a severe form characterized by dyspnea (Figure 6B) and a milder form characterized by conjunctivitis and swollen infraorbital sinuses (Figure 6A), which confirmed a previous report demonstrated by Zorman et al. (2021). A similar study conducted stated that severe instances of the illness were characterized by significant dyspnea and expectoration of blood-stained mucus, as indicated in this study (Garcia *et al*., 2013). Other studies also confirmed the present studies by Mossad et al. (2022), who detected infra-orbital sinuses, as well as hemorrhage clots in the trachea.

Oldoni (2007) reported that the main infection route is through the upper respiratory tract and conjunctiva, which aligns with present studies. A similar clinical sign was observed in Magouz et al. (2018) and Oldoni (2007), who reported gasping and conjunctivitis. A post-mortem examination revealed hemorrhage within the tracheal lumen of the infected birds, as indicated in present studies (Figure 6C). Hemorrhagic tracheitis with blood clots was also observed in this study, consistent with Hong et al. (2022), who reported similar clinical signs previously. All of the ILT-positive clinical specimens were obtained from chicken farms that had not been vaccinated against ILT, this indicated the isolated virus was wild type rather than vaccine reversions depending on the (Ojkic *et al*., 2006) reports. The virus was isolated using molecular tools, specifically PCR, which is more sensitive for ILTV detection than other methods, as described by Ou and Giambrone (2012).

Furthermore, Conventional PCR has proven to be useful in detecting ILTV-infected birds during both severe and mild forms of the disease (Williams *et al*., 1994). As Chacon & Ferreira (2009) stated, two regions of the ICP4 gene were amplified in a different protocol that has been used in veterinary diagnostic laboratories worldwide for the detection of the virus; hence, primers ICP4-1F (5´-ACT-GAT-AGC-TTT-TCG-TAC-AGC-ACG-3´) and ICP4-1R (5´-CAT-CGG-GAC-ATT-CTC-CAG-GTA-GCA-3´) amplify a 688 bp; ICP4-2F

(5´-CTT-CAG-ACT-CCA-GCT-CAT-CTG-3´) and ICP4-2R (5´-AGT-CAT-GCGTCT-ATG-GCG-TTG-AC-3´) amplify a 635 bp fragment. Hence, ICP4, which amplifies the 688 bp fragments, was used in this study. Accordingly, Molecular identification of ILTV using PCR technique for amplification of specific ICP4 gene revealed the presence of ILTV in 4 samples out of 11 pooled samples (36.3%). A similar study conducted by Emadi, et al (2021) detected ILT in 18 out of 28 (64.2%) from tracheal specimens. Three tracheal swab samples from the Lafto wereda of Addis Ababa and one positive sample from a tracheal tissue sample obtained from Mekelle were found in the current investigation. These findings imply that ILTV was mostly detected in the poultry's respiratory system, specifically the upper respiratory system. However, Oldoni et al. (2009), Wang et al. (2013), Zhao et al. (2013), and Roy et al. (2015) reported ILTV DNA has been found in several organs other than the respiratory tract and conjunctiva tissue in several investigations, and dissimilar with present reports. But Organs other than the lung, trachea, and conjunctiva were not found to have ILTV antigen as stated by Tran et al. (2021) and it is in agreement with this study.

In this study, the infected cell protein (ICP4) gene is identified using a primer available from the National Veterinary Institute. A similar study was conducted in 2015 and 2016 by Santander et al, (2022) who demonstrated the detection and molecular characterization of ILTV in Brazil by using the ICP4 gene. This gene was produced before viral genomes replicate within the infected cell, and it is in charge of the early and late gene regulation during infection. As described previously by Ghalyanchi et al. (2020), the adult ages of chickens were most severely affected by the virus. In these studies, both young and adult chickens were affected by the virus, but more of those affected were adults. A similar report was performed by Yi et al. (2024) who stated: “Birds of all ages and breeds are susceptible, although those over 4 weeks old are most affected.”

In this study, three different cell cultures CEF, DF-1, and Vero cells were tested to observe the replication of ILTV and also included the isolation of the virus from the chorioallantoic membrane (CAM) of the infected embryonic egg. In the case of the CAM, a volume of 0.2 mL supernatants of the field samples were introduced into the chorioallantoic membrane of SPF eggs that were 11 days old, and eggs were kept at a temperature of 37°C for 2-7 days and checked every day for any abnormalities as previously reported by Magouz et al (2018). However, Mossad et al, (2022) used a volume of 0.1 mL supernatant fluid of a positive sample for CAM inoculations, which was not in

agreement with current studies. Ou (2010) reported that embryonated chicken eggs and several avian cell cultures like fibroblast (CEF) Garcia and Zavala (2019) were used to propagate ILTV. In chicken embryos, it forms plaques on the chorioallantoic membrane (CAM), which is consistent with present studies. As indicated in Table 2 embryo mortality increased with the passage and the virus's density increased at passage 3 compared to passage 1, suggesting that a higher passage is necessary for the virus to propagate as previously described by Islam et al, 2010 who reported the antigen increased with the passage.

Thickened and brown opaque plaques were observed on the infected CAMs, resulting from necrosis and proliferation of the affected cells as indicated in these studies (Figure 8B) (Garcia *et al*., 2013). Similar cytopathic effects (CPE) were observed in China by Yi *et al*. (2024), who reported considerable thickening of the CAM and white pock-like necrotic plaques. On the fifth day post-inoculation, CAMs of SPF chicken embryos were examined. In the infected embryo, severe hyperemia was observed (Figure 9B), a similar post-inoculation sign previously reported by Zhao et al. (2013). As Shehata et al. (2013) described, death of the embryo occurred 3–7 days post-inoculation and was characterized by stunted growth. However, in this study, embryo death occurred 2-4 days post-inoculation, and undersized embryos were observed (Figure 9B), aligning with Garcia et al. (2013), who observed plaques and embryo death as early as two days post-inoculation.

In these studies, chicken embryo fibroblast (CEF) cell cultures were prepared using trypsinization methods from eggs aged 9 to 11 days, as described by Hughes and Jones (1988). The cytopathic effect of the Infectious Laryngotracheitis Virus (ILTV) was detected by the formation of syncytia (multinucleated cells) on the 3rd day post-inoculation, and cell detachment was observed. This differed from the findings of Magouz et al. (2018), who reported detecting CPE of CEF cells on the 5th day post-inoculation. Additionally, Vero cells were utilized in this study for the growth of a virus derived from field samples, as reported by Menendez et al. (2014). Cells that were used for inoculations of the virus were used when fully confluent after 48 to 72 hours as stated by Hughes & Jones (1988). Consequently, cytopathic effects were observed 2 to 4 days post-inoculation, similar to the findings of Portz et al. (2008). The cytopathic effects from ILTV infection in this study were characterized by cell swelling, chromatin displacement, nucleoli rounding, and syncytia formation, which align with the observations of Gowthaman et al. (2020).

6. CONCLUSION AND RECOMMENDATIONS

Infectious Laryngotrachitis virus is one of the avian respiratory illnesses and causes serious challenges to the economy of the nation in a covered-up way due to unstudied points of interest and few recorded information accessible, particularly in the location of the ILTV at the national level. The present study confirmed 4 ILTV positive results in the layer and commercial setting of chickens in Mekele and lafto wereda of Addis Ababa. Molecular isolation with conventional PCR was performed, and embryonated eggs, primary chicken embryo fibroblast cell (CEF), chicken embryo fibroblast (DF-1) cells, and African green monkey kidney (Vero) cells were used for propagation of the virus by detecting the cytopathic effect of the virus (CPE). It suggested evidence of a fairly present ILTV in the chickens of the study area. The research indicated that tracheal swab samples were more focused and more effective than tracheal tissue samples for detecting viral antigens using polymerase chain reaction. Furthermore, there were no ILT vaccines given for the sampled farm, and this suggested the isolated virus was wild-type. Additionally, this study gave clues to the problem of the disease in the study area. Therefore, the following recommendations will be forwarded based on the above conclusions:

- Infectious laryngotracheitis (ILT) vaccines should be developed to control diseases in poultry farms in the country.
- All poultry farm owners in the study areas and the whole country level should be aware of control measures for the disease.
- The genome of the virus should be sequenced to identify the viral genotype spreading in the study area.

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8. ANNEXES

Annex 1: Tracheal Tissue Sample Preparation Procedures

i. The frozen Tracheal tissue sample was removed from -80ºC and placed at room temperature until melted.

ii. The sample was washed 3 times by PBS with antibiotic (pinstripe)

iii. The tissue sample was cut into small pieces with scissors

iv. The trachea was ground with a mortar and pestle, and 1 g of suspension was taken and added with 9 ml of PBS, which contains antibiotic

v. Tracheal tissue suspension was added to the tube and centrifuged at 3000 rpm for 10 minutes. Finally, 10 ml of suspended tracheal tissue was preserved at -80 °C until DNA extraction was per

Annex 2: Virus Isolation Techniques

1. The tracheal swabs or tissue suspension were removed from -80°C and melted at room temperature.
2. The swab samples were vortexed and centrifuged, and tissue samples were treated with sterile phosphate-buffered saline solution and a pen strip inside the Biosafety Cabinet Class II.
3. SPF eggs were incubated for 11 days at 37 OC, 5% CO_2, and a humidity of 55-65.
4. Embryonated eggs were checked for life or death and labeled for inoculations on CAM with a life embryo.
5. Eggs with dead embryos were discarded, and eggs with live embryos were disinfected with 70% ethanol and put in the cabinet.
6. Each egg was labeled according to the sample code.
7. The airspace of each embryonated SPF egg was drilled except for the control.
8. 0.2 ml of suspension was inoculated in every 2 embryonated eggs, while 0.3 ml of suspension was inoculated into each other of the 2 embryonated eggs for all samples in the same way.
9. The airspace was sealed with a dropped candle on each drilled hole of the eggs.
10. Inoculated eggs were positioned horizontally, incubated at 37°C for 5 days, and candled daily for mortalities.
11. Embryo deaths within 24 hours were considered nonspecific and were discarded, and eggs with dead embryos after 24 hours were removed and stored at 4°C for chilling overnight.
12. Allantoic fluid was harvested from dead embryos by grasping them with sterile forceps.
13. Allantoic fluids were placed in sterile tubes and stored at -80°C until DNA extraction was performed.

Annex 3: DNA Extraction Protocols

1) Tracheal tissue and swabs were suspended in 1 ml virus transport media and mixed by vortexing for 15 sec
2) 20 µl of QIAGEN Protease was pipetted into the bottom of a 5 ml microcentrifuge tube.
3) 200 µl samples were added to the microcentrifuge tube.
4) 200 µl of buffer AE was added to the samples.
5) Mixed by pulse vortexing for 15 sec, then the mixtures were incubated at 56 °C for 10 min.
6) Briefly centrifuge the 1.5-ml microcentrifuge tube to remove drops from the insides of the lid.
7) 200 µl ethanol (96%) was added to the samples and mixed by pulse vortexing. After mixing, briefly centrifuge the 1.5-ml microcentrifuge tube at 6000 g (8000 rpm) for 1 minute to remove drops from the inside of the lid.
8) 620 µl mixtures were transferred carefully from step 6 to the QIAGEN spin columns (in a 2 ml collection tube) without wetting the rim, closing the cap, and centrifuged at 6000 g (8000 rpm) for 1 min, then the QIAGEN spine columns were placed in a cleaned collection tube and discarded of the collection tube contained
9) The QIAGEN spine column was opened.
10) 500 µl buffer AW1 was added without wetting the rim. Close the cap and centrifuge at 8000 rpm for 1 min, then place the QIAGEN spin column in a cleaned collection tube and discard the tube containing filtrate. The QIAGEN spine column was opened carefully and 500 µl buffer AW2 was added without wetting the rim. Close the cap and centrifuge at 14000 rpm for 3 min.
11) ml collection tubes were placed in the QIAGEN spine columns and then centrifuged at full speed (20000 g; 14000 rpm) for 1 min.
12) The QIAGEN spin column was placed in a cleaned 1.5-ml microcentrifuge, and the filtrate collection tube was discarded. The QIAGEN spine columns were opened carefully and added 50 µl buffer AE.
13) Incubated at room temperature for 5 min and centrifuged at 6000g (8000rpm) for 1min, finally, the DNA extracts were stored at +4°C until the next test was performed

Annex 4: Master Mix preparation protocol

S.N	Types of reagent	For one reaction	Total reaction (6)	Remark
1	RNAs free water	5µl	18 µl	
2	Primer- ILT-Fow-5pm/µl 5'ACTGATAGCATTTTCGTACAGCACG-3'	2 µl	12 µl	
3	Primer- ILT-Fow-Reve-5pm/µl 5'CATCGGGACTTCTCCAGGTAGCA-3'	2 µl	12µl	
4	10X Dream Taq^{TM} buffer	5 µl	30 µl	
5	4NTP mix. 2Mm each	5 µl	30µl	
6	Dream Taq^{TM} DNA polymerase	5 µl	30 µl	
7	Template (DNA)	5 µl		
	Total volume	23.5 µl		

Annex 5: PCR Amplification protocol

	Temperature	Time	Cycle	Remark
Initial denaturation	94°C	3min	1 Cycle	
Denaturation	94°C	30sec	35 Cycles	
Annealing	62°C	45sec		
Elongation	72°C	1:50sec		
Final elongation	72°C	3min	1 Cycle	
Put at	4°C	Until machine off		

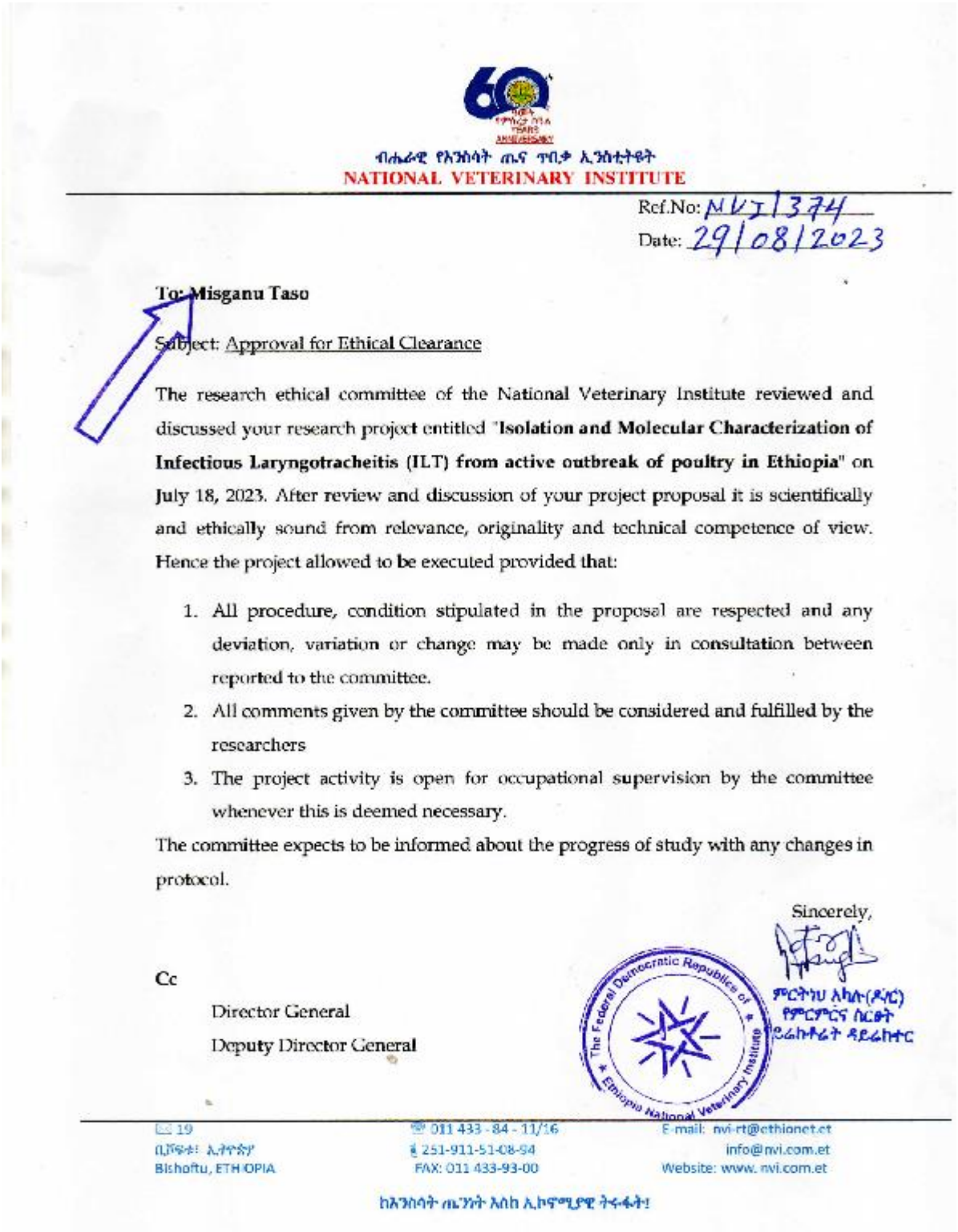
Annex 6: Gel electrophoresis procedure;

a. 1.5% Agarose gel was Prepared
b. 100 ml 1X TAE (40 mM Tris-acetate, 1 mM EDTA) was added to the agarose-containing flask. Swirl to mix.
c. The agarose/buffer mixture was melted in a hot oven at 36°C for 20min.
d. The flask was removed and the contents to mixed well. Repeat until the agarose has completely dissolved and allow the agarose to cool on the bench top
e. 1µl of pronasafe was added per 100 ml molted agarose.
f. Agarose was poured into a casting tray until agarose was halfway up the line of the comb to create the well and allowed the gel to solidify and gently removed the comb.
g. Removed the gel from the casting tray and placed it in an electrophoresis box with enough TAE to cover the gel.

   5µl loading dye was added into the PCR product tube.

h. 10µl PCR product was added into each well except the negative control well and 5µl of molecular marker (1000 bp) was added to the first well of electrophoresis.
i. Replaced the lid to the gel box, turned on the power then ran electrophoresis for about 1:20hr at 120V.
j. Removed the gel from the gel tray and exposed the gel to UV light.

Annex 7: Ethical Clearance



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NATIONAL VETERINARY INSTITUTE

Ref.No: NVI/374
Date: 29/08/2023

To: Misganu Taso

Subject: Approval for Ethical Clearance

The research ethical committee of the National Veterinary Institute reviewed and discussed your research project entitled "**Isolation and Molecular Characterization of Infectious Laryngotracheitis (ILT) from active outbreak of poultry in Ethiopia**" on July 18, 2023. After review and discussion of your project proposal it is scientifically and ethically sound from relevance, originality and technical competence of view. Hence the project allowed to be executed provided that:

1. All procedure, condition stipulated in the proposal are respected and any deviation, variation or change may be made only in consultation between reported to the committee.
2. All comments given by the committee should be considered and fulfilled by the researchers
3. The project activity is open for occupational supervision by the committee whenever this is deemed necessary.

The committee expects to be informed about the progress of study with any changes in protocol.

Sincerely,

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Director General
Deputy Director General

The Federal Democratic Republic of Ethiopia National Veterinary Institute

ቢሾፍቱ፣ ኢትዮጵያ
Bishoftu, ETHIOPIA
011 433 - 84 - 11/16
251-911-51-08-94
FAX: 011 433-93-00
E-mail: nvi-rt@ethionet.et
info@nvi.com.et
Website: www. nvi.com.et

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