# Isolation and Identification of Infectious Laryngotracheitis Virus from Outbreaks at Lipa City, Batangas, the Philippines

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## ABSTRAK

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Infectious laryngotracheitis (ILT) merupakan penyakit pernafasan akut dan sangat menular pada ayam yang ditandai dengan kesulitan bernafas dan batuk yang disertai pengeluaran eksudat berdarah. Penyakit ini disebabkan oleh virus Herpes yang termasuk dalam famili *Herpesviridae*, subfamili *Alphaherpesvirinae*. ILT tersebar luas di seluruh dunia, termasuk di Filipina sejak tahun 1980. Penyakit tersebut tidak pernah dilaporkan keberadaannya semenjak terjadinya wabah. Sampel yang digunakan untuk isolasi penyakit ILT berasal dari sembilan lokasi peternakan ayam petelur komersial yang berlokasi di kota Lipa, Batangas dari bulan Mei hingga Juli 2002. Sampel berupa trakhea dan paru-paru diproses dan diinokulasikan pada telur ayam tertunas melalui *chorio-allantoic membrane* (CAM). Lima sampel menghasilkan lesi yang khas berupa becak pada CAM setelah pasase kedua. Lesi tersebut berupa becak berwarna kekuningan dengan bagian tepi yang keruh dan tersebar diseluruh bagian CAM. Galur vaksin yang digunakan sebagai kontrol positif menghasilkan lesi berupa becak yang sama dengan lesi yang berasal dari isolat lapang. Hasil konfirmasi secara serologik dengan menggunakan uji *Agar Gel Immunodiffusion* (AGID) memperlihatkan garis presipitasi yang sangat jelas pada agar setelah bereaksi dengan antisera ILTV standar reference (anti-NS-175). Semua isolat menghasilkan garis presipitasi terhadap masing-masing isolat tersebut dan juga terhadap kontrol positif. Hasil penelitian ini membuktikan bahwa penyakit yang mewabah pada peternakan ayam petelur tahun 2002 di kota Lipa, Batangas disebabkan oleh penyakit ILT.

**Kata kunci:** *Infectious laryngotracheitis*, *agar gel immunodiffusion*, isolasi, lesi berupa bercak, identifikasi, CAM

## ABSTRACT

SAEPULLOH, M and ROVIRA H.G. 2003. Isolation and identification of infectious laryngotracheitis virus from outbreaks at Lipa City, Batangas, Philippines. *JITV* 8(2): 122-134.

Infectious laryngotracheitis (ILT) is an acute, highly contagious respiratory disease of poultry characterized by respiratory disorder such as coughing with blood exudate from the trachea. The disease is caused by *Herpesvirus* of the family *Herpesviridae* and subfamily of *Alphaherpesvirus*. ILT is worldwide distribution and has been reported to be present in the Philippines since 1980. Since then, confirmation of subsequent outbreaks were not reported. Isolation was conducted from nine commercial layer chicken farms located at Lipa City, Batangas from May to July 2002. Tracheal and lung extracts were processed and inoculated into embryonated chicken eggs by chorio-allantoic membrane (CAM) inoculation. Five samples produced typical pock lesions in CAM after the second passage. Lesions observed were yellowish pocks with opaque edges, distributed throughout the CAM. A vaccine strain of the virus used as the positive control also produced similar pock lesions. Serological confirmation using the Agar Gel Immunodiffusion (AGID) test showed sharp precipitation lines reacting to a standard reference ILTV antisera (anti-NS175). All five isolates produced lines of precipitate identity among themselves and the positive control. This study confirms that the 2002 disease outbreak in the commercial layer chicken farms in Lipa City, Batangas was due to the ILTV.

**Key words:** *Infectious laryngotracheitis* virus, agar gel immunodiffusion, isolation, pocks lesion, identification, CAM

## INTRODUCTION

Infectious laryngotracheitis (ILT) is an economically important respiratory disease of chickens caused by an alpha-herpesvirus (HANSON and BAGUST, 1991). It can also affect pheasants, partridges and peafowl (OIE, 1996). ILT is a poultry disease affecting the respiratory tract in which lesions and exudates are constantly found in the trachea and larynx, and less frequently in the other parts of the respiratory system. ILT is characterized by conjunctivitis, depression, sneezing, rales, and nasal exudate. In severe cases, birds may also show signs of gasping, dyspnea, and death (GUY et al., 1990).

ILTV is considered to have a worldwide distribution. The disease was first reported by MAY and

TITSLER (1925) who described an outbreak in fowl at Rhode Island. In Indonesia, the cases were first reported by PARTADIREDJA *et al.* (1982) who described on the field outbreaks at several commercial layer chicken located at Bogor district. Furthermore, GILCHRIST (1992) also reported that the ILT cases occurred at village chickens in Bekasi district, West Java. Based on the distribution of the ELISA titer, WIYONO *et al.* (1996) reported that the prevalence of ILT reactor among chicken in the districts of Cianjur, Tangerang and Karawang was 71%. The reactor sera were found in layer and village chicken, but not in broiler chicken. The recent report, HAMID *et al.* (2001) and SAEPULLOH *et al.* (2000) showed that 14 of 95 tracheal organ samples from layer chickens located at district of Bogor, Bekasi and Karawang were ILTV positive by mean isolation using CAMs and identified AGID test. Those findings indicated that ILT disease also occurred at layer chicken in West Java.

In the Philippines, the only documented field outbreak occurred in 1980 at several commercial layer poultry farms in Rizal. The farms had a mortality rate of 5-10% and a morbidity rate of 30-50% (RAMOS *et al.*, 1980). ILT was classified as a disease of farm concern wherein prevention and control are recommended (BAI, 1992). The cases of ILT in a broiler breeder farm was reported by CRUZ and SILVANO (1993). The farm had a 90% morbidity rate and a 75% mortality rate. Clinical signs included muco-hemorrhagic nasal exudation, gasping, and dyspnea was observed. At necropsy, cheesy plugs were seen in the tracheal openings with muco-hemorrhagic exudates in the trachea. Pinpoint to diffuse hemorrhages were seen in the tracheal mucosa. A second case was also reported in 1993 involving a fighting cock farm in Batangas. Diagnoses of these last two cases were based on clinical and pathologic signs of the animals. Confirmation and demonstration of the infectious laryngotracheitis virus were not performed.

The recent suspected outbreak of ILT in 2002 at various poultry farms in Batangas prompted the isolation of the virus from the existing cases. Affected farms had mortality rates ranging from 11 to 24%. Isolation and identification of the virus was necessary for disease confirmation, which was essential for decisions regarding appropriate vaccination programs. The aim of this study was to diagnose, based not only on clinico-pathological signs, but also on the isolation and identification of the virus.

## MATERIALS AND METHODS

### Farms used

Nine commercial layer chicken farms located at Lipa City, Batangas were visited and observed for signs of ILTV disease. The farms and their specific location were as follows: 1) Farm I, *Barangay* (Village) Tugtug, 2) Farm II, *Barangay* Ludlod, 3) Farm III, *Barangay* Galamay-Amo, 4) Farm IV, *Barangay* Galamay-Amo, 5) Farm V, *Barangay* Lumil, 6) Farm VI, *Barangay* Balagtasin, 7) Farm VII, *Barangay* Galamay-Amo, 8) Farm VIII, *Barangay* Galamay-Amo and 9) Farm IX, *Barangay* Galamay-Amo.

These farms were selected based on information from the Bureau of Animal Industry (BAI) and from a commercial poultry distributor. All the farms had a history of clinical signs similar to ILT and none had a history of vaccination against ILTV. Vaccination against Newcastles Disease (ND), Infection Bursal Disease (IBD), Fowl Pox and Infectious Coryza were used in various combinations in all the farms. The age of birds affected ranged from 9 to 56 weeks. While, the layer chickens that have ILT vaccinated history were not collected.

### Sample collection

Eighty tracheal and lung tissues as well as throat swabs were collected from 9 farms, however, only forty-nine tracheal and lung samples were processed for isolation because the embryonated eggs were not available at the Bureau of Animal Industry for a few months. Samples were collected from birds exhibiting clinical forms of respiratory distress. The samples were suspended in a transport media which contained 10 ml of nutrient broth supplemented with 1000 IU/ml Penicillin (Meiji), 1000 µg/ml Streptomycin (Meiji) and 50 µg/ml Gentamycin (SIGMA). All samples were kept in ice prior and during transport to the Animal Disease Diagnostic Laboratory (ADDL), Veterinary Teaching Hospital (VTH), Tabon Station, College of Veterinary Medicine, University of the Philippines, Los Baños. Upon reaching the laboratory they were immediately transferred to –80 ºC freezer until processing.

### Embryonated eggs

Commercial chicken embryonated eggs (9 – 12 old days) from an ILTV vaccination free flock were obtained from the Bureau of Animal Industry, Tiaong, Quezon City. These were used for isolation and propagation of the ILT virus. Six embryonated eggs were used for each sample.

### Positive and negative serum standard reference

Positive hyperimmune sera produced against ILTV strain NS-175, Japanese standard reference strain and negative control sera were produced by the National Veterinary Assay Laboratory, Tokyo, Japan and the Veterinary Drug Assay Laboratory, Bogor, Indonesia.

**Virus isolation**

Trachea and larynx samples were freezed and thawed three times and then grounded in a sterile mortar and pestle to make 10% suspension. The suspension was then clarified at 1,600 X *g* for 15 min to remove cell debris, and the supernatant was filtered through a 0.45μm millipore filter.

A volume of 0.2 ml supernatant was inoculated onto each of six CAMs of 10-12 days old embryonated chicken eggs and incubated for 6 days at 37°C. To harvest the virus, the eggs were chilled at 4°C for 24 hours then cleansed and opened aseptically. The embryos were chilled at 4°C for 24 hour. The morphology of pock formed on CAMs, such as whitish or yellowish pocks with opaque edges, depressed gray central area of necrosis, and small pocks on CAMs, which were frequently surrounded by a translucent edematous zone, was observed. The CAMs without typical pock lesions were processed and passaged two more times. If the CAMs without pock lesions on the third passage were considered a negative ILTV on the sample. The CAMs with pock lesions were then grounded and made 10% suspension in Dulbeco's Modified Eagle Medium, DMEM (GIBCO). A 10% of CAM suspension was clarified at 1,600 x *g* for 15 min at 4°C, and the supernatant was tested using rapid Haemagglutination (HA) method as described by SHORTRIDGE *et al*. (1982) to detect Newcastle disease virus (NDV). Briefly, one drop of ILTV supernatant was mixed with one drop of 10% Chicken Red Blood Cells (CRBC) on the plate. After mixing gently, the plates were allowed for 15 minutes. The supernatant that gave non-agglutination reaction was considered a negative NDV and stored at –80 °C until used.

**Identification of ILTV isolates using AGID**

*Antigen used*

A modified live ILT virus vaccine (BAL-ILT® by BESTAR Laboratories, Singapore) used in this study was an attenuated strain of the ILTV. The ILTV antigen was prepared essentially according to the methods described by IZUCHI *et al.* (1982). Briefly, the virus was propagated in embryonated chicken eggs. For our purposes, this antigen was further propagated by inoculation, incubated at 37°C and observed for dead embryos for 6 days. The embryos died on the first day of inoculation was discarded and embryos died on 2 to 6 days post-inoculation were chilled at 4°C for 24 hour. The CAM of embryonated eggs were then examined for typical pock lesions. The infected CAMs were grounded and made 10% suspension in Dulbeco's Modified Eagle Medium, DMEM (GIBCO). A 10% of CAM suspension was clarified at 1,600 x *g* for 15 min

at 4°C, and the supernatant was tested using rapid HA. The supernatant that gave a negative HA reaction was used as ILT antigen and stored at –80 °C until used.

**AGID**

In order to confirm the virus isolation results, the five samples that developed pock on CAMs were processed for further identification using the agar gel immuno-diffusion test (AGID). This serological test involved the use of specific hyperimmune antisera against ILTV strain NS175. Strain NS175 was considered the standard ILTV strain of various Japanese researchers based on the works of IKE *et al* (1978). The identification of ILTV isolates was performed using AGID according to the method described by JORDAN and CHUBB (1962). Briefly, the AGID media contained Nobel agar (1,5%), sodium chloride (8%) and sodium azide (1%), as preservative. This was prepared and autoclaved for 15 minutes. Fifteen-ml of molten agar was poured into 5 cm Petri dishes in flat and balance table. The agar was allowed to set and a pattern of wells was punched in the agar. These patterns consisted of a central well and six surrounding wells. The wells are 8 mm in diameter and 4 mm apart.

A fifty ml of hyperimmune serum as positive control was pipetted into the central well, while the surrounding wells were filled with suspect ILT virus samples. Each well pattern contained a positive control composed of the ILTV vaccine antigen. Another dish was used containing negative control sera with the same pattern of suspected virus sample and positive vaccine viral antigen. The ILT antigen dilutions that were used in this test were optimized using several dilutions of both antigen and ILTV isolates starting from undiluted and 1:2. Hyperimmune serum concentration used for the assay was also optimized by using the following dilutions: undiluted, 1:2 and 1:4. The optimal dilution determined was the concentration that formed precipitation lines mid point between the antigen and antibody wells. The optimum dilution determined for the hyperimmune serum was 1:4 thus subsequent AGID tests used this dilution. Upon filling, all petri dishes were incubated in a humid atmosphere at room temperature or at 37ºC. The agar was then examined for lines of precipitation (reaction of identity), 24-48 hours later by oblique illumination. In addition, all of the tests included uninfected CAM as negative antigen control and a negative antiserum as negative antibody control.

**Interpretation of serological response**

The mid point of precipitation line or identity reaction formed between anti-sera standard and ILTV isolates were considered a positive ILTV.

## RESULTS

### Clinical signs and lesions

Eight out of nine commercial poultry layer farms located in San Jose, Lipa City, Batangas (Table 1) were affected with ILTV-like symptoms. With the exception of Farm III, all eight farms had similar histories of sudden onset of the disease, with marked dyspnea as the predominant symptom. Birds in Farm III were not affected, having 0% mortality and morbidity. The birds affected were from 9 to 56 weeks. However, birds between 9 and 20 weeks of age appear to be slightly susceptible.

Affected birds were recumbent and most had their eyes shut. Their heads were trusted forward and upward at each inhalation. Spasmodic coughing, accompanied with expulsion of mucus, stained with blood. Coughing, sneezing and wheezing were frequently heard at night in all of the farms (See Figure 1). Drop in egg production from 2 to 15% was reported in farms IV, VI and VII. Farm IV, observed eggs of poor shell quality. Swollen faces and wattles were also observed in some birds. Conjunctivitis affecting one or both eyes were often accompanied by frothy exudates. Expectoration of blood, however, was not observed. In a poultry house with 5,000 bird's capacity, the duration of the illness was from 2 to 3 weeks. Morbidity rate was from 20 to 33% while mortality rate was from 2 to 22%.

### Pathological examination

The lesions observed in the affected chickens were confined to the pharynx, larynx and trachea. The trachea and laryngeal mucus were hyperemic and showed petechial hemorrhages especially in autopsied birds from farm IV, VI and VII (See Figures 2A, 2B, 2C and 2D). Excess mucus was found throughout the respiratory tract and caseous tracheal plugs were observed mainly in the upper part of the trachea, however, this can occasionally extend throughout the entire length. The clinical signs observed were similar to the clinical disease of field cases in Rizal Province as reported by RAMOS *et al.* (1980). Some birds showed extensive mucus accompanied by mild inflammation of the trachea. The bucal mucus membrane contained a yellow surface film, which could easily be removed leaving a raw inflamed tissue. In many cases, the eyelids were inflamed with conjunctivitis. Inflammation of the eyelids and conjunctivitis were not common symptoms and only three cases were seen in all the farms.

### Isolation

Embryonated chicken eggs were inoculated with a vaccine strain (BAL-ILT) which served as the positive control while minimum essential medium (MEM) was inoculated in order to serve as the negative control. After six days of incubation, the chorio-allantoic membranes (CAM) of the eggs were examined for pock lesions. CAM with and without lesions were collected and were subsequently passaged into other 6 of 9 - 10 day old embryonated eggs. The eggs inoculated with the vaccine virus showed pock lesions on the second egg passage. The embryonated eggs inoculated with MEM, on the other hand, did not show any pock lesions (See Figures 3A and 3B).

**Figure. 1.**  Respiratory signs such as difficulty in breathing, gasping and coughing was the most commonly observed (Arrow head)

Only forty-nine of the eighty samples collected were processed. These came from six commercial layer chickens farms. Sample processing was stopped as soon as five isolates were obtained. Five tissue extracts out of forty-nine processed samples were identified as positive. The lesions that developed in the CAMs were distinct whitish or yellowish pocks with opaque edges and depressed gray central areas of necrosis (See Table 2 and Figures 4A, 4B, 4C, 4D and 4E). Individual pocks size ranged from 1 mm to 3 mm, however extensive areas of pock coalescence were also observed.

Positive isolates were obtained from Farm IV Sample 1, Farm VI Sample C28, Farm VI Sample C29, Farm VI Sample C30 and farm VII Sample 7. These samples will be called isolates IV, VI-C28, VI-C29, VI-C30 and VII-7 subsequently. The CAM lesions varied from a few scattered foci to large coalesced lesions that could be as big as 3 cm in diameter. The pock lesions were distributed throughout the CAM and were seen to increase in size in subsequent passages. Thus the pocks observed in the third passage were bigger than those in the second passage (See Figures 4A, 4B, 4C, 4D and 4E). For isolation purposes, a total of three passages were done. Samples that did not give rise to pocks after the third passage were considered negative. None of the isolates developed pock lesions on the first passage. Embryonic death prior to 5 days post inoculation was not a common observation in both second and third passage and did not develop pock lesions in the CAM.

Other than ILTV, Newcastles disease virus was isolated from Farm I. All samples from this farm were considered negative for ILTV since no pock lesions were observed up to the third passage. All of embryonated eggs inoculated with samples from this farm were dead and haemorrhagic after 48 to 72 hours post-inoculation. The suspension of CAMs from these eggs tested positive against Newcastles disease virus (ND) when examined using the rapid plate haemagglutination test. None of the samples from the other farms developed Newcastles disease virus lesions in the embryonated eggs inoculated.

**Table 1.** Population, age, morbidity, mortality rates and vaccination histories of birds from nine farms suspected to have infectious laryngotracheitis.

| Code of farms | Population | Breed of chicken | Age (weeks) | Morbidity (%) | Mortality (%) | Vaccinations | Clinical Signs * |
|---|---|---|---|---|---|---|---|
| Farm I | 1,300 | Hy-Line | 20 | 25 | 16 | ND, IBD | Cg; Sz; Gs; La; Lw; Rep; |
| Farm II | 1,300 | Hy-Line | 12 | 20 | 2 | ND, IBD, Fowl Pox | Cg; Sz; Gs; D; La; Dp; En. |
| Farm III | 1,600 | Hy-Line | 16 | 0 | 0 | ND, IBD | Ns |
| Farm IV | 2,300 | Hy-Line | 24 | 30 | 22 | ND. IBD | La; Lw; C; Sf |
| Farm V | 5,200 | Hy-Line | 56 | 25 | 13 | ND, IBD, Fowl Pox | Cg; La; Lw; L; C; Nd. |
| Farm VI | 1,800 | Hy-Line | 9 | 20 | 6 | ND, IB | D; La; Dp; En; Lw; C; Nd. |
|  | 1,800 | Star-Cross | 25 | 30 | 17 |  |  |
|  | 1,800 | Hy-Line | 36 | 20 | 6 |  |  |
| Farm VII | 1,200 | Hy-Line | 14 | 33 | 19 | ND, IB, IBD, Coryza | Cg; D; La; Dp; En; Lw. |
| Farm VIII | 1,000 | Hy-Line | 12 | 20 | 10 | ND, IBD, Fowl Pox | Cg; Sz; Gs; D; La; Lw; Nd; C; L. |
| Farm IX | 5,500 | Hy-Line | 8 | 10 | 4 | ND, IB | Cg; D; C |

* Clinical Sign : Cg=coughing; Sz=Sneezing; Gs=Gasping; La=Lost appetite; Lw=Loss of body weight; Rep=Reduced egg production; D=Dyspnea; Dp=Depression or stand with wing spread; En=Extended neck during an inspiration effort; Sf=Sweeling face; C=Conjunctivitis; L=Lacrimation or eatery eyes; Nd=Nassal discharge; Ns=No clionical sign

**Table 2.** ILTV isolation results using 10 days old embryonated eggs inoculated via the CAM route

| Code of farm | Location (Barangay) in Lipa, Batangas | Number of samples tested | Result of isolation | |
|---|---|---|---|---|
| | | | Positive | Negative |
| I | Tugtug | 9 | 0 | 9 |
| II | Ludlod | 2 | 0 | 2 |
| III | Galamay-Amo | No samples | tested | |
| IV | Galamay-Amo | 9 | 1 | 8 |
| V | Lumil | 5 | 0 | 5 |
| VI | Balagtasin | 17 | 3 | 14 |
| VII | Galamay-Amo | 9 | 1 | 8 |
| VIII | Galamay-Amo | No samples | tested | |
| IX | Galamay-Amo | No samples | tested | |
| Total | | 49 | 5 | 44 |

**Figure 2.** A) and B) trachea showing petechial hemorrhages collected from Farm IV sample C28 and VI sample C29; C) and D) Caseous tracheal plug observed in trachea from Farm VI sample C30 and Farm VII sample 7

**Figure 3.** Negative and positive ILT controls on CAMs. A) CAM inoculated with MEM had no pock lesions, negative control; B) CAM inoculated with vaccine (ILT-BAL) showed pock lesions typical of ILT (arrow head), served as the positive control

**Viral identification using the agar gel immunodiffusion (AGID) test**

The results of the AGID are clearly demonstrated in Figure 5. Strong line of identity was revealed in all five ILTV isolates from Batangas. Similarly, the positive control ILTV using vaccine strain (ILT-BAL, BELSTAR, Singapore) showed strong line of identity.

The viral samples were diluted at 1:2 with buffer and reacted with the specific ILT antisera. Despite dilution, lines of identity were also observed with the lines in the diluted test toward the antigen containing wells. These results indicated that the concentration of antibody to antigen is proportionately greater.

**Figure 4.**   Chorio allantoic membrane showing pock lesions on the 3$^{rd}$ passage. (Arrow head). A) Isolate from Farm IV; B) Isolate from Farm VI # C28. C) Isolate from Farm VI # C29. D) Isolate from Farm VI # C30 and E) Isolate from Farm VII #7. A, B, D and E showing large coalesced pock lesions were distributed throughout the CAM and depressed gray central area of necrotic. C showing a few scattered pocks formed on the CAM

**Figure 5.** Five ILTV isolates from Batangas showing identity reaction against antisera standard (anti-ILT strain NS175) using the AGID test. The samples were undiluted (left side) and diluted at 1:2 (right side). Wells 1) isolate IV; 2) Isolate VI-C28; 3) isolate VI-C29; 4) isolate VI-C30; 5) isolate VII; 6) control positive ILTV strain vaccine and AS (central well) antisera against ILTV strain NS-175

## DISCUSSION

The clinical observations of the 2002 field outbreak in San Jose, Batangas exhibited great similarity to the clinical disease picture reported by RAMOS *et al.* (1980) in Rizal and HUGHEST *et al.* (1991) in England and Wales. These clinical signs seem more severe than those described by CURTIS and WALLIS (1983) who were faced with a milder form of the disease in England. Infectious Laryngotracheitis, like many other poultry disease, cannot be reliably diagnosed by mere observations of signs and lesions. Although some per-acute signs are characteristic, many signs are similar to other respiratory diseases of poultry (BAGUST, 1982; BAGUST *et al*., 1986). According to HANSON (1984), the following clinical signs are sufficient for diagnosis: high morbidity (90-100%) and mortality (5 – 70%), coughed-up blood, tracheal blood clots and haemorrhagic tracheitis at autopsy. All other forms of the disease require laboratory confirmation in order to determine the necessary measures for disease control.

Several questions arise as to the origin of the infection since the disease had not been reported in the Philippines in years. The disease was mainly seen in birds beginning to lay eggs, since egg laying is added stress to the animals. Transmission of the disease from one farm to the other may be due to the following reasons: 1) a break down in bio-security, 2) the close proximity of birds houses to each other making the spread of the virus rapid and 3) the possible reversion of ILT vaccine virus to its virulent state. All of the farms visited lack of bio-security and were situated very close to each other wherein neither fences nor barriers were present. COVER (1996) reported that the virus could be carried on the hands and clothing and on poultry equipment. Transmission from farm to farm could be attributed to contaminated clothes that were not appropriately disinfected (KINGSBURY and JUNGHERR, 1958). Other sources of spread include farm dogs, crows, rats and wild birds.

As a characteristic of viruses under the family *Herpesviridae*, the ILTV persists in chicken populations sub-clinically and can re-emerge when stressful conditions occur (COVER, 1996). HUGHEST *et al.* (1987) reported that the re-excretion of the ILT virus from latently infected birds follow stress of re-housing. The virus can be shed for long periods of time, thus exposing other susceptible birds. The presence of the ILT virus in the larynx and trachea of recovered chicken following natural outbreaks can persist for many years (GUY *et al*., 1990; and HUGHES *et al*., 1991).

The risk of the vaccine virus reverting to its virulent state had been reported (GUY *et al*., 1990; and HUGHES *et al*., 1991). Prior to the beginning of this study, Infectious Laryngotracheitis vaccines were sold by a commercial breeder in affected farms in the area. The vaccines were not made available a few months after April 2002 due to a directive the Bureau of Animal Industry banning importation of ILT vaccines. As a result, ILT farm vaccination programs automatically stopped due to the unavailability of ILT vaccines. In

this study, samples from commercial poultry farms that had history of ILT vaccination located at nearby disease outbreak areas were not collected. In contrast, the samples collected for this study came from farms without vaccinations against ILTV.

In countries where the disease is endemic and where the chronic type of infectious laryngotracheitis exists, widespread vaccination is practiced to reduce losses and to improve flock production. One disadvantage of vaccination especially the use of live vaccines, is the possible risk of producing carriers. The disease outbreak at Batangas probably occurred from the transmission of vaccine virus from vaccinated chicken to the unvaccinated healthy chicken. GUY et al. (1990) reported that disease outbreaks in North Carolina, U.S.A. was due to use a modified live virus vaccine. This incident clearly demonstrates that ILT outbreaks can stemmed out from the use of modified vaccine virus. Spread of the vaccine virus to non-vaccinated birds can result to the reversion of the vaccine viruses to its parental-type virulence.

Virus isolation results showed that the morphology and size of pock produced by vaccine strain were significantly different from those produced by the field isolates. The isolates had distinct pock patterns, which were different from each other. Differences in pock lesions cannot distinguish a vaccine virus from a field strain. This parameter cannot be used to differentiate virulent of ILT strains from a-virulent strains. Pathogenicity tests in chickens should be performed in order to determine virulence. In such case, a-virulent strains will not produce clinical signs, while virulent strains result to presence of pathogenic lesions indicating disease.

The pock lesions reported by RAMOS et al. (1980) were small lesions, which developed at 48 hours post inoculation. They described that pock size increased after 72 hours of incubation. In contrast, SAEPULLOH et al. (2000) stated that the pock lesion of ILTV Indonesian isolates developed after 3 times passage in SPF-embryonated eggs via CAM. In comparison, the virus isolates in this study required a longer time to develop pock lesions in embryonated eggs. The difference in results could be due to differences in the concentration of virus in the trachea and lung samples. It is also possible that the embryonated egg used in both studies varied in sensitivity. The time of pocks development can also be related to the virus' ability to adapt to embryonated eggs. It is difficult to assume that the difference is related to virulence. The mortality rate, at its highest, in Rizal (RAMOS et al., 1980), twenty two years ago, was lower (5–10%) than the mortality of this current ILTV outbreak in Batangas (2-22%). Morbidity rate, however, was higher in RAMOS et al. study, 30–50% compared the current study's morbidity rate which was at 20 to 33%.

The positive isolates came from the towns of Galamay Amo and Balagtasin. No ILT virus was isolated from Barangay Tugtug, Barangay Ludlod, and Barangay Lumil. Isolation procedures were stopped upon getting five isolates exhibiting typical ILTV lesions on CAM.

Compared to other viral detection test, the AGID test is less sensitive and is classified as a secondary binding test. Other viral detection test such as the enzyme-link immunosorbent assay (ELISA), radio-immuno assay (RIA) and virus neutralization test (VN test) are more sensitive and can detect lesser concentrations of antigen-antibody reaction. The AGID test, which is a precipitation reaction, can detect protein antigen concentrations more than 30 μg/ml (TIZARD, 2002). Even though the AGID is less sensitive than ELISA, but its method is relative simple and cheaper than ELISA, RIA and VN test.

The positive lines of identity indicated that the virus isolates from Lipa City, Batangas are of great antigenic similarity to the ILTV vaccine strain (BAL-ILT ™, Belstar). In addition, each of the five isolates had also identical antigenic moieties to each other. ABBAS and ANDREASEN (1991) compared the antigenicity of various ILTV strains and concluded that most ILT virus were antigenically homogenous even though they vary in virulence. In their study, virus neutralization (VN) and immuno-fluorescence test were utilized with polyclonal antibodies for detection. KOTIW et al. (1982) reported that ILTV has only one sero-type and two different pathotypes, a-virulent and virulent strains. Both strains were not differentiated in conventional methods such as the AGID and Indirect immunofluorescence test (IFAT). These tests were not designed to distinguish various strain pathotypes. DNA hybridization and restriction endonuclease procedures can differentiate between virulent and a-virulent of ILTV-strains (KOTIW et al., 1986; GUY et al., 1989). These methods can precisely differentiate between vaccine strains from wild virulent strains.

Despite the limitations of the AGID test, the presence of ILTV infections in poultry farms in Lipa, Batangas were demonstrated using ILTV specific antisera raised in SPF chickens. This study confirms that the 2002 disease outbreak in the commercial layer chicken farms was due to the ILTV.

## ACKNOWLEDGEMENTS

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