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Seroprevalence and molecular detection of infectiouS laryngotracheitiS viruS (iltv) in commercial chicken in gazipur diStrict of BangladeSh

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abstract

Infectious laryngotracheitis (ILT) is one of the major diseases of chickens that cause great economic losses in commercial chicken farms. It is caused by the infectious laryngotracheitis virus (ILTV). This research work was designed to investigate the seroprevalence of ILTV in commercial chicken farms in Gazipur district. A total of 590 blood sera were collected randomly from commercial chicken farms from different locations in Gazipur district. An antigen-coated indirect ELISA was performed to determine the antibody titer against ILTV in these sera. Moreover, gross and histopathological changes in different organs were investigated. Polymerase chain reaction (PCR), a molecular detection method, was performed to detect the fragment of the glycoprotein 'C' (gC) gene of ILTV in the infected chicken. Overall, 195 out of 590 serum samples were positive for anti-ILTV IgG, representing 33.05% of positive cases. The highest mean titer of ILTV antibody was found in anti-ILTV-vaccinated layer chickens with a mean titer of 3554. In seasonal variation, the highest prevalence of ILTV was recorded during the winter, followed by the rainy and summer seasons. The prevalence of ILTV was the highest at the age of 10-30 weeks in layer chickens. Necropsy findings of dead chickens showed severe congestion and fibrosis in the lungs and trachea, with occlusion of the tracheal lumen by mucus, caseous exudates and blood. Microscopically, hemorrhage and huge infiltrations of inflammatory cells were found in trachea and lungs. Moreover, the nucleotide sequence of 'gC' gene of ILTV was amplified successfully and yielded 1.26 kbp amplicons. This study suggested that ILTV was endemic in Gazipur district and the PCR technique is a useful molecular tool for diagnosis of ILTV in the commercial chicken industry.

keywords: ILTV, indirect ELISA, PCR, histopathology, chicken, Gazipur.

introduction

In Bangladesh, the poultry industry, a significant agricultural subsector, has increased employment opportunities, increased the availability of high-quality protein, ensured food security, contributed to economic growth, and decreased poverty levels in both urban and rural areas (Hamid *et al.*,

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2017; Ali & Hasan, 2018). There are several obstacles that prevent the establishment of a sustainable poultry industry, but disease is by far the biggest one. One of the most common chicken diseases that annually results in significant financial losses in commercial poultry farms is infectious laryngotracheitis (ILT). This disease is caused by infectious laryngotracheitis virus (ILTV), classified into the genus *Iltovirus* in the family *Herpesviridae* in the subfamily *Alphaherpesvirinae* (Islam *et al.*, 2010).

Taxonomically, the virus is known as *Gallid herpesvirus 1* (Roizman, 1982). It is a double-stranded DNA virus that is enveloped, non-segmented, and linear, and measures around 155 kb (Islam *et al.*, 2010; Hidalgo, 2003; Johnson *et al.*, 1991). Viral transmission occurs through respiratory secretions, which are easily transmitted through respiratory tract or mechanically by people and fomites (Menendez *et al.*, 2014). Morbidity and mortality rates from ILT vary depending on the virulence of the circulating strain (Kirkpatrick *et al.*, 2006; Oldoni *et al.*, 2009). Mortality due to ILT can be as high as 70%, leading to significant economic losses in high-density poultry-producing regions (Bagust *et al.*, 2000).

Due to increased mortality, lower egg production, delayed body weight gain, and predisposition to various respiratory diseases, ILT frequently develops in densely populated poultry production regions and results in significant economic losses (Vagnozzi *et al.*, 2012; Guy & Garcia, 2008).

However, ILTV is a significantly less studied organism that causes respiratory diseases in chickens. Confirmatory diagnosis of ILTV by molecular technique can contribute to the development of a novel, safe, and effective vaccine against ILT. Therefore, isolation and identification of the prevalent strains in an endemic area are needed to develop effective vaccines to control the disease. However, no investigation has been performed for the isolation, and molecular detection of ILTV strains available in Bangladesh. Very few investigations have been performed on the investigation of seroprevalence of ILTV in Bangladesh (Jahan *et al.*, 2012; Islam *et al.*, 2010). Also, there is no available information regarding the pathology and pathogenesis of ILT in Bangladesh. In addition, Gazipur serves as center of Bangladesh for the production of chicken. 70% of chicken and eggs are produced in Gazipur district. Considering the above facts, this study was aimed at investigating the sero-surveillance of ILTV in commercial chickens of Gazipur district in Bangladesh, and to elucidating the gross and histopathological changes in different tissues of chickens that occur due to ILTV, and molecular detection of the locally isolated ILTV strain as well.

materials and methods

ethical issues

The ethical issues of this experiment have been examined and approved by the Animal Research Ethics Committee of Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur-1706, Bangladesh (Ref. No. FVMAS/AREC/2023/10).

Sample collection

A total of 590 sera samples were obtained from several chicken farms in the Gazipur district of Bangladesh (Gazipur Sadar,

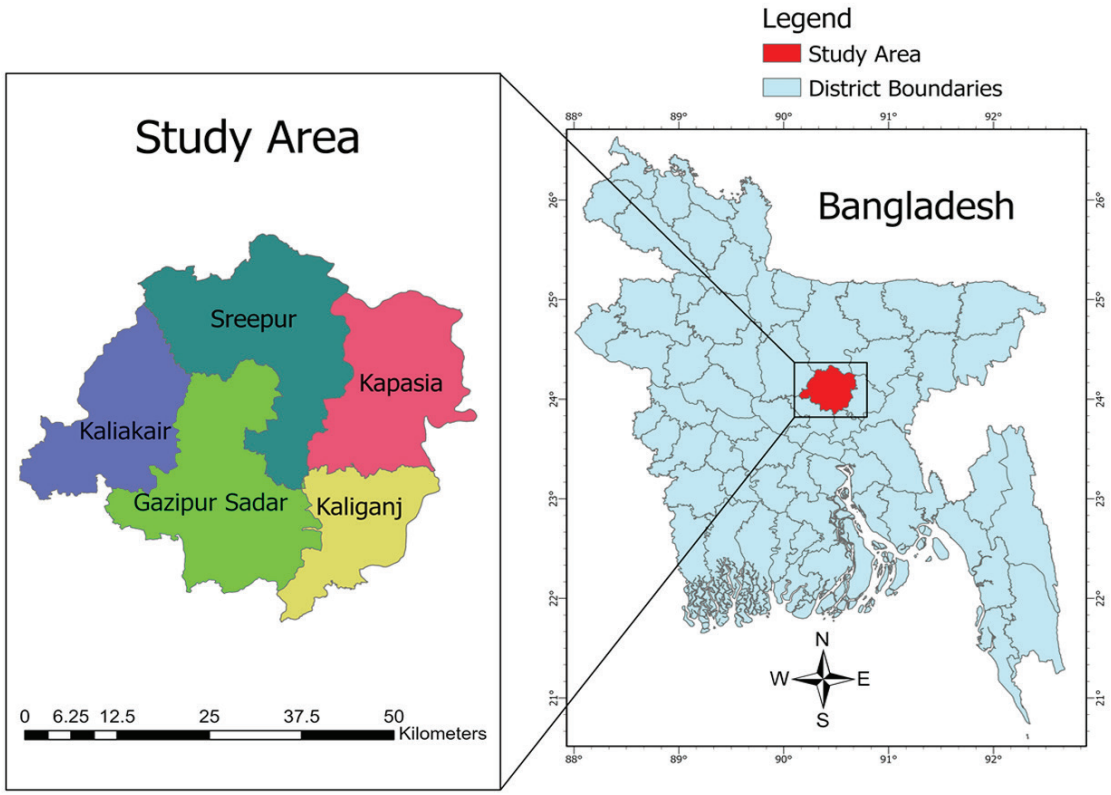

fig. 1. Study area map of Bangladesh.

Kapasia, Kaliakoir, Sreepur and Kaliganj) at various sites (Fig. 1). From these farms, dead and sick birds were collected for necropsy and assessment of gross lesions in various organs. Following necropsy, representative organs collection was made, including the trachea, lungs, comb, wattle, liver, brain, spleen, heart, kidney, cecal tonsils, intestine, proventriculus, gizzard, bursa of Fabricius, shanks, gonads, and egg follicles.

histopathological examination

All of the collected samples were cut into pieces for histopathological studies, fixed in 10% neutral buffered formalin (Gridley, 1960), dehydrated in a series of ascending grades of alcohol, cleared in a series of changes of xylene, and infiltrated with various grades of melted paraffin in a 56°C oven. The tissues were then fixed in paraffin, and finally, using a rotatory microtome, the slices were cut at a 5-m thickness. The hematoxylin and eosin staining procedure (H&E) was then used to stain the sections (Luna, 1968).

Serum preparation and indirect eliSa

To examine the seroprevalence of ILT, 590 sera samples were randomly and sterilely collected. Briefly stated, 1 ml of blood was taken from wing vein of each chicken using a sterile 2ml disposable plastic syringe without anticoagulant and the collected blood samples were subsequently stored in ice box. The samples were then transferred to the lab

for the serological test indirect ELISA. The serum and blood were then clearly separated by centrifuging the blood samples at 1,000 g for 10 minutes. Then the sera were collected in a sterile Eppendorf tube and stored at –20°C until further study. According to the manufacturer's instructions, indirect ELISA was performed on the acquired sera samples using a commercial ILT antibody test kit.

A commercial ELISA kit (Biochek, Holland) was used to perform indirect ELISA. The pre-coated ELISA plate and the reagents included in the kit were used to standardize all conditions in accordance with the manufacturer's instructions. The titer was estimated using the absorbance value of 1:500 dilution of a serum at 405 nm. The S/P value (color absorbance value of a sample to positive ratio) of samples to an end point titer is defined by the manufacturer's instructions as follows: log10titer=1.1× (log10 S/P) + 3.361. Anti-ILTV antibodies were considered to be present in serum samples with a S/P value of ≥0.501 (titer 1071 or higher), while those samples with a S/P value of ≤0.500 (titer 1070 or less) were considered to be negative.

extraction of dna and pcr

In order to collect viral DNA, scrapings from the lung and trachea of seropositive and clinically suspected non-anti-ILTV-vaccinated chickens were used following the manufacturer protocol (QIAamp DNA Mini Kit, Qiagen Inc., Valencia, CA, USA). TaKaRa Ex Taq™ (Takara Bio Inc., Shiga, Japan) was used for the PCR. ILTV glycoprotein 'C' (gC) gene was amplified from the collected DNA by using following primers: forward primer, 5' AACATGCAGCATCAGAGTACTG 3' and reverse primer, 5' CGTTTATGTTGTCTTCCAGCAC 3'. The manufacturer's instructions were followed in order to prepare the PCR reaction. The PCR conditions were as follows: 94°C for 2 min, 35 cycles of denaturation at 94° C for 30 sec, annealing at 54°C for 30 sec and extension at 72°C for 1.5 min, followed by a final cycle of extension at 72° C for 5 min (Craig *et al*., 2017). A negative control, ddH2O, was applied to each reaction. Electrophoresis on a 1.0% agarose gel with a 1-kb plus DNA ladder as a marker (Invitrogen, CA, USA) was used to identify the PCR results.

Sampling category

The obtained sera samples were divided into groups based on two (2) criteria: season of the year (winter [October to February], summer [March to May], and rainy [June to September]) and age group (10-30 weeks, 31-60 weeks, and more than 60 weeks of age).

data interpretation

Data were entered in a Microsoft Excel workbook at the conclusion of the study and exported to the SPSS 13.0 version for descriptive statistics analysis. The prevalence of ILTV in commercial chicken in several upazilas of the Gazipur district was examined using *chi*-square tests.

results

overall seroprevalence of iltv in gazipur district

A total of 590 sera samples were obtained from the Gazipur district and 195 of those were ILT-positive, making up 33.05% of all cases (Fig. 2). A total of 192 samples were collected from the Gazipur Sadar Upazila, where there were 66 ILT-positive cases and a

proportion of 34.38%, the highest percentage of ILT positive cases. The lowest number of ILT positive cases was found in Kapasia upazila, where 108 serum samples were obtained. Of these, 33 were ILT positive, making up 30.56% of the total. Among 103 samples, 35 (33.98%) positive cases were found in Kaliakoir upazila; among 75 samples, 25 (33.33%) positive cases were found in Sreepur upazila; and among 112 samples, 36 (32.14%) positive cases were found in Kaliganj upazila. According to statistics, there was no significant variation in the frequency of ILTV across the various upazilas in the Gazipur district ($p > 0.05$) (Fig. 2).

Seroprevalence of iltv in chickens without iltv vaccination

Out of the 590 sera samples, 230 were collected from layer chickens that had not received the anti-ILTV vaccination. Of these, 57 samples tested positive, making up 24.78% of the total cases (Fig. 3). The largest percentage of ILT positive cases (26.58%) was found in Gazipur Sadar upazila, where there were 21 ILTV positive cases out of 79 samples. Kaliganj upazila had the lowest percentage of positive cases (22.45%) where 11 samples were positive out of 49 samples. But the prevalence of ILTV in this chicken did not change significantly ($p > 0.05$) (Fig. 3).

Seroprevalence of iltv in vaccinated layer chicken

A total of 120 sera samples were collected from anti-ILTV vaccinated layer chickens, of which 115 were positive instances, making up 95.83% of the total (Fig. 3). In the instance of layer chickens that received the anti-ILTV vaccination, a total of 54 sera samples were obtained from Gazipur Sadar (35), and Kaliakoir (19), where every sera sample tested positive for the ILTV virus, making up 100% of the cases of ILT positivity. Kaliganj has

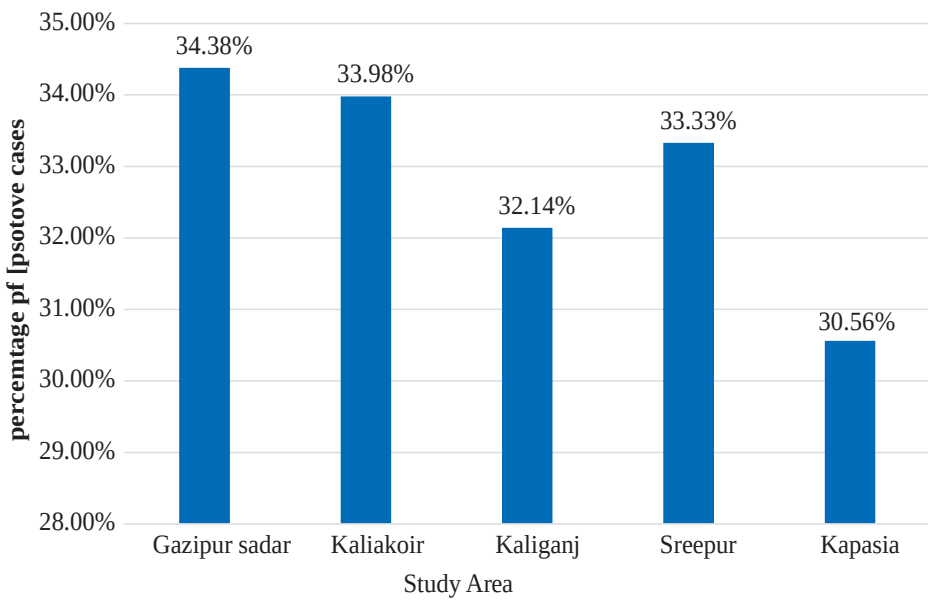

fig. 2. overall seroprevalence of iltv in study area.

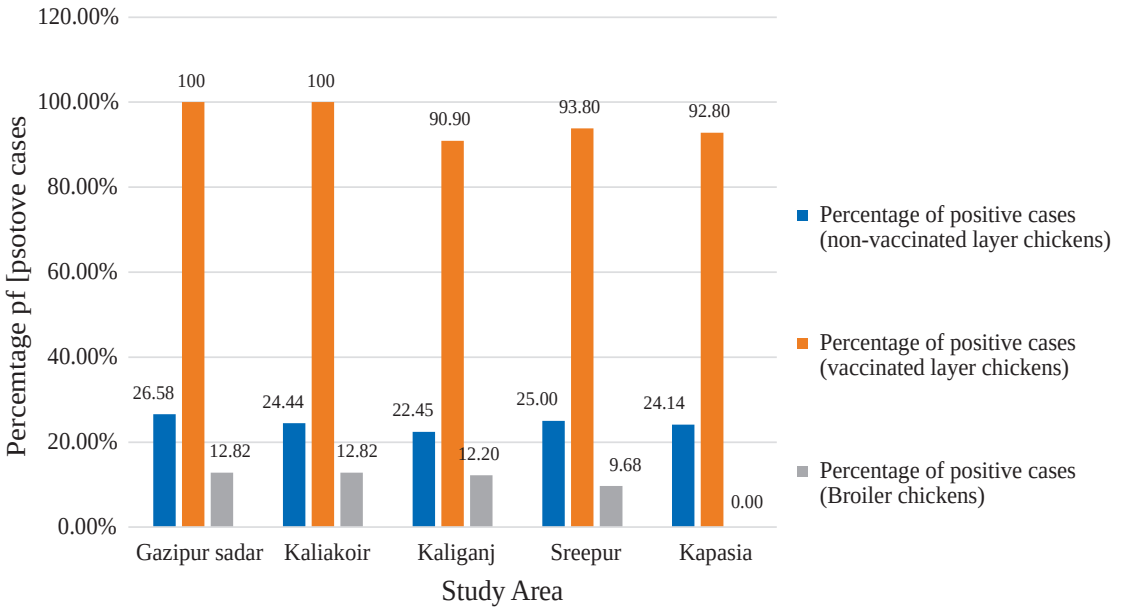

fig. 3. iltv positive cases in sera samples, collected from non-vaccinated layer chickens, vaccinated layer chickens and broiler chickens of study area.

the lowest prevalence rate (90.90%). There was no significant change in the prevalence of ILTV in this chicken ($p > 0.05$) (Fig. 3).

Seroprevalence of iltv in broiler chickens

A total of 240 sera samples from broiler chickens were obtained, of which 23 were ILT-positive, making up 9.58% of the total (Fig. 3), which is very low. The highest percentage of ILT-positive cases, 10 (12.82%) out of 78 samples, were found in Gazipur Sadar. Also, the prevalence of ILTV was 12.82% in Kaliakoir. 51 sera samples were collected and no positive cases were noted in Kapasia (Fig. 3).

Antibody titer against ILTV in different types of chickens

The layer chickens that had received the anti-ILTV vaccination had the highest mean titer of ILTV antibodies, which was 3554 (Fig. 4). The antibody titer for the seropositive samples was 1670.82-7418.16. The elevated antibody titer in this instance was linked to layer chicken inoculation with the live ILTV vaccine. In layer chickens who had not received the anti-ILTV immunization, the mean titer of the ILTV antibody was 980. The titer range of seropositive samples was 1172.95-6504.68. The mean titer for broiler chicken was 680. The titer range of the seropositive samples was 1702.75–4372.57. Titer ranges between 0 and 1070 were deemed negative (Fig. 4). All of the broiler chickens used in the study were raised without receiving any ILTV vaccines. These findings showed that ILTV is endemic in Bangladesh.

Seasonal prevalence of iltv

A total of 590 sera samples from three different commercial chickens (broiler, layer without anti-ILTV vaccination, and layer with anti-ILTV vaccination) were collected during the course of the year's three major

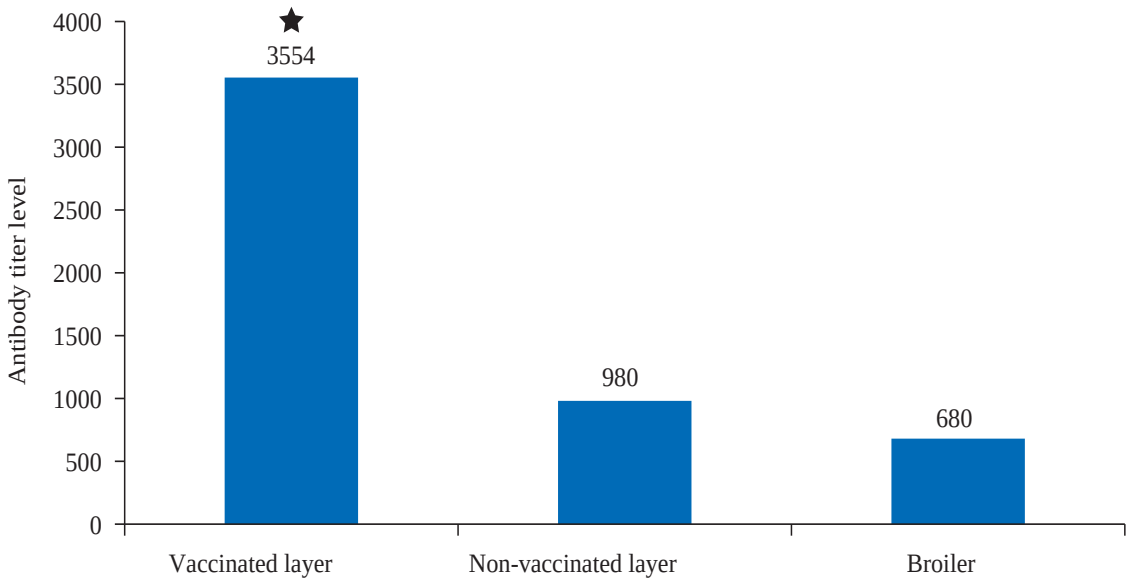

Fig. 4. Antibody titer against ILTV in different types of chickens. Significant differences are indicated with one ($p < 0.05$) asterisk in vaccinated layer chickens.

seasons—winter, summer, and rainy. For all three varieties of chicken, the winter had the highest frequency of ILTV, which was followed by the rainy and summer seasons. The prevalence of ILTV was significantly higher ($p < 0.05$) in the winter (36.05%) than in the rainy (21.13%) and summer (15.07%) seasons in the case of layer chickens lacking the ILTV immunization (Fig. 5).

Age-Specific Prevalence of ILTV

The frequency of ILTV was significantly higher ($p < 0.05$) in chickens of 10–30 weeks of age without ILTV immunization (38.75%) than in chickens of 31–60 weeks of age (20.00%) and in chickens older than 60 weeks (14.28%) (Fig. 6). Additionally, although there was no significant difference ($p > 0.05$), the prevalence rate of ILTV in layer chickens with ILTV vaccination was higher at 10 to 30 weeks of age (Fig. 6).

Clinical findings occur due to ILTV infection in chicken

The ILTV-affected chickens had moist rales and nasal discharge, followed by depression, laborious breathing, expectoration of blood-stained mucus, and pump hand respiration (Fig. 7: A & B). Some of the affected birds made a respiratory noise. Moreover, affected birds had conjunctivitis, swollen infraorbital sinuses, and closed eyes. In extreme cases, the eyelids and skin around the eye were adherent with a dry, crusty ocular discharge. Bloody mucus was expelled by several birds, and it was evident in their mouths, on their beaks, and everywhere around them. Spots of dried bloody exudates were recorded on the sides and bottom of cages in a flock that had been severely affected. On the walls of feeders and at watering sites in some farms, dried blood stains were observed. Laying chickens had lower egg production, anorexia, and signs of

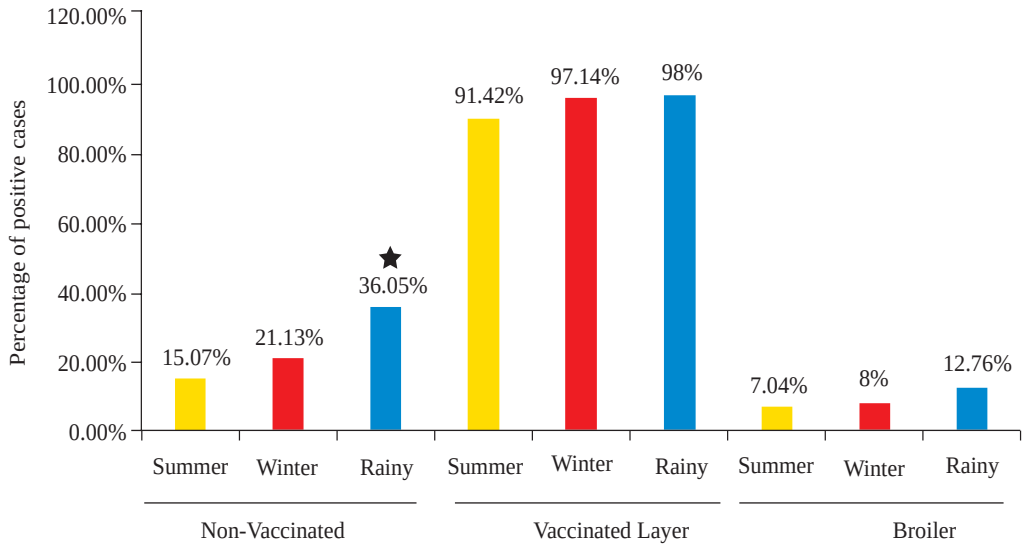

fig. 5. Seasonal prevalence of iltv positive cases in sera sample from three types of commercial chicken (layer without anti-iltv-vaccinated, layer with anti-iltv-vaccinated and broiler). In case of all the three types of chicken, the highest prevalence of ILTV was recorded during winter, followed by rainy and summer season. The prevalence of ILTV was significantly higher ($p < 0.05$) in the winter (36.05%) than in the rainy (21.13%) and summer (15.07%) seasons in the case of layer chickens lacking the ILTV immunization. Significant differences are indicated with one ($p < 0.05$) asterisk in winter season.

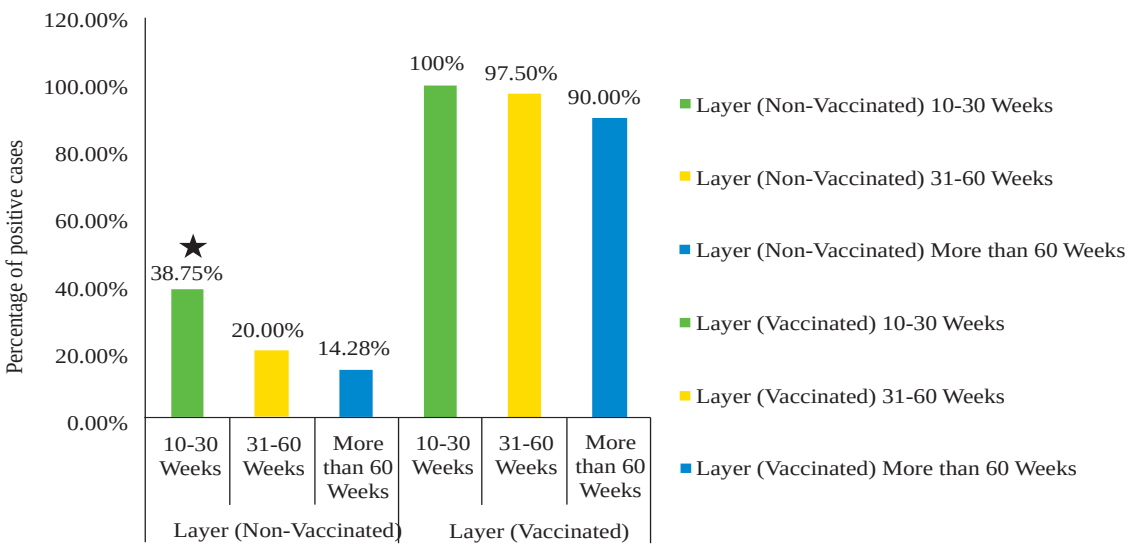

Fig. 6. Age Specific Prevalence of ILTV positive cases in sera sample from layer without anti-ILTV-vaccinated, and layer with anti-ILTV-vaccinated. Prevalence of ILTV was significantly higher ($p < 0.05$) in the chickens without iltv vaccination of 10–30 weeks of age (38.75%) than in the chickens of 31–60 weeks of age (20.00%) and in the chickens of more than 60 weeks of age (14.28%). Moreover, prevalent rate of ILTV in ILTV-vaccinated layer chickens was also higher at 10-30 weeks of age, though there was no significant difference ($p > 0.05$). Significant differences are indicated with one ($p < 0.05$) asterisk.

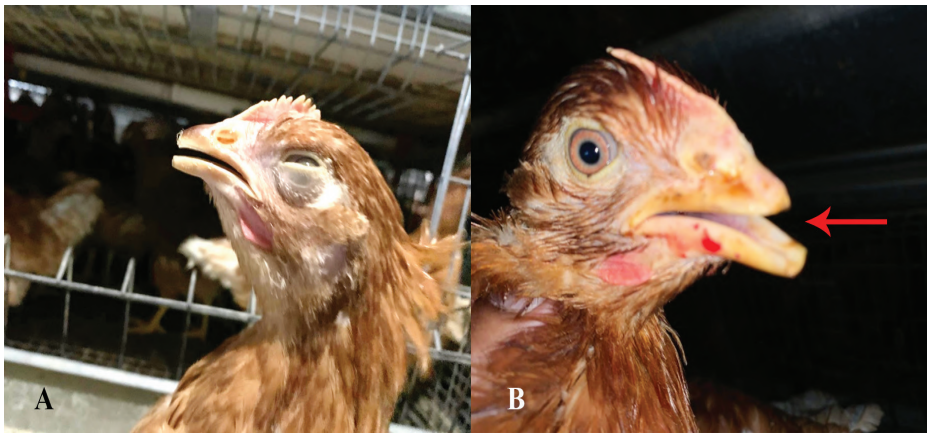

fig. 7. (a & B) ILTV affected bird, pump hand like respiration with wide open beak.

sadness. Young broiler chickens stretched out their necks as a result of dyspnea. The mortality rate gradually increased daily, and by 3 to 5 days, 30 to 40% of the flock had perished.

gross and histopathological changes in different organs and tissues in ILTV-infected chickens:

Grossly, mucus and severe hemorrhage were seen in the trachea (Fig. 8: A). The lumen of the trachea was partly obstructed by white or yellowish exudate (Fig. 8: B). The lungs had severe fibrosis and congestion (Fig. 8: C). The spleen had petechial hemorrhages (Fig. 8: D). Microscopically, hemorrhage with destruction of lining epithelial cells and mononuclear inflammatory cells infiltration were observed in the trachea (Fig. 9: A). In the lung, there was severe congestion, alveolar wall damage, and an invasion of mononuclear inflammatory cells (Fig. 9: B). Additionally, splenic hemorrhages were observed (Fig. 9: C).

molecular detection of viruse

In this study, DNA were isolated from the scrapings of trachea and lungs of serologically and clinically ILTV-positive chicken which were not vaccinated with anti-ILTV vaccine. Partial nucleotide sequences of 'gC' gene were successfully amplified from the isolated DNA. A product of the expected 1.26 kbp was successfully amplified (Fig. 10).

discussion

The research work was conducted to investigate the seroprevalence of ILTV in commercial layer chickens from Gazipur districts in Bangladesh, to establish a molecular detection method of ILTV and also to observe gross and histopathological changes in different tissues of chickens that occur due to ILTV. 590 commercial Chickens from various commercial chicken farms in various upazilas of the Gazipur district were randomly selected for sera samples in an aseptic manner. Out of 590 sera samples, 230 were collected from layer chicken farms without anti-ILTV vaccinations, 120 from anti-ILTV-vaccinated layer farms, and 240 from broiler farms without anti-ILTV vaccinations. Indirect ELISA was used to examine ILTV seroprevalence. The ELISA test was used to detect the ILTV antibodies because it is

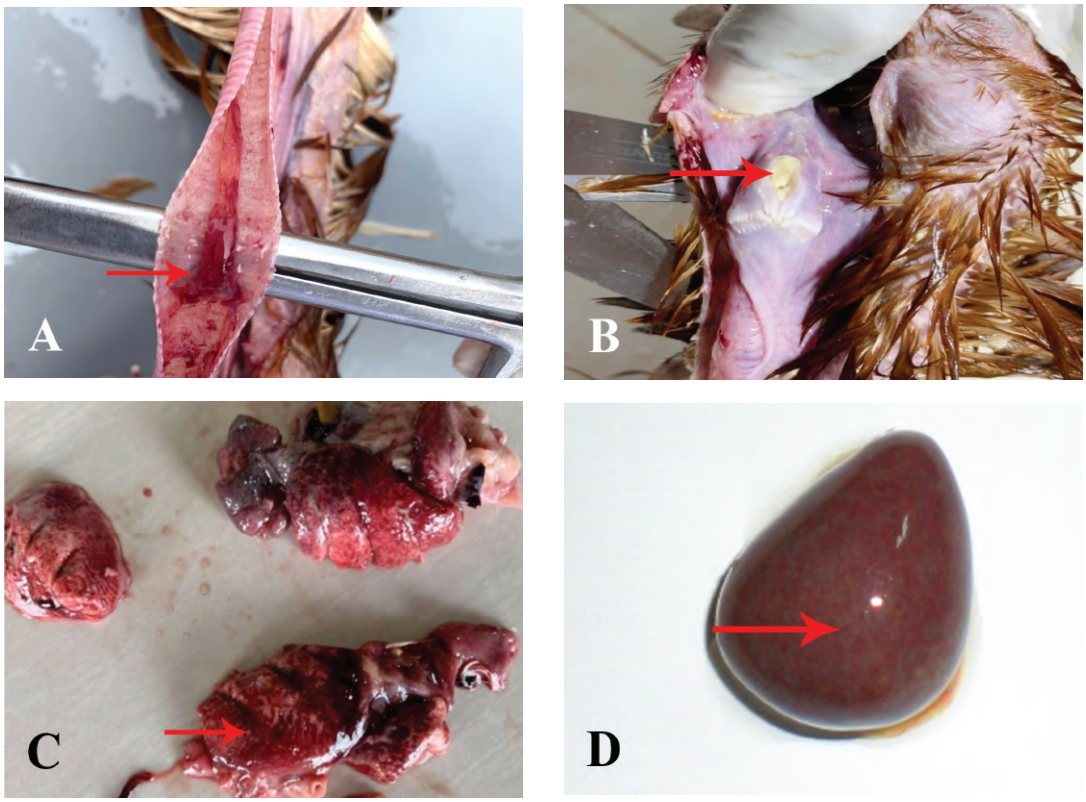

fig. 8. a. Severe hemorrhage and mucus in trachea. B. The lumen of trachea contained whitish or yellowish exudate partially occluded the lumen. C. Severe congestion and fibrosis in lung. D. Petechial hemorrhages in the spleen.

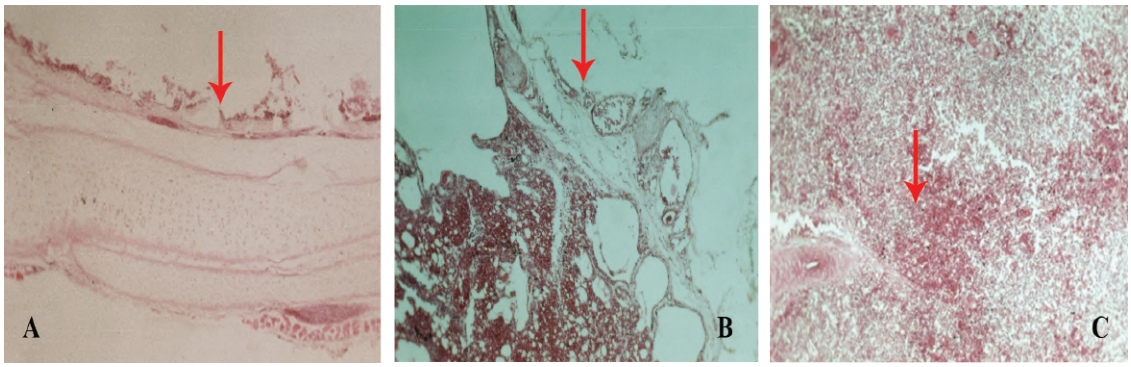

fig. 9. A. Hemorrhage with destruction of lining epithelial cells and infiltration of mononuclear inflammatory cells were found in trachea. B. Severe congestion, destruction of alveolar wall and infiltration of mononuclear inflammatory cells in lung were observed. C. Hemorrhages were also found in the spleen.

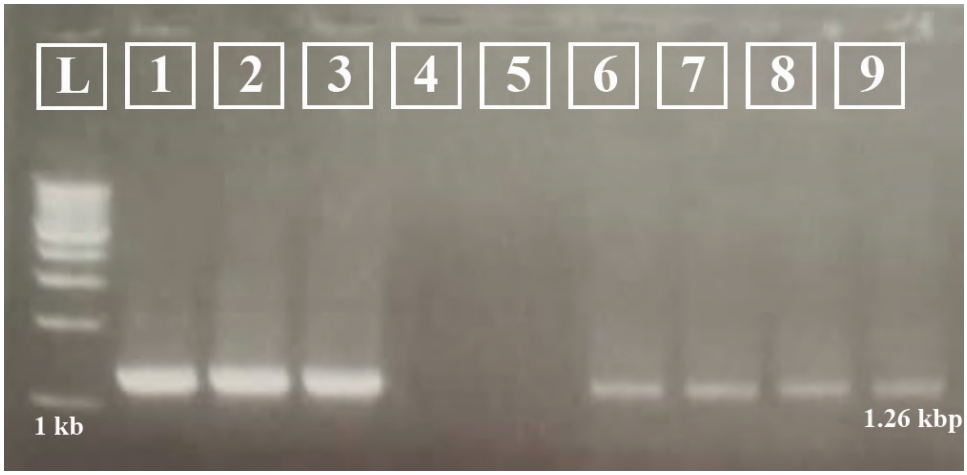

fig. 10. pcr detection of glycoprotien 'c' (gc) gene of iltv . Lane L is for 1kb ladder. Lane 1-3 and Lane 6-9 showed 1.26 kbp long amplicon of 'gC' gene of ILTV. Scrapings from trachea (Lane 1-3) and lung (Lane 6-9) of seropositive and clinically suspected layer chicken which were not vaccinated with anti-ILTV vaccine, were used to isolate viral DNA. Lane 4-5 were used for negative control.

more sensitive than serum neutralization (SN), fluorescent antibody (FA), and agar gel immunodiffusion (AGID) (Adair *et al*., 1985).

In this study, the total seroprevalence of ILTV in the Gazipur district was 33.05%. All of the sera were obtained from chickens without a history of clinical ILTV infections. According to earlier research, the seroprevalence of ILTV in the commercial layer was 81.47% in the Gazipur districts (Rahman *et al*., 2018) and 92.28% in various parts of Bangladesh (Jahan *et al*., 2012). In these instances, all of these flocks had a prior history of clinical ILTV infection (Rahman *et al*., 2018; Jahan *et al*., 2012). In the Chattogram district, where there was no record of an ILTV immunization campaign, the seroprevalence of ILTV was 17.33% (Uddin *et al*., 2014).

Among the upazillas of Gazipur district, the highest prevalence of ILTV was recorded in Gazipur Sadar area (34.38%). The seroprevalence of ILTV in non-anti-ILTV-vaccinated layer chickens were the highest in Gazipur Sadar within Gazipur district. Moreover, seroprevalence of ILTV in broiler chickens was also the highest in Gazipur Sadar and Kaliakoir. Actually, Gazipur Sadar is a region with a lot of poultry. Farmers in this region were maintaining a lower standard of biosecurity. However, in the Gazipur district, the prevalence rate of ILTV in broiler chickens was just 9.58%, which is extremely low. On the other hand, there were no records of ILTV immunization programs being carried out in broiler farms in this region. In Iran, broiler flocks were shown to have a 13% seroprevalence of the infectious laryngotrachitis virus (Ghalyanchi *et al*., 2020). In comparison to younger flocks, ILTV outbreaks are more severe in older chickens. In this study, it was shown that for all types of experimented chickens, the frequency of ILTV was much higher in the winter season than in the rainy and summer seasons. It

might be because of the colder temperatures, which cause the virus to survive longer and spread more rapidly in the winter (Robertson & Egerton, 1981). This study validated the findings of other studies (Bagust & Johnson, 1995; Pattison & Jordan, 1996; Fulton *et al*., 2000; Uddin *et al*., 2014). This variation might be brought about by modifications in the state of maternal antibodies, vaccination effectiveness, biosecurity, mechanical carriers, host variables, etc. (Uddin *et al*., 2014). Furthermore, it has been reported that cold, dry weather enhances the stability of viruses and promotes their spread. Low temperatures resulted in an increase in the death rate from respiratory diseases (Ghalhari & Mayvaneh, 2016).

The sera samples used in this experiment were divided into three age categories: 10–30 weeks, 31–60 weeks, and more than 60 weeks. The frequency of ILTV was significantly higher in chickens without ILTV vaccination at the ages of 10-30 weeks (38.75%) compared to those at 31-60 weeks of age (20.00%) and more than 60 weeks of age (14.28%). Furthermore, although there was no significant difference, the prevalence rate of ILTV in layer chickens with ILTV vaccination was also higher at 10 to 30 weeks of age. In the previous investigation, it was reported that the prevalence of ILTV was 25% in chickens of 20–30 weeks of age (Bauer *et al*., 1999). However, it was higher than the results of another study (Fahey *et al*., 1990), which found that the prevalence of ILTV antibody was 31% in chickens aged 10 to 30 weeks and 37.1% in chickens aged 15 to 35 weeks, respectively.

Seropositive and clinically infected chickens that underwent necropsy investigation revealed significant hemorrhage in the lungs and trachea as well as occlusion of the tracheal lumen by copious amounts of blood, mucus, and caseous exudates. Numerous chickens had tiny blood clots that stuck to their tracheal and laryngeal mucosa. Tracheal lumens were shown to be blocked by whitish or yellowish exudate in some cases. Lungs were found to be fibrosed and congested. Microscopically, trachea and lungs had hemorrhage and huge infiltration of inflammatory cells. The pathology seen in this ILT investigation is consistent with the earlier work (Sivaseelan *et al*., 2014).

Moreover, the 'gC' gene of ILTV was partially amplified for molecular detection. Although the detection of the 'gC' sequence was insufficient to fully characterize the circulating strains, it did aid in the rapid identification and diagnosis of the disease from locally circulating isolates. Molecular detection of ILTV has already been carried out in India using the envelope glycoprotein G gene (US4 gene) (Gowthaman *et al*., 2014). The typical clinical symptoms and pathology of ILT seen in birds are correlated with the PCR identification of ILTV. In order to confirm the ILTV, the PCR assay would be a helpful tool. Viral isolation has been reported to be less sensitive than PCR (Williams *et al*., 1992), particularly when other contaminating viruses as adenoviruses are present (Alexander & Nagy, 1997).

Diagnosis of disease is very important for proper treatment of a disease. For the diagnosis of a disease, clinical signs, gross tissue changes, and histopathological examination are very important. However, ILTV is significantly less studied than several

other human and animal herpesviruses. The findings of this study will open a new area for the prevention and control of ILTV in the poultry industry in Bangladesh. This result will be used for the sustainable development of poultry production, which will help the production of more chicken meat and eggs and will ensure the protein demand of the people of Bangladesh. Thus, the study could improve the livelihood of poor people and the lifestyle of society.

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