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Research Article

# Isolation and Molecular Characterization of Infectious Laryngotrachitis Virus from Poultry in and Around Bishoftu Town and the Bordering District Liban Cuqala

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## Abstract

Infectious Laryngotrachitis is an important respiratory disease of chicken caused by *gallid herpes virus-I* belonged to family *Herpesviridae*, subfamily *Alphaherpesvirinae,* genus *Iltovirus* The disease has little or no previous documented data in the country. The study was conducted from November 2022 to June 2022 by a cross-sectional study design with purposive sampling strategy. In this study, a molecular detection of ILTV were conducted generally in 12 pooled samples out of the total 40 poultries sampled from peasant Associations (PAs) of in and around Bishoftu town and the bordering district Liban Cuqala. Swab samples from upper trachea and tracheal tissue samples were collected from the selected PAs in the study area. From the total 12 pooled samples, 3 samples were positively detected for the presence of Infectious Laryngotrachitis. The study revealed an overall PCR detection of 25%. Three strains of ILT namely ICP4, TCO low and TCO high were detected with the general master mix that can bind with all of the ILT strains. Generally, ILTV were one of the serious avian respiratory pathogen challenging the study areas resulted in high economic losses. Control and prevention measures through vaccination programmed should schedule within the viral strains.

## Keywords

Bishoftu, Characterize ILT, Detect, Isolate, Nested-PCR

## 1. Introduction

From the total livestock population in Ethiopia, chickens are the largest with an estimated number of 57 million [1]. Annually, about 99% of chicken products for domestic consumption are supplied from chickens, which are raised under small-scale production system, whereas the remaining 1% is taken by chickens under intensive production system [2]. Nevertheless, due to population growth and a change in living standard in the country, it is expected that the demand for chicken meat and egg will increase by 268% and 737%, respectively between 2012 and 2050 [3]. However, lack of awareness regarding bio-security and its importance to poultry farming is still challenging the industry, such that the potential role of those centers in spreading communicable avian diseases to the vast majority of backyard poultry and to the rural population is likely to be underestimated [4].

One of the chicken respiratory diseases, affecting the

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Ethiopian economy is infectious laryngotracheitis (ILT) virus. Infectious laryngotracheitis (ILT) is a viral disease of poultry species caused by infectious laryngotracheitis virus (ILTV) and is an important acute contagious respiratory disease of chicken caused by *gallid herpes virus-I* belonged to family *Herpesviridae*, subfamily *Alphaherpesvirinae,* genus *Iltovirus* [5]. It is an enveloped, nonsegmented and linear double-stranded DNA virus. It shows high morbidity and mortality [6]. It causes in notable economic losses due to decreasing the growth rates, egg production, and increasing the mortality in commercial poultry, especially layer flocks, and usually, outbreaks are more severe in older birds than in younger flocks [7, 8]. They produce pathogenically important proteins such as envelop, tegument, capsid, glycoproteins including gL, gM, gH, gB, gC, gK, gG, gJ, gD, gI, gE, thymidine kinase (TK), transcriptional regulator and even non-structural proteins. gG is a conserved molecule among most alpha *Herpesviruses* that binds to and modulates the biological activity of chemokines [9, 10].

The Herpesviridae have genomes ranging in size from 124 to 241 kbp and been classified into three subfamilies, the Alpha-, Beta- and Gammaherpesvirinae. Infectious laryngotracheitis virus (ILTV, gallid herpesvirus 1) belongs to the subfamily Alphaherpesvirinae (genus Iltovirus) and causes acute upper respiratory tract disease in chickens of adult age especially [11, 12]. ILTV is an enveloped, non-segmented and linear double-stranded DNA virus. ILT viral particles are icosahedral in shape with hexagonal nucleocapsids about 80-100 nm in diameter consisting of 162 elongated hollow capsomeres. Envelopes surrounding the nucleocapsids have a diameter of 195- 250 nm [13].

There are two forms of disease including; Severe epizootic forms of infection which are characterized by respiratory distress such as gasping and expectoration of bloody mucus, high morbidity, and moderate-to-high mortality through occlusion of trachea, Milder enzootic forms of infection which occurs often in developed poultry industries and appear as mucoid tracheitis, sinusitis, conjunctivitis, general unthriftiness, and low mortality [7]. Herpesviruses are highly host-specific double stranded DNA viruses that have been isolated from most animal species, including mammals, birds, reptiles and fish. The virus mainly affects chickens as a primary host, although infections can occur in pheasants, peacocks, turkeys, and guinea fowl [8].

There is no proven transmission to humans or other mammals. The direct transmission of ILT is completed horizontally via respiratory secretions, feces, and dust, whilst the exposure of birds to infected carriers and fomites, equipment, litter, and personnel contributes to the indirect transmission of ILTV. Recovered birds become long-term carriers of the virus, which enters a latent state, mainly in the trigeminal ganglia [8].

Laboratory diagnosis of ILTV can be routinely performed by propagation of the virus in ECE and a variety of avian cell cultures such as chicken embryo liver (CEL), chicken embryo lung, chicken embryo kidney (CEK) and chicken kidney (CK) cell cultures. Molecular techniques, including conventional and real-time polymerase chain reaction assays, have been used efficiently in the diagnosis of ILTV [14].

Statement of the Problem

The Ethiopian poultry industry is growing quickly; however, high mortality and morbidity during production due to diseases are becoming major constraints. As poultry production expands and grows within Ethiopia, poor bio security has increased the emergence of different poultry viral diseases like infectious Bronchitis and ILT virus. These diseases have little or no previous history in the country. Despite the prevalence, there are no any Molecular reports on ILT, most commercial poultry farms in Ethiopia are never allowed to use any types of ILT vaccines [15]. Respiratory infections have major impacts on poultry welfare and productivity, yet are largely unstudied in most African countries, where attention has only be focused on the notifiable avian influenza (AI) and Newcastle disease (ND) viruses [16]; but also ILT is another challenging respiratory infectious disease to the Poultry of the country, Ethiopia.

As country's level, there is very rare documented data available concerning with the very challenging economic losses regarding an Infectious Laryngotrachitis of poultry till 2019 published by [15, 17] with the only seroprevance reports. This study was the first for the Isolation and Molecular Characterization of ILT as Country level. Although it is known that infectious respiratory pathogens, including infectious laryngotracheitis virus (ILTV), are a major threat to poultries Worldwide, there are very little data currently available on the Molecular detection of ILTV in Ethiopia except the only negative report by [16]. Therefore, in this study, a molecular detection of ILTV were conducted generally in 12 pooled samples out of the totally 40 poultries sampled from PAs of In and around Bishoftu town and the bordering district Liban Cuqala.

General Objective of the Research

To isolate, detect and molecularly Characterize of ILTV among the most avian Viral respiratory pathogenic disease that commonly causes serious challenges to the poultry industries especially in the Study Areas. And then to show the morbidity and mortality rates of the respiratory infectious Laryngotrachitis and indicating the virulence serotypes of this respiratory pathogen by inoculating into the embryonated [fertilized] Coryoallantoic membranes of the chickens' eggs.

*Specific Objectives:*

1. To isolate, detect and molecularly Characterize of ILTV from poultries in the study areas;
2. To recommend the control and prevention measures of the ILT Viral disease through confirming the disease.

## 2. Materials and Methodology

### 2.1. Study Area

The study was conducted from November 2022 to June

2022 in and around Bishoftu town and the bordering district Liban Cuqala of eastern Shoa zone of the Eastern country located at 60 km from the center of Country's city, Finfine. The town has an elevation of 1,999 meters, latitude of 8°45' ° N, and longitude of 38°58' E. It is the capital town of Ada'a District, the largest District in East Shoa Zone. The town is surrounded by Dire, Dhankaka, and Godino rural kebeles of Ada'a District in the north, the east and the south, respectively, and Dukam Town in the west. The district is the most agricultural important area in the central highlands of Ethiopia and is characterized by crop-livestock mixed farming system. Teff, barley, wheat, sorghum, maize, beans, peas, chick pea, lentils, linseeds, nug and rape seed annual crops grown in the area. cattle, sheep, goats, horse, mules, donkey, Pigs and poultry are the main livestock species raised in the zone [18].

The area receives an annual mean rainfall of around 789 mm with medium seasonal variability and bimodal pattern as stated by [19] which is 974 mm and average temperature of 18.6°C as of Regassa in 2014. An annual mean rainfall of the two becomes 881.5 mm. The "Belg" or the shorter season's rain, which is quite small to support crop production, usually occurs during the periods from the second week of March to second or third week of May. The long rainy season extends from the second week of June to the last week of September. The length of growing period (LGP) in the main rainy season in the district ranges from 112 to 144 days with a mean of 129 days [20]. The period includes the duration of the time that 100 mm of soil moisture reserve has already been evapotranspired after the end of the rainy season [1, 21].

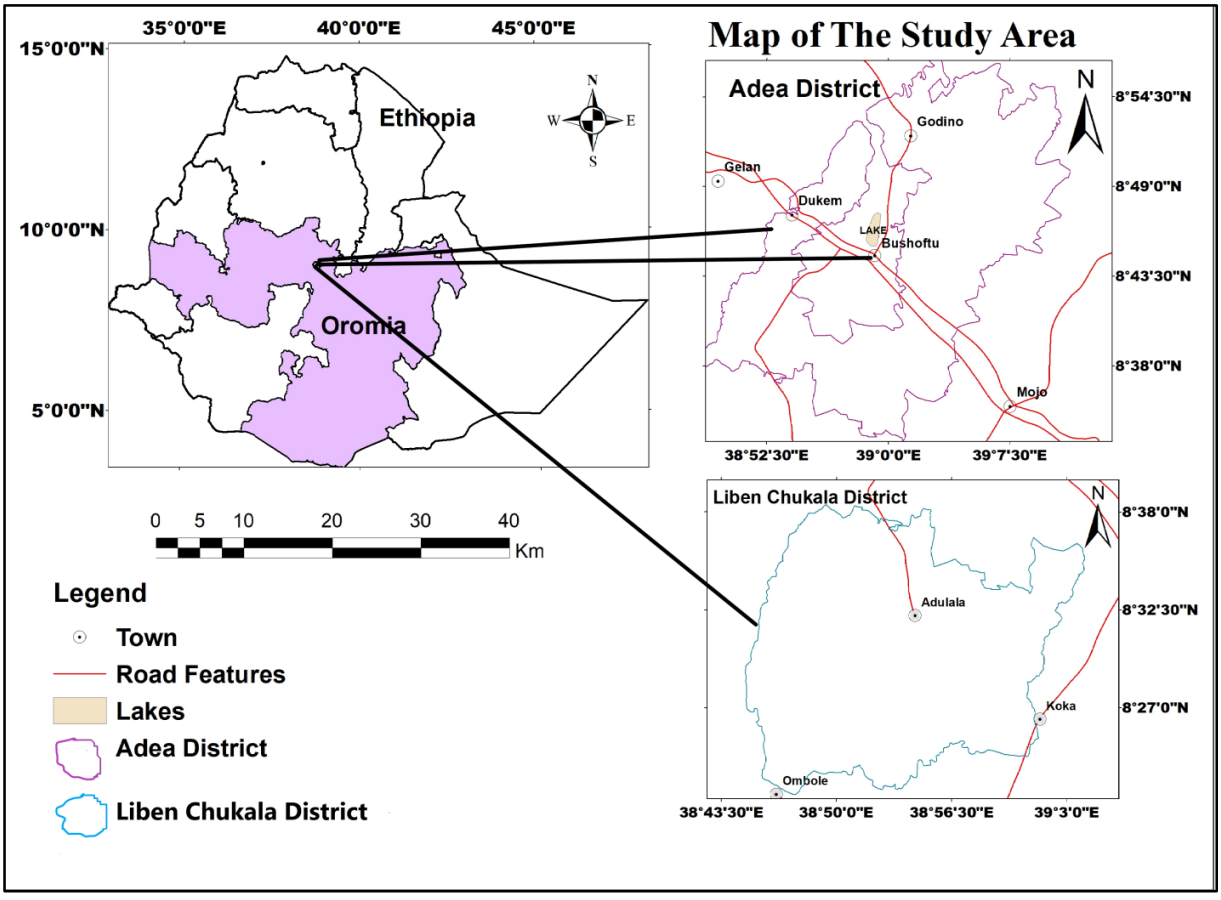



***Figure 1.*** *Map of the Study Areas.*

## 2.2. Study Population

The study animals were Intensive [small scale and medium scale] farms of Poultries of all ages, sexes and breeds, suspected by ILTV found in the district of the study area. Specifically, even if single diseased chicken or past history of the disease found were included as the study population. Eight medium scale farms in Bishoftu town of which one of the farm coded by F2 was sampled for 5 swab samples and 2 tracheal tissues and totally 20 swab samples and 5 tracheal tissues samples were sampled from this study area. Then two medium scale farms in Ade'a district [7 poultries] and also two medium scale farms in Liban Cuqala district [8 poultries] and totally 12 medium scale farms were included in the sampling farms and generally out of those 12 medium scale farms, totally 40 poultries were sampled.

## 2.3. Study Designs, Sample Size and Sampling Techniques

35 Swab samples from upper trachea and 5 tracheal tissues of poultry were collected from intensive [Small Scale] poultries' Farms of peasant associations and Medium scale farms in the study areas. The study design was conducted through cross-sectional study, and intended purposely. After collection, the samples were kept in viral transport medium (VTM) [in which the buffer solution is used to maintain pH at 7.3 +/- 0.2. Phenol red is the pH indicator] containing antibiotics and then transported to the Virology Laboratory, National Veterinary Institute, Bishoftu, Ethiopia, and stored at -80 ℃ until tested. This system, specifically achieved from 7 poultry farms of Bishoftu town [Bishoftu farm coded by F2 was sampled for both tracheal swabs and tracheal tissues] and from countryside kebeles of Liban Woreda 2 small backyards and from Adea Woreda 2 small backyards of the PAs were sampled. From each backyards and the larger farms, 50% and 25% of the diseased respectively were sampled purposely based on the size of population.

*Sample Collection and Transportation*

The clinical sample was collected by using deep swabs and Tissues from upper trachea's of poultries. Then placed into test tubes containing viral transport media (VTM), a sterilized pestles in the presence of phosphate buffered saline and the container were also inserted into Icebox having the temperature of +4°C. Then it were transported to Laboratory of NVI by the day of collection and then inserted into deep refrigerator adjusted at -20°C and stayed there until next day of transportation for processing in Virology Laboratory room and then stored there at -80°C until tested.

## 2.4. Laboratory Analysis

### 2.4.1. Preparation of Samples

The transported samples with the help of icebox were inserted into deep refrigerator having the temp. of -80°C and stayed there for 96 hrs. The swab samples were grinded and homogenized, and 10% suspension was prepared by using phosphate buffer solution (PBS). The suspension was centrifuged at 1500 rpm for 10 min for the collection of supernatant; and genomics DNA was extracted by QIAamp DNA Mini Kit, according to the manufacturer's guidelines. Then Nested-PCR was performed for the confirmation of ILTV positive samples by reference primer and probe. Then after it were processed as follows.

*Tissue sample homogenization:* Tissue sample were taken from -80°C deep freezer and stayed in room temperature for activation of the suspected microorganism. Then 1 gm of the tissue sample were cut off and transferred to petridish and then washed 3 times by Phosphate buffered saline solution containing antibiotics for the sake of reducing bacterial contamination in 2nd level Bio-safety cabinet. This procedure were done separately for each individual samples. After washing off, each individual samples were cut off into pieces by scissors by the help of rat toothed forceps in mortal and lastly grinded into very pieces by mortal's pistil. Totally 9 ml of PBS were added to it and after homogenization, it were transferred to bottle. Then after three of the samples separately prepared and 2 of them were pooled into 1, 4 of the suspension with 1 another bottle containing the same volume of water were inserted in automated centrifuge and centrifuged at 1500 rpm for 10 minutes. Then the centrifuged suspension were returned back to II level BSC and at there 1.5 ml~1 ml of the supernatant were taken and transferred into cryovial separately for each individual samples.

*Tracheal Swab Sample Processing:* Firstly 35 total swab samples were pooled into 8 samples that were prepared for molecular detection and characterization of Infectious Laryngotrachitis (ILT) Virus. Then from each cryovial, it were washed by PBS and again added or transferred to the total five (5) in one pooled samples. Then it were centrifuged by manually hold centrifuge till total homogenization were formed. Lastly 1.5 µl ~1 ml of the suspension were transferred to cryovial for each eight (8) samples. At the most end that days procedure, the eight (8) swab samples and four (4) of the tissues samples, totally of twelve (12) samples were processed and transferred to -80°C deep freezer for preservation and next days procedures of inoculation into embryonated chicken egg and molecular detection and characterization of ILTV.

### 2.4.2. DNA Extraction with Extraction Kit (QIAGEN)

*The Reagents Used to Extract the DNA of ILTV was:* Lysing buffer (Buffer AL) (Chem. Content: Guanidinium chloride @ 25-50%, Non-Hazardous Proprietary Ingredients @ Balance), Buffer AE, Protein Kinase K (Proteinase K) [Chem. Content: Proteinase @ <1%, Non-Hazardous Proprietary Ingredients @ Balance], Buffer AW2 ethanol (Chem. Content: Sodium Azide @ 0.1%, Non-Hazardous Salt Buffer @ 99.9%)

The prepared sample were taken from -20°C refrigerator. Then the prepared or processed sample were thawed/heated for activation. Then 200 µl of lysing buffer were added to the adjusted cryovial separately for each of the total 12 processed samples. Again 200 µl of processed ILT sample were again separately added to each of the cryovials containing the 200 µl of lysing buffer and mixed together. Then 20 µl of protease K (used to release unwanted contaminants from sample) were again added and the totally of 420 µl the suspension were added and mixed together by pulse-vortexing for 15 seconds. Then after that it were incubated at 56°C for 10 minutes. After incubation, 200 µl of ethanol were added and pulse-vortex-ed (centrifuged). Then after, it were transferred to minister centrifuge and centrifuged at 8000 rpm for 1 minute. After centrifugation, the total 620 µl of suspension were transferred to QIAMPSpin column cryovial and again centrifuged at 8000 rpm for 1 minute. Then after DNA of ILT was attached to the column of cryovial w/c is –vely charged; the total 620 µl of suspension were descanted and the DNA were then left on

cryovial column. Then 500 µl of AW1 (which have +ve charge) were added to the column and hence the two charges were attracted each other. And again it were centrifuged at 8000 rpm for 1 minute by mini spin centrifuge. Then it were transferred to Bio-safety cabinet and all the suspension (500 µl of buffer AW1) was descanted at there. Again 500 µl of AW2 were taken and transferred to the same vials and centrifuged at 14,000 rpm for 3 minutes. Then it were transferred to the same free vials after descanting the 500 µl of AW2 and transfer the column to 14,000 rpm for 1 minute for sake of drying only. Then after, that column were transferred to the new free vials having top securer and then at there, 50 µl of AE buffer were added to the membrane of the column spin to release the pure DNA and incubated at room temperature for 5 minutes and then centrifuged at 14,000 rpm for 1 minute. At this point DNA is released to the bottom of vials and the column spin were discarded at this stage and the vials containing DNA were ready for RT-PCR processing in the next class of Amplification room and stored at -20°C till next day of master mixing.

### 2.4.3. Multiplex Master Mixing

*Reagents used:*

***Table 1.*** *Reagents used for the total Master Mix of ILTV.*

| S/N | Types of reagents | For one reaction | Total reaction 16 | Remark |
|---|---|---|---|---|
| 1 | RNase free water | 3 µl | 48 µl | |
| 2 | Primer-ILT-Fow-5 pm/ µl 5'ACTGATAGCTTTTCGTACAGCACG-3' | 2 µl | 32 µl | Those used as multiplex master Mix [might be used for all strains of ILT] |
| 3 | PrimerILT-Reve-5 pm/ µl 5'CATCGGGACATTCTCCAGGTAGCA-3' | 2 µl | 32 µl | |
| 4 | 10 x Dream Taq™ buffer | 5 µl | 80 µl | Thermoscientific 20 mM ($Mgcl_2$) |
| 5 | dNTP mix, 2 Mm each | 5 µl | 80 µl | |
| 6 | Dream Taq™ DNA polymerase | 1.5 µl | 24 µl | |
| * | Total Rxn of Master mix | 18.5 µl | | |
| 7 | ILTV Template (DNA) | 3 µl | | In Amplification room |
| | Total volume | 21.5 µl | 296 µl | |

*Master Mix:* 90 pM (picomol) of depicted polycarbonate treated water was taken into each of the 14 cryovials [12 for sample equivalency and 2 for +ve and –ve controls].
NB.: 10 pM=10 µmol (Micromole) [in primers].

10 picomol of primers (Forward and Reverse) were pippetted and transferred to each of the 14 vials having 90 pM depicted polycarbonate and totally 100 µmol were prepared. After that 48 µl of RNase free water [for dilution and increasing of solution amount] and poured to the vials. Then 80 µl of $MgCl_2$ (buffer) [to keep PH and osmolarity] and again transferred to the same vials. Then after, 32 µl forward (Primer-ILT-Fow-5 pm/µl 5'ACTGATAGCTTTTCGTACAGCACG-3') and Reverse primers (Primer ILT-Reve-5 pm/ µl 5'CATCGGGACATTCTCCAGGTAGCA-3') were added to the same vials. After that 24 µl of Dream Taq™ DNA polymerase were also added to the same vials. Then 12 vials for the 12 samples and 2 for +ve controls and 1 –ve control were filled with the total suspension of 18.5 µl as [22] and transferred to Amplification room; and at there they were mixed together as the following:

### 2.4.4. Nested-PCR

The 14 totally Multiplex Master Mixed for the 12 samples of ILT extracted DNA with two positive and negative controls were homogenized together as follow. The 12 samples of ILT DNA extracted was taken from -20°C and pulse-vortex ed for activation and again mini-spin centrifugation for homogenization with multiplex master mixed. The 5 µl of extracted DNA of ILT was taken from each samples and transferred to the equivalented total number of multiplex master mixed vials. Then again pulse-vortex-ed and centrifuged with mini-star centrifuging machine for homogenization. Lastly the homogenized mixture of ILT and Primers into PCR runner machine by adjusting the initial denature at 94°C /3 minutes for 1 cycle; denaturation at 94°C /30 seconds for 35 cycles; annealing temperature at 60°C /45 seconds for 35 cycles; elongation at 72°C /1:30 sec for again 35 cycles and final elongation at 72°C /3 minutes. Samples were transferred to ILT Real

time PCR kit (Genekam®): This ready to use amplification kit has been manufactured by Genekam biotechnology AG, Germany, to detect ILT virus in real time PCR. The kit need DNA which can be isolated from nasal swab, respiratory swab, cell culture, vaccine, blood, lung tissue and other tissue and anybody fluid [23].

### 2.4.5. DNA Amplification with Amplification Kit (Genekam®)

The 12 ILT DNA samples were taken from -20°C and pulse-vortex ed and again centrifuged by minispin for homogenization. Then 5 µl of ILT DNA from each 12 samples, were transferred by micropipette to the total master mixed vials of 12 [100 µmol of master mix+5 µl of ILT DNA] without adding to the 1+ve & 1-ve controls. Again pulse-vortexing and centrifugation by minister were carried out for the sake of homogenization. After strictly homogenized, the suspension were transferred into PCR runner machine's grooves prepared for vials and after insertion, the tops of that machine was secured and adjusted as the following table with start time at 10:56 Am [4:56 LT] and end time at 1:30 PM [7:30 LT] just for 2:75 hrs (3:15 hrs).

*Table 2. Procedures of Amplification with its Temp, Time and Numbers of Cycles.*

| Procedure | Temperature | Time | Cycles |
|---|---|---|---|
| Initial denaturation | 94°C | 3 minutes | 1-cycle |
| Denaturation | 94°C | 30 sec | 35-cycles |
| Annealing | 60°C | 45 sec | |
| Elongation | 72°C | 2:30 sec | |
| Final Elongation | 72°C | 3 minutes | 1-cycle |
| Put at | 4°C | Until machine off | |

Key: The total 105 µl PCR product in each vials of the 12 vials with 2 positive and negative controls was inserted into the internal grooves of PCR machine. Then adjusted with the initial denaturation temperature of 94°C/3 minutes for 1-cycle and 94°C/30 seconds denaturation for 35-cycles; annealing temperature at 60°C/45 seconds for 35-cycles and elongation temperature at 72°C/2:30 seconds with final elongation temperature of 72°C/3 minutes for 35-cycles for both. At the end of the total cycles, the machine was adjusted at the temperature of 4°C and lastly after the total time of about 2:30 hrs, the machine was turned off.

### 2.4.6. Gel Electrophoresis

Then after, the PCR products were transferred to gel electrophoresis running room. Then 1.5% agarose gel powder were measured by ABT 220-4M machine (sensitive balance). Also 100 ml of 1 x TAE buffer solution were measured by 500 ml cylinder and transferred to sterilized flask with gel powder and mixed together and transferred for sterilization and homogenization to the sterilizer machine (IAEA [Internatiional Atomic Energy Agency] нь BOSCH) by ray for 10 minutes. Then the three very important dyes: loading dye, intercalating dye (Pronasafe) and molecular ladder were took out of refrigerator and pulse-vortex-ed. At this stage, the sterilized gel suspension were taken out of the machine with the help of bandages and brought to the working area and 5 µl of intercalating dye was added, and then mixed together strictly. Then after homogenization, it were poured on gel tray [container] which is adjusted with comb and then stayed for 15 minutes until gel is solidified.

After gel was solidified, it was transferred to gel tanker (BIORAD SUB-CELL®GT) containing $NaCl_2$ solution with its container. Then after that, the PCR product were pulse-vortex ed by Wagtech vibro Gene® SA4 machine and the cryovials tips opened and 5 µl of loading buffer added which is used for increasing the gravity of NA of that microorganisms, and leave the micropipette tips in each cryovials containing the PCR-product and mixed together by sucking and releasing redundantly. After that, the gel comb were taken out of the gel in upward manner and 10 µl of PCR and Loading buffer mixture of each product were transferred to the groove of gel formed by comb. Then 6 µl of molecular ladder were added at the 1st and end free grooves. Lastly, the total top securer was strictly secured and the electric source was switched on at 120 v for 1:20 hrs. After 1:20 hrs, the electric source were switched off and the gel was transferred to UV-light reading of PCR-product (UNIVARET®). Then the prepared gel with sample insertion was placed on the sensor area base of the UV-light linked with its computer software for reading.

### 2.4.7. Isolation of ILT Virus by CAM Inoculation

The 3 detected samples for ILTV by nested-PCR out of the total 12 pooled preserved field samples which were in sterile PBS were diluted approximately 1/5 in nutrient broth containing penicillin and streptomycin, and agitated vigorously. The suspension was then centrifuged at 3000 rpm for 30 minute for clarification. Supernatant fluid were then treated with broad-spectrum antibiotics and anti-fungal (Gentamycin 50 µg/ml, penicillin 2000 units/ml, streptomycin 2 mg/ml and mycostatin 1000 units/ml) at room temperature for one hour. The sterility of the inoculum were then checked on blood agar according to [10]. The resulting suspension were centrifuged at low speed to remove debris, and 0.1 ml of the supernatant fluid were inoculated on to the dropped CAM of 5 embryonated chicken eggs of 10–12 days' incubation for second passages. The eggs were then sealed with paraffin wax and incubated at 37 ℃ for up to 7 days. They will again candled daily and the CAMs of dead embryos or of those surviving for 7 days were examined for typical pocks according to [22, 23].

*Figure 2. Candling of Coryoallantoic membrane (CAM).*

Alternatively, 5 confluent CEL or CEK cell monolayers, with their medium removed, were inoculated and allowed to adsorb for 1–2 hours. Cultures were then overlaid with fresh medium, incubated for up to 7 days and examined daily under the microscope for evidence of a typical syncytial cell or cytopathic effect (CPE).

### 2.4.8. Extraction of DNA from Isolated Virus

DNA was extracted using Wizard Genomic DNA Purification Kit as per manufacturer's instructions of the kit. The primer pair targeting the envelope glycoprotein-G gene (US4 gene) of GaHV-1 as described by [24] was designed and that were used in the PCR reaction. The reaction were carried out using HotStar Taq Master Mix, with an initial denaturation at 95°C for 15 min, followed by 35 cycles at 95°C for 1 min, 61°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min. Two micro litres of the PCR product were analyzed by electrophoresis in 1.5 % agarose gel. PCR products were purified employing ExoSAP-IT (Affymetrix, USA) and sequenced through commercial source as Scigenom, Cochin.

### 2.4.9. Amplification of Isolated Virus

The total 14 master mixed for the 12 ILT extracted DNA samples with 2 positive and negative controls were transferred to amplification room and inserted to Biosafety cabinet level III. Then after, the 12 extracted DNA sample were taken from -20°C and thawed by minispin Pulse-vortexing wagtech vibro gene®SA4 and centrifugated by ministar® centrifugation machine for homogenization and transferred to the same Biosafety cabinet level III. Then at there, 5 µl of the extracted DNA from each of the 12 vials (that used as a template) were pipeted and transferred to the 12 of the total 14 master mixed (except +ve &-ve controls) with their equivalented codes. The positive control were taken from the previous. Then again pulse-vortexed and centrifuged. Lastly the homogenized viral DNA with forward and reverse primers were transferred to Real-Time PCR runner machine and adjusted its initial denaturation at 94°C/3 minutes [for 1-Cycle]; denaturation at 94°C/30 sec, annealing temp. at 60°C/ 45 sec, elongation at 72°C/$1^{1/2}$ minutes [for 35 Cycles] and final elongation at 72°C/3 minutes [for 1-Cycle] as [22].

### 2.4.10. Sequencing of Isolated Virus

The process of ILTV gene sequencing is ongoing process after sent to outside of the country. It may take 2-3 months to be replied and the result will be included in the publishment of the paper. The field isolate, (isolated virus) and purified amplicon at the virology laboratory of NVI; will be sequenced using BigDye Terminator V3.1 (Applied Biosystems) in accordance with the manufacturer's recommendations and this might be the first duty in Ethiopia. Resulting sequences will be analyzed using the nucleotide Basic Local Alignment Sequence Tools (BLASTn) (http://www.ncbi.nlm.nih.gov/BLAST/) at the National Center for Biotechnology Information [25] and the multiple sequence alignment program (ClustalW2) at the European Bioinformatics Institute (http://www.ebi.ac.uk/Tools/clustalw2/).

## 2.5. Data Analysis

Collected raw data were stored in a Micro soft Excel database system used for data management. SPSS Windows latest 20 version was used for data analysis. Mean ILTV mortality and related results of the study were expressed using descriptive statistics (mean ± standard error of mean, percentage, and graph).

# 3. Results

Out of the total 40 samples that were pooled to the total of 12 prepared for molecular tests [35 of tracheal swab samples pooled to 8 and from 5 tracheal tissues 2 were pooled and the 3 rests were taken directly], three samples coded by number 3, 4 [Bishoftu, Gibayu farm and Girar farm], and 5 [Liban Cuqala farm-2] or 1, 4 and 7 as shown by figures 4 and 5 respectively were positively detected for ILTV by one step RT- PCR at base pairs of 688 (688 bp) with a minimal slight band formation here in Ethiopia for the first time up to date except for the only 19.4% seroprevalence were observed by [26]. The present studies indicate that out of the total seven (7) medium scale farms and five small scale farms, three poultry farms named as Bishoftu Girar farm, Bishoftu Gibayu farm and Liban Cuqala farm-2 were detected for the presence of ILTV by nested PCR at the base pairs of 688 as indicated by table 3 and gel electrophoresis figures 4, 5 and 6.

*Table 3. Nested PCR Results of the total samples.*

| S.N | Study areas | Farm name | Types Of Sample | Tot № of sample taken | Sample Code | After pooling sample code | Nested PCR Result |
|---|---|---|---|---|---|---|---|
| 1 | Bishoftu town | F1 | Tracheal Swab | 5 | 1-5 | 01 | x |
| | | F2 | Tracheal Swab | 5 | 6-10 | 02 | x |
| | | Girar* | Tracheal Swab | 5 | 11-15 | 03 | + |
| | | Gibayu* | Tracheal Swab | 5 | 16-20 | 04 | + |
| | | Barberetera | Tracheal tissue | 1 | 36 | 09 | x |
| | | Gebeya | Tracheal tissue | 1 | 37 | 10 | x |
| | | F2 | Tracheal tissue | 2 | 38-39 | 11 | x |
| | | Gara-beru | Tracheal tissue | 1 | 40 | 12 | x |
| 2 | Liban Cuqala (Adulala) district | F1 | Tracheal Swab | 5 | 28-32 | 06 | x |
| | | F2* | Tracheal Swab | 3 | 33-35 | 05 | + |
| 3 | Ade'a district | F1 | Tracheal Swab | 3 | 21-23 | 07 | x |
| | | F2 | Tracheal Swab | 4 | 24-27 | 08 | x |

Key: X=Negative samples; +=Positive samples

As stated by [27], the standard base pair [bp] to read the ILTV positivity is 688 bp and 635 bp by the fragments of the ILT ICP4 gene. With this standard, 3 samples were positives for ILT. Those samples were number 3, 4 and 5 or 1, 4 and 7 as shown by the figures 4 and 5 below respectively.

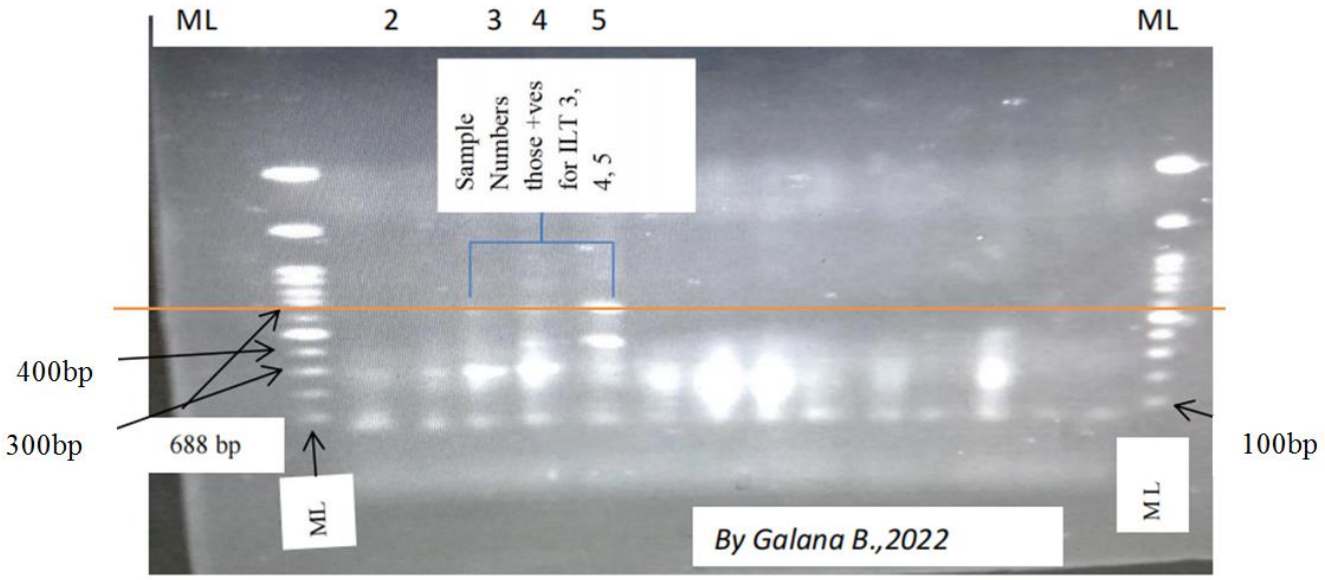



*Figure 3. Visualization of PCR product run on a 1.5% agarose gel of PCR.*

Key: ML-Molecular Ladder, 688 bp- position of base pairs at which ILTV was positives, 3, 4, 5 were numbers of samples those positives for ILT.

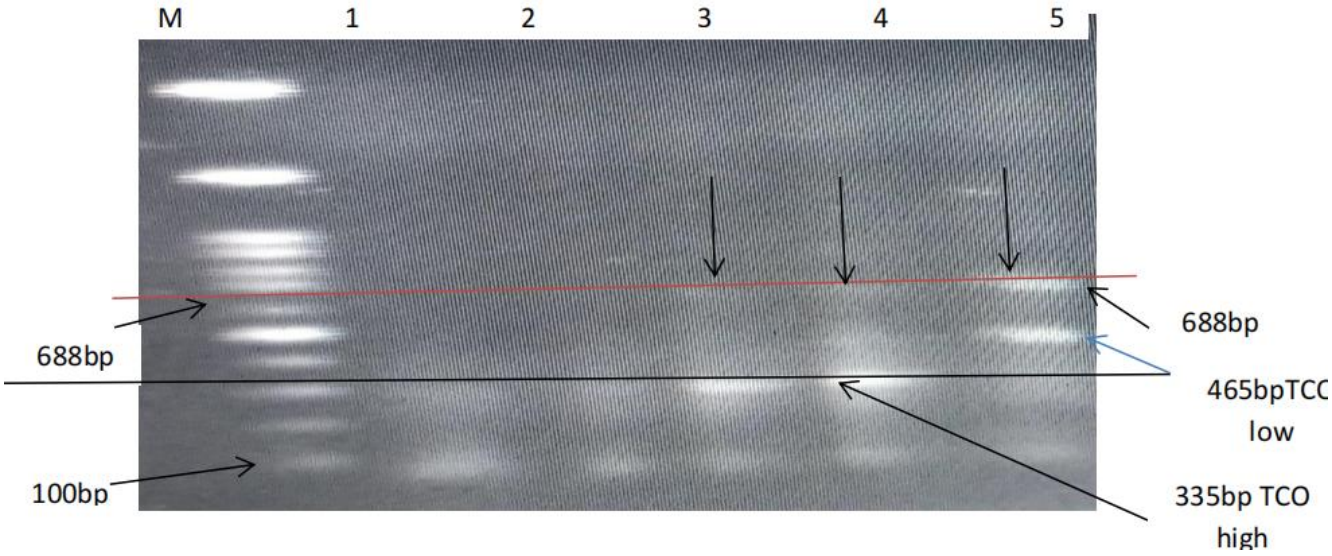
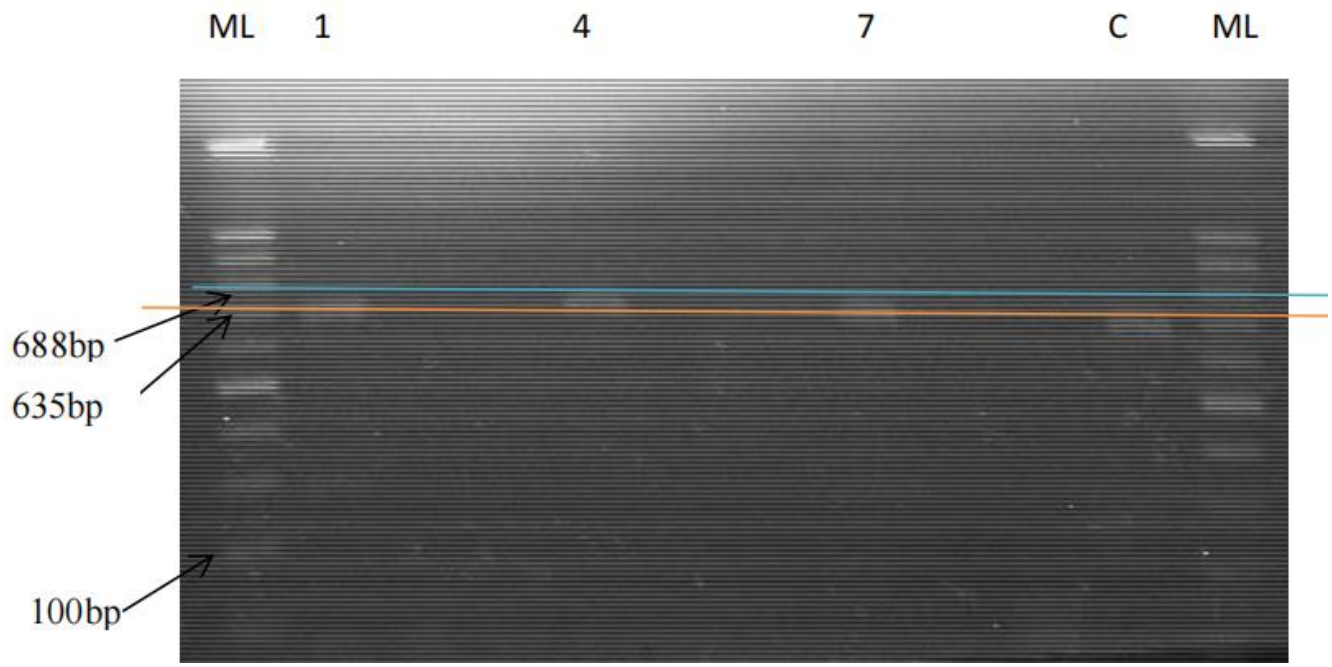



***Figure 4.*** *Progress of PCR product Optimization run on a 1.5% agarose gel of PCR.*

Key: ML-Molecular Ladder, 688 bp- position of base pairs at which ILTV was positives, 3, 4, 5 [indicated by arrows] were the numbers of samples those positives for ILT. Three field strains of ILTV, ICP4, TCO low and TCO high were detected.




Key: ML:- Molecular Ladder, C:- Control, 1, 4 and 7 are ILTV positive samples

***Figure 5.*** *After Optimized Visualization of PCR product run on a 1.5% agarose gel of PCR.*

Generally the figures 3, 4 and 5 above indicate that the visualization attempts of gel electrophoresis band formations by different annealing temperatures of 60°C 45 sec, 65°C/30 sec [there were no any band formed and left from attachment], 62°C/45 sec, 59°C/45 sec respectively.

Then after 2 days of the sample preparation, eggs of SPF (specific pathogen free) were pierced by piercer (needle tip) on free space on Chorioallantoic sac and inoculated with 0.2 ml and 0.3 ml. I.e 4 ECE inoculations per each sample of ILTV sample suspension into 48 of 10 days embryonated hen's eggs with 2 controls and totally 48 ECE of w/c one sample were inoculated into two for the case of increasing chance of viral existence and propagation with. Lastly of inoculations, all except control were sealed by candle. At the end it were again incubated at 37°C after candling and stayed for 24 hrs. After 24 hrs of incubation, again candled and

checked for the death existence of ECE and no any death of ECE were seen; i.e. all were allives. After 48 hrs (after 2 days of 1st incubation), 1 embryo were died which were labeled by code 03 of swabs inoculated with 0.3 ml of sample suspension. After 96 hrs (after 4 days of 1st incubation), the result were as follows:

***Table 4.*** *Processed and Inoculated in CAM samples result.*

| S.N. Of Samples | Types of samples | Sample code | Amount of inoculation (ml) | Inoculation Result after | | |
|---|---|---|---|---|---|---|
| | | | | 24 hrs | 48 hrs | 96 hrs |
| 1 | Tracheal Swabs | 01 | 0.3 | X | X | X |
| | | | 0.3 | X | X | Died |
| | | | 0.2 | X | X | Died |
| | | | 0.2 | X | X | Died |
| 2 | | 02 | 0.3 | X | X | X |
| | | | 0.3 | X | X | Died |
| | | | 0.2 | X | X | Died |
| | | | 0.2 | X | X | Died |
| 3 | | 03 | 0.3 | X | X | Died |
| | | | 0.3 | X | Died | * |
| | | | 0.2 | X | X | X |
| | | | 0.2 | X | X | X |
| 4 | | 04 | 0.3 | X | X | Died |
| | | | 0.3 | X | X | Died |
| | | | 0.2 | X | X | Died |
| | | | 0.2 | X | X | X |
| 5 | | 05 | 0.3 | X | X | Died |
| | | | 0.3 | X | X | X |
| | | | 0.2 | X | X | X |
| | | | 0.2 | X | X | X |
| 6 | | 06 | 0.3 | X | X | X |
| | | | 0.3 | X | X | X |
| | | | 0.2 | X | X | Died |
| | | | 0.2 | X | X | X |
| 7 | | 07 | 0.3 | X | X | X |
| | | | 0.3 | X | X | X |
| | | | 0.2 | X | X | Died |
| | | | 0.2 | X | X | X |
| 8 | | 08 | 0.3 | X | X | X |
| | | | 0.3 | X | X | X |
| | | | 0.2 | X | X | X |
| | | | 0.2 | X | X | X |

| S.N. Of Samples | Types of samples | Sample code | Amount of inoculation (ml) | Inoculation Result after | | |
|---|---|---|---|---|---|---|
| | | | | 24 hrs | 48 hrs | 96 hrs |
| 9 | Tracheal Tissues | 09 | 0.3 | X | X | Died |
| | | | 0.3 | X | X | Died |
| | | | 0.2 | X | X | Died |
| | | | 0.2 | X | X | Died |
| 10 | | 10 | 0.3 | X | X | Died |
| | | | 0.3 | X | X | X |
| | | | 0.2 | X | X | X |
| | | | 0.2 | X | X | X |
| | | | 0.3 | X | X | X |
| 11 | | 11 | 0.3 | X | X | Died |
| | | | 0.2 | X | X | Died |
| | | | 0.2 | X | X | Died |
| 12 | | 12 | 0.3 | X | X | X |
| | | | 0.3 | X | X | X |
| | | | 0.2 | X | X | X |
| | | | 0.2 | X | X | X |

Key: X=survived embryos, only one embryo was died after 48 hrs of post inoculation coded by 03 swab sample's code from the total number of 12 homogenized samples and 21 embryos were died after 96 hrs of post inoculation [13 from tracheal swab samples and 8 from tracheal tissues].

In each instance, up to three passages of CAM for Viral Isolation, Incubator, Petridish, forceps, scalpel blade, fluorescence microscope, candler and the like were the necessary materials before a specimen was considered to as a negative. During the first and second inoculation there were no lesions observed but at the third passages, there were an Embryonic changes observed as follow.

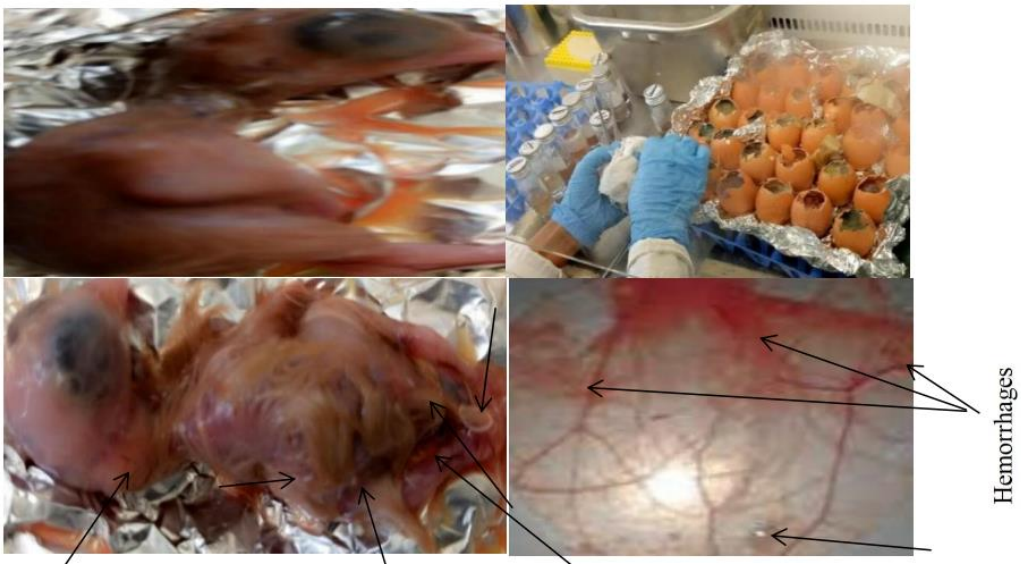



***Figure 6.*** *After 3rd passages Whitish Pock-sack and hemorrhages are Observed.*

Generally out of the total 12 homogenized samples [35 of tracheal swab samples pooled to 8 and from 5 tracheal tissues 2 were pooled and the 3 rests were taken directly], three (3) tracheal swab samples coded by 03, 04 and 05 given for the Bishoftu Girar farm-A1, Bishoftu Gibayu farm-A2 and Liban farm-2 respectively were positively detected by PCR. The rest tracheal swab samples coded by 01, 02, 06 07 and 08 given for Bishoftu farm-1, 2, Ade'a farm-1, 2 and Liban farm-1 respectively and the four (4) tracheal tissues coded by 09, 10, 11 and 12 taken from Barberetera, Gebeya, Bishoftu farm-2 and Gara-Beru respectively were negatively resulted. Out of the total 48 10 days fertilized chicken eggs inoculation through CAM, totally 22 Embryos were died after 96 hrs of incubation in 37 ℃ of Incubator. After the total third passages of CAM fluid to the 10 days fertilized chicken eggs, whitish pock sack, hemorrhages and dysmorphia were observed.

The present study reveals an overall PCR detection of 25%. Polymerase chain Reaction (PCR) techniques were the best techniques to detect infectious Laryngotrachitis virus even from direct samples. With this truth, 3 samples of the total 12 pooled samples were directly detected before viral propagation in ECE.

## 4. Discussion

It is clear from the present findings that ILT is one of the major poultry viral diseases circulating in the study areas confirmed from 3 of the total 12 pooled samples out of total 40 samples. There were high percentages of infectious laryngotrachitis that can easily disseminate to the neighboring farms even to the country level.

The viral disease was causing very high mortality in chickens and prevalent in different production systems of poultry in and around Bishoftu town and the bordering district Liban cuqala where there was no history of vaccination against ILT in-lined with [30, 15]. Adult and young ages of the chicken were most severely attacked by infectious Laryngotrachitis which partially disagreed with [7, 8] due to they reports as the adults were the majorly attacked groups. ILTV causes an acute and chronic respiratory disease in poultry leading to significant economic losses to the poultry industry in-agreed with [13].

Transmission of the viral antigen through chicken to chicken contact, environmental contamination and poor management systems were the key routes. The virus is not easy to control, because it is highly contagious, can cause latent infections, and has many transmission sources in-lined with [31, 32]. Clinical and pathologic findings were consistent with previously reported severe form of infectious laryngotracheitis. This form is characterized by severe dyspnea, high mortality and marked hemorrhagic and/or diphtheritic laryngotracheitis and are attributed to wild-type ILTV in agreed with [10].

Nested-PCR and Real-time PCR technique showed high sensitivity to detect ILT virus before isolation of the virus into cell culture and after isolation into CEF cell culture by amplification of matrix gene in-lined with (Zaid *et al.,* 2017). The strains ICP4 at 688 pb, TCO low at 465 bp and TCO high at 335 bp (Ou and Giambrone, 2012) were found in the same wells of gel electrophoresis that were be due to the problem of pooled samples taken from different chicken of the same farm.

9-10 days of fertilized Chicken eggs passages upto 3 directly from CAM to CAM increased the density of infectious laryngotrachitis antigen chance of isolation by forming small whitish pock sacks in-lined with [5, 15]. When samples taken from live birds for virus isolation, tracheal swabs were superior to oropharyngeal or conjunctival swabs and tracheal tissues in-lined with [27, 28]. Any prolonged storage of infected tracheal tissues and tracheal swabs were at –70 ℃ to minimize loss of virus titter. Repeated freezing and thawing were avoided as thus reduces virus infectivity in-lined with [23].

Generally from the present study, tracheal swab samples were the highly targeted and superior for the viral antigen detection cross-checked by polymerase chain reaction, viral isolation and propagation in 10 days fertilized chicken eggs and gel electrophoresis. The present study reveals an overall detection of 25% out of the total 12 small scales to medium scales farms of the study areas. Out of the total 22 died embryos, 7 died embryos were the directly suspected ILT since included in the positive samples cross-checked by the gold standard test PCR.

## 5. Conclusions and Recommendations

Infectious Laryngotrachitis virus is one of the avian respiratory diseases and Causes serious challenges to the economy of the country in hidden manner due to unstudied in details and no well documented data available especially in detection of the ILTV to the country level. In few negative reports of molecular detection of ILT virus in Ethiopia has long been considered nonexistent and a disease of no concern in the country. Three strains of ILTV were circulating in the study areas causing serious challenges to the poultry industries. There is a high variation in the genetic properties between strains, these variations may play a role determining the antigenic and pathological characteristics of the viruses present in the field. Tracheal swab samples were the highly targeted and superior than tracheal tissue samples for the viral antigen detection cross-checked by polymerase chain reaction, viral isolation and propagation in 10 days fertilized chicken eggs and gel electrophoresis. The steps and types of techniques, equipments and the temperatures of storage followed were the mandatory remindations of post negatively detection of the samples. Based on the above Conclusions the following recommendations were forwarded.

Commercial farms, backyards and Peasant associations of

the study areas even the whole country level should awared about control measures of the disease.

Village chickens should be vaccinated against most infectious diseases including ILTV.

The persistence time of ILTV maternal antibodies (MAb) on unvaccinated SPF progeny flocks should be investigated to design an optimum vaccination schedule.

Detail study about the disease to its molecular level should be carried out.

Infectious laryngotrachitis disease (ILTV) virus molecular epidemiology should be studied with a planned interval to assess the antigenic diversity of the ILTV virus.

## Abbreviations

| | |
|---|---|
| CAMs | Chorioallantoic Membranes |
| CEF | Chicken Embryo Fibro-blast |
| CEK | Chicken Embryo Kidney |
| CEL | Chicken Embryo Liver |
| CK | Chicken Kidney |
| CSA | Central Statistical Agency |
| CTLs | Cytotoxic T-lymphocytes |
| ECE | Embryonated Chicken Eggs/Egg Cell Embryo |
| ELISA | Enzyme Linked Immuno Sorbant Assay |
| ILTV | Infectious Laryngo Trachitis Virus |
| NKC | Natural Killer Cells |
| NVI | National Veterinary Institute |
| OIE | Office International des Epizooties |
| Pas | Peasant Associations |
| PCR | Polymerase Chain Reaction |
| RFLP | Restriction Fragment Length Polymorphism |
| RT-PCR | Real Time Polymerase Chain Reaction |
| SPF | Specific Pathogen Free |
| VN | Viral Neutralization |

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## Author Contributions

**Galana Biratu Marga:** Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing

**Dereje Abera Wako:** Supervision, Visualization

**Belayneh Getachew:** Resources, Validation

## Conflict of Interests

The authors declare no conflicts of interest.

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