

Molecular Characterization of Infectious Laryngotracheitis Virus (ILTV) Isolates from Outbreaks Cases at Lipa City, Batangas Province, The Philippines

MUHARAM SAEPULLOH

Research Institute for Veterinary Science, PO Box 151, Bogor 16114
m.saepulloh@balitvet.org

ABSTRAK

SAEPULLOH, M. 2004. Karakterisasi molekuler isolat virus infectious laryngotracheitis (ILTV) yang berasal dari wabah penyakit di daerah Lipa, Propinsi Batangas, Filipina. *JITV* 9(1): 26-36.

Penelitian ini bertujuan untuk mengetahui karakter molekuler isolat virus *infectious laryngotracheitis* (ILT) yang berasal dari peternakan ayam petelur komersial di daerah Lipa, Propinsi Batangas, Filipina dengan menggunakan teknik *Western blotting*. Virus ILT diisolasi pada selaput korio allantoik (CAM) telur ayam bertunas. Lima isolat (#IV, #VI-C28, #VI-C29, #VI-C30, dan #VII) menghasilkan lesi berupa plak yang berwarna kekuning-kuningan di permukaan CAM pada pasase kedua. Lebih lanjut, kelima isolat virus tersebut dikarakterisasi dengan menggunakan teknik *Western blotting* dengan konsentrasi akrilamid 7,5%. Hasil karakterisasi menunjukkan bahwa antisera ILTV standar (NS-175) yang diproduksi pada ayam dan antisera ILTV galur vaksin yang diproduksi pada kelinci bereaksi dengan isolat #VI-28, #VI-29, #VI-30 dan #VII menghasilkan 4 protein virus utama dengan berat molekul 205, 160, 85 dan 60 kDa. Sementara itu, isolat # IV hanya menghasilkan 2 protein virus utama, yaitu 205 dan 85 kDa. Dengan dikenalnya keempat protein ILTV oleh kedua antisera ILT, menunjukkan bahwa telah terjadi reaksi silang antara protein virus asal galur vaksin dan isolat lokal yang berasal dari kota Lipa, Provinsi Batangas, Filipina. Terjadinya reaksi silang ini akan menjelaskan lebih lanjut bahwa pemakaian galur vaksin di lapangan akan memberikan proteksi yang baik pada unggas.

Kata kunci: Infectious Laryngotracheitis, CAM, western blotting, ayam

ABSTRACT

SAEPULLOH, M. 2004. Molecular characterization of infectious laryngotracheitis virus (ILTV) isolates from outbreak cases at Lipa City, Batangas Province, The Philippines. *JITV* 9(1): 26-36.

Investigations were carried out to identify molecular character of infectious laryngotracheitis virus (ILTV) isolates from commercial layer chicken farm located at Lipa City, Batangas Province, the Philippines using western blotting. The virus was first isolated in chorio allantoic membrane (CAM). A-total of five isolates (#IV, #VI-C28, #VI-C29, #VI-C30, and #VII) produced typical plaque lesions in CAM at second passages such as yellowish plaques with opaque edges. Furthermore, five isolates were then characterized by western blotting on 7.5% of acrylamide. These results showed that Chicken antisera to the ILTV strain NS-175 (as standard sera), and rabbit antisera to vaccine strain (BAL-ILT) recognized four major viral protein with molecular weight of 205, 160, 85 and 60 kDa of isolates #VI-C28, #VI-C29, #VI-30 and #VII. While the isolate # IV produced viral protein of 205 and 85 kDa. The same four viral proteins were recognized by both ILTV antisera, indicating that the viral proteins of the vaccine strain and ILTV local isolates from Lipa City, Batangas Province, the Philippine had cross-reactivity. Thus, this cross reactivity may cause the effective protection afforded by the vaccine strain in the field.

Key words: Infectious laryngotracheitis virus, CAM, western blotting, chicken

INTRODUCTION

Infectious laryngotracheitis virus (ILTV) is a herpes virus belonging to the *Alphaherpesvirinae* subfamily. The virus classified as *Gallid herpesvirus* 1 (ROIZMAN, 1982), causes an acute disease of chickens characterized by respiratory depression, gasping, and expectoration of blood exudates (ABBAS and ANDREASEN, 1996). Strains of ILTV vary considerably in their virulence (HANSON and BAGUST, 1991). The disease is found worldwide and sporadic outbreaks occur in which the severity of clinical symptoms may vary considerably. RAMOS *et al.*

(1980) reported that a milder form of infectious laryngotracheitis has become widespread in Rizal Province. In the recent years, a milder form of ILT was also found in commercial layer chicken farm at district of Bogor, Bekasi and Tangerang, West Java, Indonesia in 2000 (SAEPULLOH *et al.*, 2003) and in Lipa City, Batangas Province, Philippines in 2003 (SAEPULLOH and ROVIRA, 2003). Although the local isolates of ILT virus from field cases has already been isolated, but molecular characterization of those isolates have not been reported yet both in the Philippines and also in Indonesia.

Although minor antigenic variation has been reported among isolates of ILTV, virus neutralization (VN) and immuno-fluorescence performed with polyclonal antibodies have shown most strains to be antigenically homogenous even though they vary in virulence (ABBAS and ANDREASEN, 1996).

YORK *et al.* (1987) showed that chicken anti-sera to ILT vaccine strain (SA-2) and to a virulent isolates immuno-precipitated five major viral glycoproteins of 205, 160, 115, 90 and 60 kiloDaltons (kDa), respectively. An additional glycoprotein bands are recognized by immune chicken and rabbit sera in Western blotting using glycoprotein fraction purified from extract of virus infected cells. WATARI *et al.* (1987) reported that the envelope glycoproteins of herpesvirus, which are located both on the outside of the virus and on the surface of the virus-infected cells, are important immunogens capable of inducing humoral and cellular immune response. The possible involvement of other virion components in the immune response has not yet been investigated (YORK *et al.*, 1987).

Further work using monoclonal antibodies defined the five major glycoproteins into two groups: the 205 complex (205, 160, 115 and 90 kDa glycoprotein) and the 60 kDa glycoproteins (YORK and FAHEY, 1990). YORK *et al.*, (1987) reported that immune chicken serum and serum from rabbits hyperimmunized either with whole virus or with a glycoprotein fraction gave essentially the same profile in Western blotting, indicating that the ILTV glycoproteins are both highly antigenic and immunogenic.

The objective of this research was to identity molecular character of five ILTV local isolates from Batangas compare with vaccine strain (ILT-BAL, Singapore) using western blotting technique.

MATERIALS AND METHODS

Isolates

Five ILTV isolates from field cases in Lipa City, Batangas, the Philippines were propagated in chorio allantoic membrane (CAMs) of 10-12 day-old embryonated chicken eggs. All of isolates showed typical pocks lesion in 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.

Antigen

A commercial attenuated live ILT virus vaccine (BAL-ILT) from BESTAR Laboratories, Singapore was propagated in 10-12 day-old embryonated chicken eggs.

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 were 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 Dulbecco'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.

Positive and negative serum standard reference

Hyperimmune sera against ILTV strain NS-175 (Japanese standard reference strain) was obtained from Dr. Osamu ITOH (National Veterinary Assay Laboratory, Tokyo, Japan) and from Dr. Ida Lestari Soedidjar (Veterinary Drug Assay Laboratory, Bogor, Indonesia). These sera were produced in Specific Pathogen Free (SPF) of chickens. Negative control sera prepared in SPF of chicken were also provided by the above individuals.

Production of rabbit hyperimmune sera against ILT virus

Hyperimmune sera were prepared for each ILTV isolate in order to determine cross reactivity among the various isolates and the vaccine virus as well. The ILTV vaccine virus (BAL-ILT, Bestar, Singapore) and five ILTV local isolates were grown in CAMs of embryonated chicken egg and were passaged up to the 5th embryo-passage. The virus was titrated using method of GELENEZEI and MARTY (1964) and calculated using the method of REED and MUENCH (1938).

A-crude ILTV isolate and the vaccine strain containing of log 10^{4.3} EID₅₀/ml was used for inoculation into each New Zealand white rabbits. Another rabbit was injected with CAM suspension (serve the negative sera as control). Virus was first inactivated using beta-propiolactone at final concentration of 0.2% (v/v) (FAHEY *et al.*, 1983). The viability of this virus was then tested using the egg inoculation test, and immunologic capability was determined using the AGID. Inactivated ILT virus was then emulsified in an equal volume of Freund's Complete Adjuvant, FCA (SIGMA) and was injected 1 ml subcutaneously into rabbits. Booster was given 2 weeks later by 1 ml crude virus suspensions

intravenously. Three weeks later, the rabbit was inoculated by the same isolate intravenously at weekly interval. Each rabbit was bled at 2, 3 and 4 weeks post immunization for the detection of titer antibody. On the fifth week, the rabbits were bled and sacrificed. The collected blood was processed in order to separate serum from other blood components. A total of seven different hyperimmune sera were obtained from seven rabbits. Antibody titers of the sera collected were determined using the AGID test. Sera produced from one virus isolate containing 1:16 antibody titer was pooled.

Concentration and purification of infectious laryngotracheitis (ILT) virus

The concentration and purification procedures used for the five ILTV local isolates and vaccine strain was modifications of protocols reported by SCHLOER and BREESE (1982). Infected CAMs were ground to make crude virus preparations for each ILTV isolate and vaccine strain. In order to concentrate the virus in the suspension, Polyethylene Glycol (PEG #8000) and NaCl were added to make 8 and 3% (w/v) solution, respectively. These suspensions were centrifuged at 3,000 rpm for 1 hour. The pellets were collected and re-suspended with 1/10 volume in Tris-Saline Buffer (TSB) pH 7.4. Purified virus was separated from the rest of the cell debris through 20-60% sucrose-density gradient. Single band formation was achieved by 200,000 x g for 5 hours (SW 28 rotor, Beckman). The bands were individually collected and dialyzed in PBS for 12 hours at 4°C to remove the sucrose. The protein contents were determined by spectrophotometric reading at 280 nm. Results of the readings were recorded. These readings were eventually used for adjusting protein concentrations in subsequent SDS-PAGE studies.

SDS-PAGE of protein

Sodium Dodecyl Sulphate–Polyacrylamide Agar Gel Electrophoresis (SDS-PAGE) was performed using 7.5% acrylamide resolving gel with 5% stacking gels using the continuous system of LAEMMLI (1970). Briefly, approximately 100 µg of protein was loaded per well. The electrophoresis, staining, and de-staining of the gels were performed as described by HAMES (1981). Briefly, gels were run with constant voltage at 50 volts at room temperature for 2 hours, stained for 1 hour at room temperature with 0.5% (w/v) Coomassie Brilliant Blue in 25% of methanol: 10% of glacial acetic acid and de-stained in several changes with 25% of methanol; 10% of glacial acetic acid.

Western blotting

The transfer of proteins from the separation gel to a membrane surface was performed as described by TOWBIN *et al.* (1979) and YORK *et al.* (1987) with modification. Briefly, protein was separated under reducing conditions by discontinuous polyacrylamide gel electrophoresis (PAGE) and transferred to a nitrocellulose membrane (7-cm x 10-cm, 0.45 µm, N-8142, Lot #10B148142, SIGMA) by electrophoresis with constant voltage at 50 volt using a BIORAD Miniprotean™ for 2 hours. The nitrocellulose from the electroblot apparatus was rinsed in Phosphate Buffered Saline (PBS) for 5 minutes, and soaked in blocking solution (20 mL of 5% skim milk, Amersham Life Science) at room temperature for 60 minutes with gentle agitation. The blocking solution was discarded and the membrane was rinsed three times in PBS (5 minutes/rinse). Optimization of antibody concentration was performed using the following dilutions: 1:10, 1:100, 1:200 and 1: 400, respectively.

The membrane was then incubated in 10 mL of polyclonal antibody standard against ILTV as primary antibody (1:100 in 1% of gelatin) for 5 hours at 37°C while gently shaking. The membrane was rinsed twice in Tween 20-PBS (TPBS) and once in PBS only, 5 minutes each rinse. The blots were incubated with secondary Rabbit anti-Chicken IgG horseradish peroxidase (HRP) labeled antibody conjugate (SIGMA). Conjugate dilution was also optimized from 1:100 to 1:1000 dilutions. Further test used a diluted 1:500 conjugate in 1% gelatin for 2 hours at 37°C while gently shaking. The membrane was again rinsed twice in TPBS and once in PBS, 5 minutes each rinse, and treated with 3,3' Diaminobenzidine (DAB) substrate (Roche Diagnostic GmbH, Germany) until dark brown/black color developed. Typical incubations were from 5 to 15 minutes. The SDS-PAGE high range standards (49 to 205 kDa) (SIGMA) was used as molecular weight standard.

RESULTS

Hyperimmune production and antibody titers

Rabbit antiserum titers against ILTV were determined using the AGID test. The immunization program had 6 bleeds at the follow dates: day 0, 14, 21, 28, 35 and 42. At day zero, none of the rabbits gave positive precipitation lines in the AGID test and can be considered to have no prior exposure to the ILT virus. These rabbits were unrelated to each other and were obtained from various sources in Lipa City, Batangas. Their response to this immunization protocol was varied as can be demonstrated in Table 1.

Table 1. Detection of antibody titers of rabbits immunized against ILT at various points in the immunization schedule using the AGID test

Virus	Rabbit No.	Titer of virus (Au*)					
		Day 0	Day 14	Day 21	Day 28	Day 35	Day 42
Vaccine strain	1	0	0	4	16	16	16
Isolate # IV	2	0	0	2	4	2	1
Isolate # VI-C28	3	0	0	4	4	4	4
Isolate # VI-C29	4	0	0	4	16	4	4
Isolate # VI-C30	5	0	0	8	16	16	4
Isolate # VII-7	6	0	0	2	16	16	4
Uninfected CAM	7	0	0	0	0	0	0

*AGID Unit

The rabbits inoculated with the vaccine strain, isolate # VI-C29, isolate # VI-C30, and isolate # VII-7 did not show an antibody response after the 7th and the 14th day of the immunization period. It was possible that the antibody levels mounted by these animals during this period were low and not detected by the AGID test. Since the animals used in this study have no previous exposure to the antigen, it was expected that they will take several days to mount up a good antibody response.

Concentration and purification of infectious laryngotracheitis (ILT) virus

The result of concentration and purification to all of five ILTV local isolates demonstrated that purified ILTV had a single band around the middle of the tubes (Figure 1). The vaccine virus strain similarly had the same band location as the isolates. This indicated that all of virus isolates as well as the vaccine positive control had the same buoyant density (1.704 g/mL). The protein contents were determined by spectrophotometric reading at 280 nm. Results of the readings were recorded in Table 2. These readings were eventually used for adjusting protein concentrations in subsequent SDS-PAGE studies.

The results of the spectrophotometric readings showed that the vaccine strain had the highest protein content at 0.7400 mg/mL. Concentration of the ILTV isolates from highest to lowest were # VI-C28 at 0.6486 mg/mL, isolate #VI-C30 at 0.5871 mg/mL, isolate # IV at 0.5142 mg/mL, isolate # VI-C29 at 0.4421 mg/mL and isolate #VII-7 at 0.1807 mg/mL. All ILTV isolates had the right concentration necessary for SDS-PAGE studies. These studies require a concentration of 0.5 to 5 µg per lane (20µL) in order for the protein to be detected by coomassie blue staining (WILSON, 1983).



Figure 1. Purification ILTV by sucrose gradient centrifugation. A single band was obtained by density gradient centrifugation in 20-60% sucrose, centrifugation 200,000 x g for 5 hours at 4°C

Sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE)

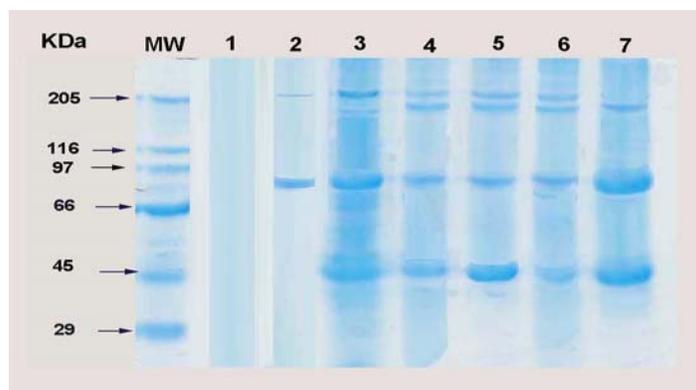
The presence of ILTV specific proteins was determined by SDS-PAGE using 7.5% acrylamide gel. Major protein bands were identified and these migrated at the following molecular weights: 205, 160, 85 and 60 kDa. The banding patterns, however differed slightly among the isolates and vaccine strain. Table 3 and Figure 2, summarizes the protein profile of the vaccine strain and each of the five field isolates. These results revealed that the common band at 85 kDa in all isolates as well as the vaccine strain. Isolates # VI-C28, # VI-C29, #VI-C30 and #VII had the same banding patterns, which included all four major proteins: 205, 160, 85 and 60 kDa. In contrast, isolate # IV had only two major bands and lacked of protein bands 160 kDa and 60 kDa. In comparison, the banding profile of the vaccine strain virus showed the lack of protein band 205 kDa.

Table 2. Protein content of ILT virus after sucrose gradient (20-60%) purification

Virus	Absorbance reading at 280 nm	Protein content	
		mg/mL	µg/20 µL
Vaccine strain	1.036	0.7400	14.80
Isolate # VI-C28	0.908	0.6486	12.97
Isolate # VI-C29	0.616	0.4421	8.84
Isolate # VI-C30	0.822	0.5871	11.74
Isolate # IV	0.720	0.5142	10.28
Isolate # VII-7	0.253	0.1807	3.61

Table 3. Protein profile of purified ILTV isolates from Batangas and vaccine strains analyzed by SDS-PAGE on 7.5% gel

Virus	Proteins (kDa)			
	205	160	85	60
Isolate #IV	+	-	+	-
Isolate #VI-C28	+	+	+	+
Isolate #VI-C29	+	+	+	+
Isolate #VI-C30	+	+	+	+
Isolate #VII	+	+	+	+
Vaccine ILT-BAL	-	+	+	+



MW = molecular weight standard, 1) Purified CAMs (negative control), 2) isolate from Farm # IV, 3) isolate from Farm VI # C28, 4) isolate from Farm VI # C29, 5) isolate from Farm VI # C30, 6) isolate from farm VII #7 and 7) vaccine ILT-BAL (positive control)

Figure 2. Purified of ILTV isolates from the Philippines and vaccine strains analyzed by SDS-PAGE on 7.5% gel

Western blotting

Optimization of primary and secondary antibody

In order to minimize the background and increase specificity, the primary antibody (polyclonal antibody) concentration was optimized. Optimization experiments were performed using the ILTV vaccine virus as

antigen, and the rabbit anti-ILTV raised against the vaccine virus as primary antibody. The result demonstrated that the optimum conditions determined for both primary antibody and the rabbit anti-chicken IgG-HRP conjugate were 1:100 and 1:500, respectively.

Characterization of ILT specific viral protein present in virus-infected cells

The ILT viral proteins demonstrated by SDS-PAGE were reacted to antibodies that were produced against the crude form of the virus. Assessment of this reaction was through western blotting using purified isolates # IV, #VI-C28, #VI-29, #VI-C30, #VII and vaccine strain as antigen. The viral proteins were separated by SDS-PAGE, transferred to nitrocellulose, and probed with rabbit anti-sera against isolate #IV, isolate #VI-C28, isolate #VI-29, isolate #VI-C30, isolate #VII, ILT vaccine strain and chicken anti-sera to ILTV strain NS-175.

Major protein bands recognized by the anti NS-175 sera (Table 4). The specific viral proteins 250, 160, 85, and 60 kDa of isolates #VI-C28, #VI-C29, #VI-C30, and #VII were all recognized. Although raised using a different strain, the anti-ILTV NS-175 sera also immunologically recognized protein bands 205 and 85 kDa of isolate #IV and all three proteins bands (160, 85 and 60 kDa) of the ILTV vaccine.

Purified isolates # IV, #VI-C28, #VI-29, #VI-C30, #VII and vaccine strain were reacted with rabbit anti-

sera to raised against isolate # IV (Table 5). From the SDS-PAGE results, isolate # IV demonstrated only two major protein bands despite this, however, the rabbit anti-sera against isolates # IV recognized all the specific viral proteins of 205, 160, 85 and 60 kDa from viral isolates #VI-C28, #VI-C29, #VI-C30 and #VII. Only viral proteins of 160, 85 and 60 kDa from the ILT vaccine can be recognized by anti-isolate #IV. Anti-isolate #IV against its homologous protein recognized both 205 and 85 kDa proteins of the isolate. Despite the absence of bands 205 and 60 kDa in the SDS-PAGE protein profiles of isolate #IV, the anti-sera produced against this virus recognized these bands in the other isolates (#VI-C28, #VI-C29, #VI-C30 and #VII) and in the vaccine virus strain as well.

Immunological recognition of ILTV Batangas isolates and ILTV vaccine against anti-isolate #VI-C28 revealed four major consistent bands with the following molecular sizes (kDa): 205, 160, 85 and 60 in ILTV isolates # VI-C28, #VI-C29, #VI-30 and #VII (Table 6). The protein bands of isolates #VI-C29, #VI-30 and #VII were also recognized but with a lesser degree of intensity relative to the homologous strain. Reaction

Table 4. Immunologic recognition of ILTV Batangas isolates and ILTV vaccine virus against anti-serum standard (anti-ILTV strain NS-175) in Western Blot

Virus	Proteins (kDa)			
	205	160	85	60
Isolate #IV	+	No Band*	+	No Band*
Isolate #VI-C28	+	+	+	+
Isolate #VI-C29	+	+	+	+
Isolate #VI-C30	+	+	+	+
Isolate #VII	+	+	+	+
Vaccine ILT-BAL	No Band*	+	+	+

* No protein band demonstrated in SDS-PAGE gels

Table 5. Immunologic recognition of ILTV Batangas isolates and ILTV vaccine virus against anti-isolate #IV in Western Blot

Virus	Proteins (kDa)			
	205	160	85	60
Isolate #IV	+	No Band*	+	No Band*
Isolate #VI-C28	+	+	+	+
Isolate #VI-C29	+	+	+	+
Isolate #VI-C30	+	+	+	+
Isolate #VII	+	+	+	+
Vaccine ILT-BAL	No Band*	+	+	+

* No protein band demonstrated in SDS-PAGE gels

was less especially to the 85-kDa protein. The ILTV vaccine has three major protein bands (160, 85 and 60 kDa), were also identified clearly. The anti- #VI-C28 hyperimmune sera recognized only one of the major protein (85kDa) of isolate #IV. The reaction to this protein band was very weak compared to the other band reactions.

Hyperimmune sera against isolate #VI-C29 similarly recognized all four major protein of isolates #VI-C28, #VI-C29, #VI-C30, and #VII with molecular weight of 205, 160, 85 and 60 kDa (Table 7). Unlike the previous blots described above, anti - #VI-C29 sera, recognized the bands of isolates #VI-C28, #VI-C30, and #VII with the same intensity as the homologous strain. The anti-isolate # VI-C29 anti-sera recognized both proteins of isolate #IV (MW 205 and 85 kDa).

Hyperimmune sera against isolate # VI-C30 similarly recognized all four major protein of isolates #VI-C28, #VI-C29, #VI-C30, and #VII with molecular weights of 205, 160, 85 and 60 kDa (Table 8). Unlike the previous blots described above, anti-#VI-C30 sera, recognized the bands of isolates #VI-C28, #VI-C29, and #VII with the same intensity as the homologous strain. The anti-isolate #VI-30 recognized both proteins of isolate #IV (MW 205 and 85 kDa). Recognition of the 85-kDa protein was seen, however, the reaction for 205-kDa was recognizably less. In the vaccine strain, all proteins were recognized sharply.

Similarly, hyperimmune sera against isolate #VII recognized all four major proteins of isolates #VI-C28, #VI-C29, #VI-C30 and homologous antigen, #VII with molecular weights of 205, 160, 85 and 60 kDa (Table 9). Unlike the previous blots described above, anti-#VII sera, recognized the bands of isolates #VI-C28, #VI-C29, and #VI-30 with the same intensity as the homologous strain. The anti-isolate #VII anti-sera recognized both proteins of isolate #IV (MW 205 and 85-kDa). Most distinct was the strong reaction for the 85-kDa protein. The reaction for the 205-kDa, however, was recognizably less. All proteins in the vaccine strain were recognized sharply.

The hyperimmune sera against ILTV vaccine virus strain recognized three major protein bands (160, 85 and 60 kDa) of isolates #VI-C28, #VI-C29, #VI-C30, #VII (Table 10) and all bands of the homologous vaccine antigen. The recognition of the bands in isolates, #VI-C28, #VI-C29, #VI-C30 and #VII were of similar intensity as the homologous strain. The anti-vaccine strain anti-sera only recognized one protein of isolate #IV, the 85-kDa protein. Interestingly, this hyperimmune serum did not recognize protein band 205 kDa, which is only present in the field isolates. Unlike the field strains, this protein band was absent in the SDS-PAGE profile of the vaccine strain virus. Therefore, the immunized rabbit against protein 205 kDa formed no antibodies.

Table 6. Immunologic recognition of ILTV Batangas isolates and ILTV vaccine virus against anti-isolate #VI-C28 in Western Blot

Virus	Proteins (kDa)			
	205	160	85	60
Isolate #IV	No Rxn [^]	No Band*	+	No Band*
Isolate #VI-C28	+	+	+	+
Isolate #VI-C29	+	+	+	+
Isolate #VI-C30	+	+	+	+
Isolate #VII	+	+	+	+
Vaccine ILT-BAL	No Band*	+	+	+

* No protein band demonstrated in SDS-PAGE gels

[^] No reaction in this Western Blot

Table 7. Immunologic recognition of ILTV Batangas isolates and ILTV vaccine virus against anti-isolate #VI-C29 in Western Blot

Virus	Proteins (kDa)			
	205	160	85	60
Isolate #IV	+	No Band*	+	No Band*
Isolate #VI-C28	+	+	+	+
Isolate #VI-C29	+	+	+	+
Isolate #VI-C30	+	+	+	+
Isolate #VII	+	+	+	+
Vaccine ILT-BAL	No Band*	+	+	+

* No protein band demonstrated in SDS-PAGE gels

Table 8. Immunologic recognition of ILTV Batangas isolates and ILTV vaccine virus against anti-isolate #VI-C30 in Western Blot

Virus	Proteins (kDa)			
	205	160	85	60
Isolate #IV	+ (weak)	No Band *	+	No Band*
Isolate #VI-C28	+	+	+	+
Isolate #VI-C29	+	+	+	+
Isolate #VI-C30	+	+	+	+
Isolate #VII	+	+	+	+
Vaccine ILT-BAL	No Band*	+	+	+

* No protein band demonstrated in SDS-PAGE gels

Table 9. Immunologic recognition of ILTV Batangas isolates and ILTV vaccine virus against anti-isolate #VII in Western Blot

Virus	Proteins (kDa)			
	205	160	85	60
Isolate #IV	+ (weak)	No Band*	+	No Band*
Isolate #VI-C28	+	+	+	+
Isolate #VI-C29	+	+	+	+
Isolate #VI-C30	+	+	+	+
Isolate #VII	+	+	+	+
Vaccine ILT-BAL	No Band*	+	+	+

* No protein band demonstrated in SDS-PAGE gels

Table 10. Immunologic recognition of ILTV Batangas isolates and ILTV vaccine virus against anti-ILT vaccine in Western Blot

Virus	Proteins (kDa)			
	205	160	85	60
Isolate #IV	No Band [^]	No Band*	+	No Band*
Isolate #VI-C28	No Band [^]	+	+	+
Isolate #VI-C29	No Band [^]	+	+	+
Isolate #VI-C30	No Band [^]	+	+	+
Isolate #VII	No Band [^]	+	+	+
Vaccine ILT-BAL	No Band*	+	+	+

* No protein band demonstrated in SDS-PAGE gels

[^] Protein band not reacted by antibodies in anti- Vaccine ILT-BAL

DISCUSSION AND CONCLUSION

In order to produce hyperimmune sera, at the highest antibody titers were produced after the third injection (21-days), and blood was collected at 7 days after immunization. The data showed that the rabbits inoculated with the vaccine strain, isolate # VI-C29, isolate # VI-C30 and isolate # VII-7 produced high

titers that reached 16 AU (AGID unit). Meanwhile, the rabbits inoculated with isolate #IV and isolate # VI-C28 produced moderate titers (4 AU) at 21 days.

On the 5th week (35 days), only rabbits inoculated with the vaccine strain, isolate #VI-C30 and isolate # VII-7 maintained a high titer (16 AU) and the rabbits inoculated with isolate #VI-C29 decreased in titer (4 AU). On the other hand, the rabbit immunized with

isolate #VI-C28 maintained the titer of 4 AU. Antibody titers of the rabbit inoculated with isolate # IV were very low titer, but detectable at 2 AU. The rabbit inoculated with CAM suspension induced no detectable antibody in the duration of this experiment.

At 35th days onward, antibody titers dropped, except for the rabbit inoculated with the vaccine strain. This rabbit maintained high levels of antibody titer (16 AU) until the 42nd day. It was unfortunate that the rabbit inoculated with isolate # IV dropped to an antibody titer of 1AU. Each bleed that tested 4 AU and above were pooled for each animal and used for western blotting since this test require large volume of hyperimmune serum. The control negative anti-sera were none reactive to the ILT antigen through the whole duration of the test. All animals were bled and sacrificed on the 6th week (42 days) of the immunization schedule.

Differences in antibody titers may be due to the rabbits used for hyperimmune production. There are 2 rabbits that injected with isolate #IV and #VI-C28 showed scabies symptoms after 2 weeks post immunization (data not shown). Thus, they are not only lack of drink and eat but also loss of body weight. This condition could affect the antibody responses demonstrated.

Previous works on ILTV characterization vary in their reports of number of protein bands demonstrated in SDS-PAGE. YORK *et al.* (1987) reported that ILTV propagated in primary chicken kidney (CK) cells have 6 major proteins of 205, 160, 115, 90, 85, and 60 kDa. ABBAS and ANDREASEN (1996), however, reported that the ILTV grown in chicken embryo liver cells have 5 major proteins of 205, 160, 100, 90 and 70 kDa. This study used the ILTV propagated in chorio allantoic membrane (CAM) and demonstrated 4 major proteins of 205, 160, 85 and 60 kDa.

Differences in such findings could be due to 3 factors. The factors are: (1) it could be occur because the antibody recognizes a determinant that is common to more than one polypeptide. Alternatively, the smaller proteins could be cleavage products of larger proteins and could be related by precursor-product relationships, or could represent forms of the protein which are glycosylated differently. Similar phenomenon has been observed for many monoclonal antibody directed against herpesvirus antigens (PEREIRA *et al.*, 1984; COLLINS *et al.*, 1984; and HAMPL *et al.*, 1984); (2) The differences in the cells used for virus propagation. Being an enveloped virus, ILTV contains various components from its host cells. This includes lipid bilayer, glycoproteins and protein, all of which play a great role in the virus structure; and (3) Differences in the protein extracting process applied. Several authors working with viruses under the family *Herpesviridae* used various detergents to extract the viral proteins. YORK *et al.* (1987), and SPEAR and ROIZMAN (1972)

used Nonidet P-40 (NP-40) as detergent to breakdown the viral proteins in the infected cell. ABBAS and ANDREASEN (1996) similarly used NP-40 in extracting the ILTV in infected chicken embryo liver cells. These authors added tunicamycin to NP-40 prior to analyses in SDS-PAGE. The rationale behind the use of tunicamycin was the claim that this reagent can prevent degradation of protein. DOLYNIUK *et al.* (1976) in their study of the Epstein Barr virus, used Phenyl-methyl-sulfonyl-fluoride (PMSF), an anti-protease, to prevent breakdown of virus by cell-associated proteases. OLSHEVSKY and BECKER'S (1970) study on the *Herpes simplex* virus recommended that non-ionogenic detergent NP-40 followed by sonic treatment (1.5 A), for 1.5 min in the ultrasonic vibrator should be used in order to study the nature of the proteins present in the virus enveloped.

This study did not use any extraction detergent and neither was anti-proteases added to the sample. The only protein dissolving detergent present was sodium dodecyl sulfate, a reagent in the sample buffer and the acrylamide gel. The focus of this work was to maintain as much as possible the native state of the proteins. The preservation of immunologic characteristics of viral surface proteins was essential in order to demonstrate their reaction with antibodies. Certain detergents act on various proteins bands, thus breaking them into smaller peptides. Mild detergents may also change tertiary structures, thus results to none recognition by antibody. Compared to the previous works on ILT proteins, this study report the least number of proteins band, four major proteins. This could be due to the absence of detergents in the extraction process. The proteins demonstrated were of same molecular weights of proteins reported by YORK *et al.* (1987). The interesting of this study was limited to proteins located on the envelope of the virus and not those in the core, capsid or tegument. Maintenance of proteins in their native state was the goal of the study, thus use of detergent was limited. Sodium dodecyl sulfate was the only detergent present. Its mechanism of action is focused only on the sulfide bonds. Slightly denaturalization of protein conformation was expected, however, the size of the protein would still be the same as in its native state.

There were two kinds of primary polyclonal antibodies were used for ILTV characterization. One of the primary antibodies used was from SPF chickens, immunized against ILTV strain NS-175, while the other primary antibody used was from rabbits, immunized against ILTV suspected isolates from Batangas as well as the ILTV vaccine strain. One secondary antibody used for detection of both primary antibodies. This was produced in rabbits immunized against anti-chicken IgG. It was a commercial preparation (SIGMA) and the antibodies were conjugated to horseradish peroxidase

(HRP). As seen in the immunoblot reactions, both of chicken and rabbit antibodies were detected by the secondary rabbit anti-chicken IgG peroxidase conjugated. Similarly, YORK *et al.* (1987) reported western blot detection of viral proteins 205, 160, 115, 90, 85, 70 and 60 kDa. The secondary antibody, a rabbit anti-chicken IgG by Cappel Laboratories (Cochranville, PA), was used to detect rabbit anti-ILTV antibodies. Some of the proteins detected in YORK *et al.* (1987) study, coincided with the molecular weights of the proteins demonstrated in this experiment, namely, the 205, 160, 85 and 60 kDa proteins. The western blot protocols followed were similar to that used in this work. Although anti-chicken conjugated antibodies were used to detect rabbit antibodies, this did not hinder the detection of major ILT viral proteins in both studies. TIZARD (2002) explained that evolutionary relationships among the major of vertebrate immunoglobulin heavy chain could have been preserved over time. One example is the C α gene of the chicken, which is found to be similar to their homolog in mammals such as rabbits, mouse and gorilla. This probably explains why the rabbit anti-chicken conjugate by SIGMA and CAPPEL laboratories reacted with the rabbit anti-ILTV antibodies. Later immunoblot studies by ABBAS *et al.* (1996) on their ILT virus isolates involved the use of monoclonal antibodies. Proteins 205, 160, 100, 90, and 70 kDa and several proteins less than 49 kDa were detected. Monoclonal antibodies were used as primary antibody and were detected by a goat anti-mouse peroxidase labelled antibody (Hyclone Laboratories Inc., Logan, Utah). Similarity of detection of 205 and 160 kDa proteins were seen here. Differences in the proteins detected may be caused by differences in the protein extraction method using NP-40 as detergent.

The following highlights can be concluded from the Western blotting results of this study: (1) There are cross-reactivity of the viral protein of the vaccine strain and viral isolates of ILTV may explain the effective protection afforded by the vaccine strain in the field; and (2) Standard ILT serum (anti NS-175) and serum from rabbit hyperimmunized reacted with whole ILTV isolates gave essentially the same profile in Western blotting, indicating that the ILTV proteins are both highly antigenic and immunogenic.

ACKNOWLEDGEMENTS

I would like to thank Dr. Osamu ITOH, National Veterinary Assay Laboratory, Tokyo, Japan and Dr. Ida Lestari Soedidjar, Veterinary Drug Assay, Bogor, Indonesia for providing the ILTV antisera standard; and Dr. H.G. Rovira, Department of Veterinary Paraclinical Science, College of Veterinary Medicine, UPLB, the Philippine for her advises and constructive comments during conduct this research.

This study was financially supported by Agency for Agricultural Research and Development (AARD), Department of Agriculture, Indonesia via PAATP-ADB Loan No. 1526-INO.

REFERENCES

- ABBAS F and J.R. ANDREASEN Jr. 1996. Comparative of diagnostic test for infectious laryngotracheitis. *Avian Disease* 40: 290-295.
- ABBAS, F., J.R. ANDREASEN JR., R.J. BAKER, D.E. MATTON and J.S. GUY. 1996. Characterization of monoclonal antibodies against infectious laryngotracheitis virus. *Avian Dis.* 40: 49-55.
- COLLINS, J.K., A.C. BUTCHER, C.A. RIEGEL, V. MCGRANE, C.D. BLAIR, Y.A. TERAMOTO and WINSTON. 1984. Neutralizing determinants defined by monoclonal antibodies on polypeptides specified by bovine herpesvirus 1. *J. Virol.* 52: 403-409.
- DOLYNIUK, M., R. PRITCHETT and E. KIEFF. 1976. Proteins of epstein-barr virus: I. Analysis of the polypeptide of purified enveloped epstein-barr virus. *J. Virol.* 17(3): 935-949.
- FAHEY, K.J., T.J. BAGUS and J.J. YOTK. 1983. Laryngotracheitis herpesvirus infection in the chicken: I. The role of humoral antibody in immunity to a graded challenge infection. *Avian Pathol.* 12: 505-514.
- GELENEZEI, E.F. and E.W. MARTY. 1964. Studies on a tissue culture modified infectious laryngotracheitis virus. *Avian Dis.* 8: 105-122.
- HAMPL, H., T. BEN-PORAT, L. EHRLICHER, K.O. HABERMEHL and A.S. KAPLAN. 1984. Characterization of the envelope proteins of pseudorabies virus. *J. Virol.* 52: 583-590.
- HAMES, B.D. 1981. An introduction to polyacrylamide gel electrophoresis. *In: Gel Electrophoresis of Proteins: A practical approach.* B.D. HAMES and D. RICKWOOD (Eds.). IRL Press, Oxford. pp. 1-92.
- HANSON, L.E and T.J. BAGUST. 1991. Infectious laryngotracheitis. *In: Disease of Poultry.* B.W. CALNEK *et al.* (Eds.). 9th editions. Ames, Iowa State University Press.
- IZUCHI, T., A. HASEGAWA and T. MIYAMOTO. 1982. Studies on a live virus vaccine against infectious laryngotracheitis of chickens: I. Biological properties of attenuated strain C7. *Avian Dis.* 27(4): 918-926.
- LAEMMLI, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*, 227: 680-685.
- OLSHEVSKY, U and Y. BECKER. 1970. Herpes simplex virus structural proteins. *Virolog.* 40: 948-960.
- PEREIRA, L., M. HOFFMAN, M. TATSUNO and D. DONDERO. 1984. Polymorphism of human cytomegalovirus

- glycoproteins characterized by monoclonal antibodies. *Virolog.* 139: 73-86.
- RAMOS, M.E., C.V. YASON and E.A. RUBIO. 1980. A study on laryngotracheitis of poultry in the Philippines. *Phil. J. Vet. Anim. Sci.* VI (4): 506-513.
- REED, L. J. and H. MUENCH. 1938. A simple method of estimating fifty per cent endpoints. *The American Journal of Hygiene.* 27(3): 493-497.
- ROIZMAN, B. 1982. The family of herpesviridae. General description, taxonomy and classification. In: *The Herpesviruses.* B ROIZMAN (Ed.), vol. 1. Plenum, Press, New York. pp. 1-23.
- SAEPULLOH, M and H.G. ROVIRA. 2003. Isolation and identification of infectious laryngotracheitis virus from outbreak at Lipa City, Batangas, the Philippines. *JITV* 8: 122-133.
- SAEPULLOH, M., H. HAMID dan DARMINTO. 2003. Isolasi dan identifikasi agen penyebab penyakit *infectious laryngotracheitis* (ILT) pada ayam petelur di Kabupaten Bogor, Bekasi dan Tangerang. Pros. Seminar Nasional Teknologi Peternakan dan Veteriner. Bogor 29-30 September 2003. Badan Penelitian dan Pengembangan Pertanian, Pusat Penelitian dan Pengembangan Peternakan.
- SCHLOER, G.M. and S.S. BREESE. 1982. Purification of malignant catarrhal fever virus using a two-phase aqueous polymer system. *J. Gen. Virol.* 58: 101-110.
- SPEAR, P.G. and B. ROIZMAN. 1972. Protein specified by herpes simplex virus: V. Purification and structural proteins of the herpesvirus. *J. Virolog.* 9(1): 143-159.
- TIZARD, I.R. 2002. *Veterinary Immunology: An Introduction.* Sixth edition. W.B. Saunders Company. pp. 180-190.
- TOWBIN, H., T. STAHELIN and J. GORDON. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci.* 76: 4350-4354.
- WATARI, E., B. DIETZSCHOLD, SZOKAN and E. HEBERT-KATZ. 1987. A synthetic peptide induce long term protection from lethal infection with herpes simplex virus2. *J. Exp. Med.* 165: 459-470.
- WILSON, C.M. 1983. Staining of proteins on gels: comparison of dyes and procedures. *Methods in Enzymol.* 91: 236-247.
- YORK, J.J., and K.J FAHEY. 1990. Humoral and cell-mediated immune responses to the glycoproteins of infectious laryngotracheitis. *Arch. Virol.* 115: 289-297.
- YORK, J.J., S. SONZA and K.J. FAHEY. 1987. Immunogenic glycoproteins of infectious laryngotracheitis herpesvirus. *Virolog.* 161: 340-347.