Improved Detection and Control of Infectious Laryngotracheitis Virus on Poultry Farms

by

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Abstract

Infectious laryngotracheitis virus (ILTV) is an economically important disease of chickens. The disease control relies on rapid and accurate diagnosis. Traditional ILTV diagnostic and PCR methods are either time consuming or less sensitive. Therefore, a TaqMan® labeled probe real-time PCR and a novel DNA detection method, loop-mediated isothermal amplification (LAMP), were developed for ILTV DNA detection. Primers and probe for the real-time PCR and LAMP assays were designed from the same region of the ILTV ICP4 gene. They detected both chicken-embryo origin (CEO) and tissue-culture origin (TCO) propagated vaccine viruses without cross-reaction. DNA plasmids were constructed from a partial sequence of the ILTV ICP4 gene. The sensitivity of real-time PCR was 10 copies/ µl and statistical analysis indicated excellent reproducibility. Six specific primers were used for the LAMP assay and the sensitivity was 60 copies/ µl. Although the sensitivity of LAMP assay was lower than real-time PCR, it was easier to perform, less expensive, and less time consuming. The LAMP assay can be optimized for the detection of ILTV in poultry diagnostic laboratories.

Water lines in commercial chicken houses can transmit avian pathogens. Biofilms in water lines can protect microorganisms from physical and chemical damage and release them in the water. In the current study, biofilms in water lines were shown to harbor ILTV that was transmitted to susceptible birds. Four sanitizers were tested for their ability to remove and/or inactivate ILTV in the water lines. Results indicated that two routinely used sanitizers, sodium hypochlorite and citric acid, were unable to inactivate ILTV. In contrast, live ILTV was

inactivated by two more expensive commercial sanitizers that contained either sodium hydrogen sulfate or hydrogen peroxide. Selecting appropriate sanitizers for water line disinfection is critical for ILTV prevention and control. Water line disinfection is important because many live viral and bacterial vaccines are administered in the drinking water and without proper sanitization, these vaccines can inadvertently infect subsequent flocks.

Darkling beetles, their larvae (lesser mealworm), and rodents are ubiquitous in chicken farms. They are known to transmit avian bacteria and viruses. Beetles and their larvae from ILTV infected chicken farms were collected. ILTV was detected in the insects by real-time PCR and virus isolation. The lung of one rat, captured from an ILTV positive house, was positive for ILTV DNA by real-time PCR. Results indicated that live ILTV could be isolated from the insects at least 42 days after the disease occurred on the farms. The PCR restriction fragment length polymorphism (RFLP) showed that the viruses from the infected poultry farms were of vaccine origin. This was the first investigation to demonstrate that darkling beetles, their larvae, rats, and drinking water may be contaminated with ILTV. Therefore, they might be important sources for ILTV transmission.

The incidence and severity of ILTV has been reduced in many commercial farms in the US. In house composting for 5 days, litter treatment, heating house at 38°C, and rigorous biosecurity can help reduce ILTV in infected houses. However, ILT is still prevalent in chicken industry intensive regions. These experiments developed rapid, accurate, and economical methods to improve ILTV DNA detection. They also provided evidence that water lines, beetles, and rodents can carry ILTV. Appropriate sanitizers for water line disinfection and reducing the population of insects and rodents are important for the prevention and control of ILTV transmission.

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CHAPTER I

GENERAL INTRODUCTION

Infectious laryngotracheitis (ILT) is an important respiratory disease of chickens and it annually causes significant economic losses world-wide. In the United States (US), ILT was first reported in the 1920's; however, this disease may have existed in chickens much earlier (May and Tittsler, 1925). ILT virus ILT (V) belongs to the *Herpesviridae* family, *Alphaherpesvirinae* subfamily, *Iltovirus* genus, and the *Gallid herpesvirus 1* species (McGeoch *et al.,* 2000; McGeoch *et al.,* 2006). In nature, herpesviruses have been identified in more than 200 different species of animals and humans. The *Alphaherpesvirinae* includes four genera, two of which are two avian herpesviruses, the genera *Mardivirus* and *Iltovirus*. Marek's disease virus (MDV) and herpesvirus of turkeys (HVT) belong to *Mardivirus*; ILTV and *Psittacid herpesvirus 1* (PsHV-1) are members of *Iltovirus*. (McGeoch *et al.,* 2000; McGeoch *et al.,* 2006). Although ILTV, MDV, and HVT belong to *Alphaherpesvirinae,* neither the nucleotide sequence nor the deduced amino acid sequence of glycoprotein D and ICP27 are similar. According to phylogenic analysis, ILTV could be an early type of alphaherpesviruses (Johnson *et al.,* 1995).

Traditional ILT diagnosis relied on clinical signs, gross and microscopic lesions, virus isolation, serologic tests, and fluorescent antibody (FA) test on tracheal lesions. The characteristic histopathologic lesions of ILT infection are intranuclear inclusion bodies in tracheal epithelial cells (Nair *et al.,* 2008; Guy and Garcia, 2008). Virus isolation can be

performed in several primary chicken tissue cultures, such as chicken embryo liver (CEL), chicken embryo kidney (CEK), and chicken kidney (CK) cell cultures (Chang *et al.,* 1973; Hughes and Jones, 1988). The typical cytopathic effect (CPE) of ILTV is multinucleated giant cells (syncytia) in cell culture. However, inclusion bodies are present only during the early stages of the infection and ILTV isolation takes at least a week. These tests are time consuming and have low sensitivity. ILTV DNA detection methods are rapid, accurate, and sensitive. Different gene segments have been used to detect ILTV DNA by PCR (Abbas *et al.,* 1996; Chang *et al.,* 1997; Clavijo *et al.,* 1997; Alexander *et al.,* 1998). PCR was more sensitive than virus isolation and electron microscopy for ILTV detection (Williams *et al.* 1994). Nested PCR provided a more sensitive detection in clinical and subclinical infections than PCR (Humberd *et al.,* 2002). Recently, developed real-time PCR assays allow qualitative and quantitative method for ILTV DNA detection (Creelan *et al.,* 2006; Callison *et al.,* 2007). Because, real-time PCR assay does not need electrophoresis in an agarose gel, it has less opportunity for contamination than PCR or nested PCR. However, prior real-time PCR assays for ILTV detection had low sensitivity and cannot predict the numbers of viral particles in broad range. Furthermore, a previously established SYBR Green I based real-time PCR had non-specific primer dimers and producing non-specific amplification products.

In chicken flocks, ILTV transmission occurs via respiratory and ocular routes. Sources of ILTV include clinically affected chickens, latent infected carrier chickens, and contaminated fomites (Bagust *et al.,* 2000; Guy and Garcia, 2008). Dogs, crows, and cats might transmit ILTV mechanically (Kingsbury *et al.,* 1958). ILTV may exist as an acute or latent infection in chickens whether it is a vaccine or wild type virus. Dormant viruses can reside in the chicken trigeminal

ganglia. When infected chickens are stressed, latent ILTV can become active and spread to susceptible birds (Bagust, 1986; Williams *et al.,* 1992).

Drinking water is an important source for disease transmission. In drinking water systems, bacteria and algae can form a layer of biofilm, which shelters pathogens from chemical and physical damages (Jessen and Lammert, 2003; van der Wende *et al.,* 1989; Reid, 1999). Water lines of commercial broiler houses have been indicated as the source for *Campylobacter jejuni*, *Pseudomonas* spp., *Escherichia coli,* and *Salmonella* spp. transmission (Trachoo *et al.,* 2002a and 2002b; Silagyi *et al.,* 2009; Marin *et al.,* 2009). Viruses can also attach to biofilm in water lines and be transmitted to susceptible animals via the drinking water, e.g. poliovirus has been shown to attach to biofilms in the water systems and be transmitted through the water (Quignon *et al.,* 1997). H9N2 avian influenza virus (AIV) was isolated from the drinking water in Hong Kong chicken farms (Leung *et al.,* 2007). In a report, H5N1 AIV infected ducks transmitted virus through the drinking water (Sturm-Ramirez *et al.,* 2004). Therefore, drinking water systems in poultry farms might be an important source for ILTV transmission.

ILT vaccine is usually administered by the drinking water. It is possible that the vaccine virus is maintained in the biofilm of water lines and be transmitted subsequently to susceptible birds. This is a problem since vaccine virus has been shown to increase in virulence after back passage in chickens. Therefore, appropriate use of sanitizers to remove biofilm from the water lines may be important for ILT control.

Darkling beetles (*Alphitobius diaperinus*), ubiquitous in poultry farms, harbor several avian pathogens and serve as vectors for disease transmission. Chickens had signs associated with infectious bursal disease (IBDV) when the birds were fed darkling beetles from IBDV positive

farms (McAllister *et al.,* 1995). Darkling beetles in IBDV positive chicken farms carried the virus for several weeks (Snedeker *et al.,*1967). Darkling beetles have also been shown to carry avian leucosis virus, Marek's disease virus (MDV), fowl pox virus, avian reovirus, and Newcastle disease virus (NDV), which were transferred to birds via the consumption of contaminated beetles (De Las Casas *et al.,* 1973, 1976; Goodwin, 1996; McAllister *et al.,* 1995; Eidson *et al.,* 1996). Darkling beetles can transmit *Campylobacter* spp. and *Salmonella* spp. in chicken farms (Skov *et al.,* 2004; Hazeleger *et al.,* 2008). Investigation of possible carriers is necessary for improved ILT control in endemic areas. Evaluation of darkling beetles as potential carriers of ILTV will help to improve biosecurity and reduce the virus spread from infected farms.

The current study had the following specific objectives: 1) develop a specific and sensitive real-time PCR for ILTV detection; 2) develop a novel nucleic acid detection method—loop-mediated isothermal amplification (LAMP) assay for ILTV detection; 3) compare the efficiency of the LAMP assay with real-time PCR; 4) determine if water lines are a reservoir for ILTV transmission and test the ability of sanitizers to remove ILTV from water systems; and 5) determine if ILTV can survive in darkling beetles (*Alphitobius diaperinus*).

CHAPTER II

LITERATURE REVIEW

2.1 The history of infectious laryngotracheitis

In 1925 May and Tittsler reported a new chicken disease characterized by clinical signs which included nasal exudate, swelling around the eyes, and labored breathing. Lesions consisted of stringy mucus and blood in the pharynx, epiglottis, larynx, and trachea. They named the disease tracheo-laryngitis (May and Tittsler, 1925). However, the disease is believed to have existed earlier. Several names have been used to describe this disease including infectious bronchitis, tracheo-laryngitis, infectious tracheitis, and avian diphtheria (Beach, 1926; May and Tittsler, 1925; Gibbs, 1931; Gwatkin, 1925). The name infectious laryngotracheitis (ILT) was adopted in 1931. ILT cases have been reported around the world, especially in areas of highly intensive poultry rearing. ILTV is an important pathogen, which causes significant economic losses worldwide. Continual research on ILTV is important for prevention and control of ILT.

The etiology of ILT was demonstrated to be a virus (Beach, 1930). In 1934, a report showed that ILTV could be propagated in the chorioallantic membrane (CAM) of 10-day-old chicken embryos (Burnet, 1934). The virus was identified as a herpesvirus (Cruickshank *et al.,* 1963).

2.2 General biology of ILTV

ILTV belongs to the family *Herpesviridae*, subfamily *Alphaherpesvirinae* and is a member of the genus *Iltovirus* (McGeoch *et al*., 2000; McGeoch *et al.,* 2006). The virus belongs to *Gallid herpesvirus 1* species (Davison *et al.,* 2005). The particles of ILTV have icosahedral symmetry and are 200-350 nm in diameter. The spherical virion comprises core, capsid, tegument, and envelope. The viral genome is a single, linear, double-stranded DNA molecule packaged into a capsid protein. The nucleocapsid of ILTV contains 162 capsomers, which contain 150 hexons and 12 pentons, and the triangulation number (T) is 16. There are 960 copies of the capsid. The diameter of the virion is about 195-250 nm. The envelope surrounding the nucleocapsid is a lipid bilayer, which is associated with the outer surface of the tegument. It contains integral viral glycoproteins (Davison *et al*., 2005). The genome of ILTV is a linear 155 kb double-stranded DNA, which consists of long and short unique regions (U_L, U_S) and two inverted repeat sequences (internal repeat, IRs; terminal repeat, TRs) that flank the U_S regions (Fig 2.1). The genome forms two isomers, which are different from the orientation of the U_S regions (Davis *et al*., 1973; Leib *et al*., 1987). This genome structure is designated as type D herpesvirus genomes (Roizman *et al*., 2001).

ILTV is an enveloped virus and sensitive to ether, chloroform, and other lipolytic solvents (Meulemans *et al*., 1978). ILTV can be inactivated by ether after 24 hours (Fitzgerald *et al.,* 1963). At 55°C for 10-15 minutes or 38°C for 48 hours, ILTV was readily inactivated (Schalm *et al.,* 1935). However, different strains of ILTV have different resistance to heat. Meulemans *et al.* in 1978 reported that the Belgian strain had partial infectivity at 56°C for 1 hour. In chicken tracheae and CAMs, ILTV was destroyed in 44 hours at 37°C, or inactivated in 5 hours at 25°C (Cover *et al.,* 1958). It has been shown that the virus was destroyed in 1 min by treating it with

3% cresol or a 1% lye solution (Meulemans *et al.,* 1978). On a chicken farm, 5% hydrogen peroxide mist administered with fumigation equipment completely inactivated ILTV (Neighbour, 1994). At lower temperatures, ILTV maintains infectivity for a long period. The virus survived for 10-100 days in tracheal exudates and chicken carcasses at 13-23°C (Jordan, 1966). When stored at -20°C to -60°C, ILTV was viable for months to years. Storage media containing glycerol or sterile skim milk greatly increases the infectivity in tracheal swabs (Bagust *et al.,* 2000; Schalm *et al.,* 1935).

2.3 Characteristics of the ILTV genome

The genome of ILTV consists of double-stranded linear DNA. Thuree and Keeler in 2006 reported that when 14 different published ILTV genome sequence data from different laboratories were analyzed, ILTV contains 148,665 base pairs, and that the G + C content was 48.16%. It was predicted that the genome had 77 open reading frames (ORFs). Of these ORFs, 63 were homologous to herpes simplex virus-1 genes (McGeoch *et al.,* 2000; Thuree and Keeler, 2006).

2.4 ILTV proteins and their functions

Herpesvirus genes can be classified into 3 groups: immediate early (IE), early (E), and late (L). The IE genes are transcribed after infection and are viral genome regulators and activators. The E gene products are involved in viral DNA replication and nucleic acid metabolism. Most of the L gene products are virion components, such as capsid proteins, tegument proteins, and envelope glycoproteins (Wagner and Hewlett, 2004). Many viral proteins have been identified by monoclonal antibodies, monospecific antisera, or directly in viral particles. The function and

distributions of ILTV gene products were assigned by Thuree and Keeler in 2006 and are summarized in Table 2.1.

Fig 2.1: The genome map of ILTV. The ILTV genome contains long unique (U_L), short unique (U_S) regions and 2 inverted repeats (internal repeat, IRs; terminal repeat, TRs). The IR and TR flank the U_S region. (Adopted from Fuchs *et al.,* 2007).

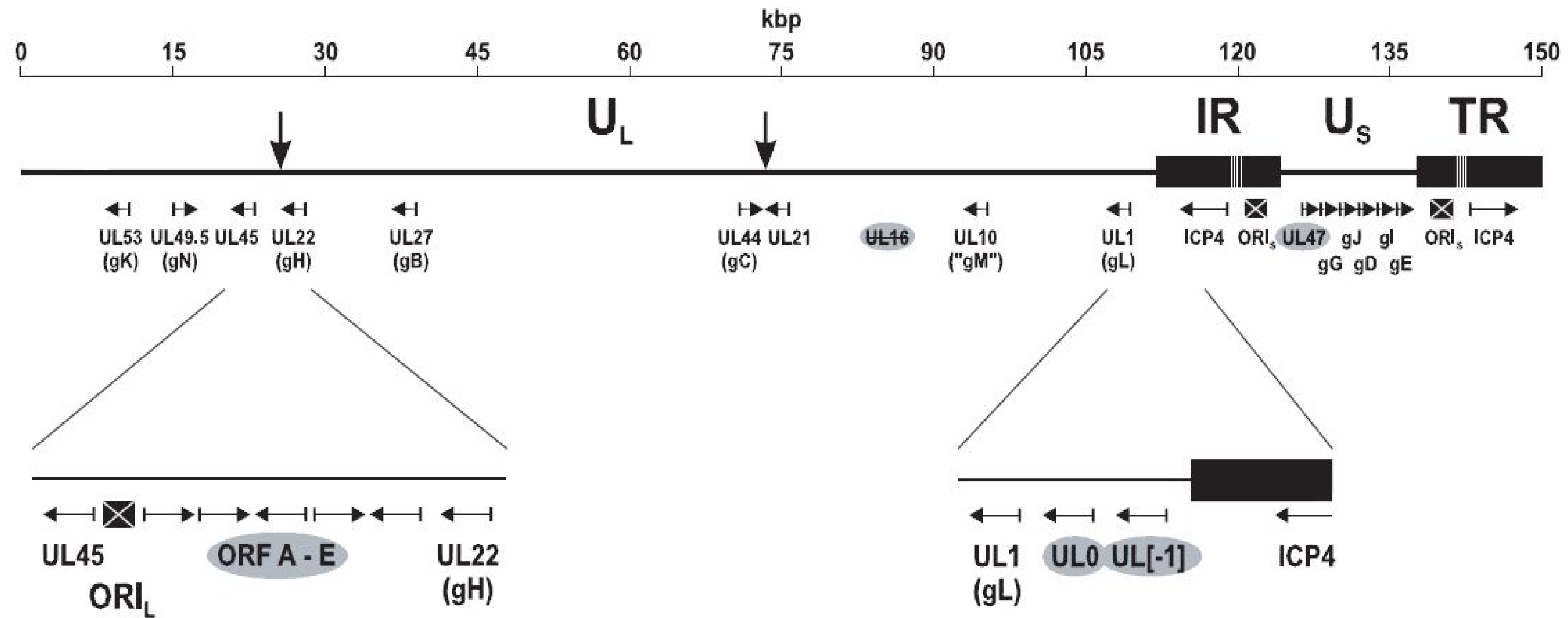

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Table 2.1: Comparison of the proteins and their locations between PsHV-1 and ILTV (Thuree and Keeler, 2006).

PsHV-1		ILTV		Length (amino acid)		% Identity	
ORF	Nucleotide position	ORF	Nucleotide position	PsHV-1	ILTV	to ILT	Putative function
UL54	4295-2376	UL54	12082-10787	639	420	38	Post-translational regulator of gene expression
UL53	6461-5385	UL53	13916-12855	358	337	21	Glycoprotein K; exocytosis
UL52	10335-6454	UL52	17176-13847	1293	1110	42	DNA helicase-primase
UL51	10319-11101	UL51	17189-17875	260	229	55	Unknown
UL50	12476-11232	UL50	19182-17935	414	416	46	Deoxyuridine triphosphatase
UL49.5	12667-13014	UL49.5	19336-19686	140	266	50	Putative viral membrane protein
UL49	13184-14038	UL49	19749-20546	283	117	50	Viral tegument protein
		UL48	20695-21882		396		Viral tegument protein (α-TIF)
UL46	14500-16323	UL46	21888-23558	606	539	44	Tegument phosphoprotein; α-TIF modulation
UL45	17426-16521	UL45	24559-23663	301	281	26	Tegument/ envelope protein
ORF A	21070-22260	ORF A	25275-26405	396	376	27	Hypothetical protein
ORF B	22726-23829	ORF B	26448-27467	367	340	30	Hypothetical protein
ORF C	25174-24056	ORF C	28529-27528	372	334	33	Hypothetical protein
ORF D	25554-26654	ORF D	28639-29760	366	374	38	Hypothetical protein
ORF E	28424-27054	ORF E	31067-29838	456	410	28	Hypothetical protein
UL22	31254-28834	UL22	33539-31128	828	779	34	Glycoprotein H; fusion complexes with gL
UL23	32569-31550	UL23	34667-33576	339	363	40	Thymidine kinase
UL24	32533-33489	UL24	34556-35416	318	287	41	Unknown
UL25	33628-35511	UL25	35392-37107	627	572	47	DNA packaging protein
UL26	35709-37382	UL26	37288-39045	557	586	33	Capsid protein p40
UL26.5	37199-37891	UL26.5	38428-39045	230	206	13	Virion scaffold protein
UL27	40995-38260	UL27	41747-39099	911	873	59	Glycoprotein B
UL28	43743-41164	UL28	44013-41722	859	537	47	ICP18.5; cleavage/ packaging

PsHV-1		ILTV		Length (amino acid)		% Identity to ILT	Putative function
ORF	Nucleotide position	ORF	Nucleotide position	PsHV-1	ILTV		
UL29	47426-43860	UL29	47094-44098	1188	999	59	Major single-strand DNA binding protein
UL30	47858-51103	UL30	47271-50291	1081	1007	54	DNA polymerase
UL31	52171-51134	UL31	51483-50467	345	339	68	Nuclear phosphoprotein
UL32	54020-52164	UL32	53233-51479	618	582	48	Envelope glycoprotein
UL33	54019-54393	UL33	53190-53579	138	119	58	DNA packaging
UL34	54616-55440	UL34	53611-54486	300	290	62	Membrane-associated phosphoprotein
UL35	55567-55992	UL35	54515-54886	141	124	50	Capsid protein
UL36	65672-56085	UL36	62584-54917	3209	2556	36	Major tegument protein
UL37	69349-66437	UL37	65881-63212	970	890	34	Tegument protein
UL38	69618-71078	UL38	65973-67280	486	412	41	DNA binding; capsid protein
UL39	71339-73774	UL39	67618-69972	818	785	50	Large-subunit ribonucleotide reductase
UL40	73851-74792	UL40	69827-70915	313	310	69	Small-subunit ribonucleotide reductase
UL41	76206-74884	UL41	72176-70983	440	398	73	Virion host shutoff
UL42	76680-78182	UL42	72398-73693	519	432	34	Processivity factor for DNA polymerase
UL43	78100-79626	UL43	73756-74970	508	300	11	Unknown
UL44	80418-81806	UL44	75683-76924	462	414	29	Glycoprotein C
UL21	83761-82052	UL21	78611-77016	569	532	28	Nucleocapsid protein
UL20	84089-84835	UL20	78782-79477	248	232	30	Membrane protein
UL19	85082-89323	UL19	79664-83872	1413	1403	65	Major capsid protein
UL18	89684-90649	UL18	84059-85015	321	319	64	Capsid protein
UL15a	92293-90773	UL15a	86212-85103	513	764	66	Terminase; DNA packaging

PsHV-1		ILTV		Length (amino acid)		% Identity to ILT	Putative function
ORF	Nucleotide position	ORF	Nucleotide position	PsHV-1	ILTV		
UL17	92187-94634	UL17	86355-88505	815	341	38	Tegument protein
UL16	94538-95605			355			Capsid assembly
UL15b	96940-95588	UL15b	89761-88598	450	764	43	Terminase; DNA packaging
UL14	96939-97529	UL14	89595-90353	196	196	45	Unknown
UL13	97403-98785	UL13	90212-91606	486	465	45	Serine/ threonine protein kinase
UL12	99023-100585	UL12	91750-93366	566	526	58	Alkaline deoxynuclease
UL11	100585-100782	UL11	93259-93502	48	80	42	Myristoylated tegument protein
UL10	10228-101047	UL10	94758-93580	413	393	46	Glycoprotein M
UL9	102386-105028	UL9	94653-97382	880	892	53	Ori binding protein
UL8	105636-107582	UL8	97378-99762	648	795	34	Helicase-primase component
UL7	108856-107690	UL7	100889-99816	388	358	41	Unknown
UL6	110909-108561	UL6	102807-100669	782	713	47	Minor capsid protein
UL5	110993-112560	UL5	102795-105314	855	840	58	Helicase-primase component
UL4	113781-114539	UL4	105403-105936	252	178	81	Unknown
UL3	114921-115523	UL3	106948-106349	200	196	66	Unknown
UL2	115671-116759	UL2	107950-107060	265	297	50	Uracil DNA glycosylase
UL1	117253-116705	UL1	107920-108279	182	131	32	Glycoprotein L
		UL0	111514-110171		447		Unknown
UL(-1)	118632-117331	UL(-1)	111670-112026	463	501	23	Unknown
ICP4a	127595-121494	ICP4	118888-114500	2033	1463	35	Gene regulation
US10	133103-133948	US10	122103-122936	281	278	32	Unknown
		sORF4/ 3	124190-123309		293		Unknown
US2	134374-134634	US2	125011-124325	85	118	34	Unknown
US3	136263-134785	US3	125100-126527	498	471	48	Protein kinase
sORF1	136535-138352	UL47	126616-128484	605	623	45	UL 47
US4	138546-139388	US4	128651-129526	280	292	24	Glycoprotein G
sORF2	139667-142642	US5	129739-132693	991	985	18	Glycoprotein J

PsHV-1		ILTV		Length (amino acid)		% Identity to ILT	Putative function
ORF	Nucleotide position	ORF	Nucleotide position	PsHV-1	ILTV		
US6	142740-143891	US6	132441-133805	383	434	28	Glycoprotein D
US8	145663-147369	US8	135198-136694	568	499	27	Glycoprotein G
		US9	136704-137483		259		Unknown
sORF4 /3	148377-149249	sORF4/ 3	137535-138461	290	322	34	Unknown
		US10	138704-139402		232		Unknown
ICP4b	154577-160678	ICP4	142837-147225	2033	1463	35	Gene regulation

Function or property as demonstrated for ILTV and /or HSV-1 homolog.

Protein assignment and functions by Thuree and Keeler, 2006.

Glycoprotein B (gB): gB is the most conserved structural protein (Pereira, 1994). The amino acid sequence of ILTV gB and its function are similar to other herpesviruses (Griffin, 1991; Kongsuwan *et al.,* 1991). The gB is essential for virus attachment and penetration to host cells. It is involved in cell-to-cell spread and syncytium formation (Liang *et al.,* 1991; Okazaki, 2007). The gB induced humoral and cell-mediated immune responses and therefore ILTV gB subunit vaccine protected chickens from clinical signs and viral replication. Tong *et al.* reported that the ILTV gB cloned into fowl pox virus protected chickens from ILTV challenge (York *et al.,* 1991; Tong *et al.,* 2001).

Glycoprotein C (gC): gC is a structural and non-essential protein for viral replication in cell culture. This glycoprotein involves viral attachment and virulence. In most alphaherpesviruses, gC mediates viral attachment by interacting with the cellular heparan sulfate receptor (Mettenleiter *et al.,* 1990). However, according to gC sequence analysis and protein expression research, ILTV gC lacked about 100 amino acids at the N-terminal end of the protein. This protein is the heparin-binding motif, but does not affect ILTV infectivity. It indicates that ILTV uses a different mechanism to attach to host cells (Kingsley *et al.,* 1994; Kingsley and Keeler, 1999).

Glycoprotein E and glycoprotein I (gE and gI): gE and gI are non-essential proteins. They form a heterdimer with non-covalent binding. In HSV-1, gE and gI assists in virus cell to cell spreading. The gE/gI heterdimer promotes cell-to-cell spreading by binding cellular receptor at cell junctions (Dingwell *et al.,* 1994; Collins *et al.,* 2003). The gE/gI plays a significant role in cell to cell spread of ILTV (Devlin *et al.,* 2006a).

Glycoprotein G (gG): gG is non-essential and conserved in most alphaherpesviruses. This glycoprotein is not assembled into viral particles, but is secreted from infected cells (Helferich *et al.,* 2007). The gG of ILTV serves as a virulence factor. gG-deficient ILTV has reduced pathogenicity, produce less clinical signs, has less effect on weight gain, and produces less mortality (Devlin *et al.,* 2006b). This gG-deficient ILTV has the potential as a marker vaccine to differentiate vaccinated and naturally infected birds with field strains (Devlin *et al.,* 2007; Devlin *et al.,* 2008).

Glycoprotein J (gJ): gJ is the product of the ORF 5 gene located in the U_S region. It is a non-essential and structural glycoprotein. This protein was named gp60, because it was identified as a 60,000 D glycoprotein (Kongsuwank *et al.,* 1993). ILTV gJ contains *N*- and *O*-linked carbohydrate chains, and two different translation products are formed by non-spliced and spliced mRNA (Veits *et al.,* 2003a). gJ-deficient ILTV has shown minor effects on cell to cell spread; however, the virus replication rate was reduced. Moreover, the gJ-deficient ILTV has reduced virulence, compared with viral challenge with the parental strain. Therefore, gJ deleted ILTV might serve as a marker vaccine for serological differentiation between vaccinated and field virus infected birds (Fuchs *et al.,* 2005a).

Glycoprotein M and Glycoprotein N (gM and gN): gM and gN are gene products of UL10 and UL49.5 in ILTV. They are important for replication of alphaherpesviruses in cell culture (Roizman and Knipe, 2001). gM and gN form a heterdimer combined with each other by disulfide-linkage. gM and gN of many herpesviruses are *O*-glycosylated. However, in ILTV, gM is not modified by glycosylation (Fuchs *et al.,* 1999; Fuchs *et al.,* 2005b).

Thymidine kinase (TK): TK gene is located in the U_L region. It is not essential for propagation in cell culture, but is a virulence factor for ILTV. TK negative alphaherpesviruses reduce the rate of re-activation from latent infection (Efstathiou *et al.,* 1989). Chickens receiving TK-deficient ILTV had reduced clinical signs and it induced protection against virulent ILTV (Schnitzlein *et al.,* 1995; Han *et al.,* 2002). TK-deficient ILTV may provide a mechanism for latent infection, reactivation, and as a vaccine candidate.

2.5 Infectious laryngotracheitis virus replication

Replication of ILTV is similar to other alphaherpesviruses, such as pseudorabies virus and herpes simplex virus (Prideaux *et al.,* 1992). The virus attaches to receptors on the cell surface. The envelope fuses with the cell membrane and virus nucleocapsid is released into the cytoplasm. Viral DNA is then transported into the nucleus. Transcription of the viral genome, replication of viral DNA, and assembly of new virions occurs in the nucleus (Roizman and Knipe, 2001).

2.6 Pathobiology of ILTV

2.6.1 Hosts

All ages of chickens are affected, but chickens older than 3 weeks are most susceptible to ILTV (Fahey *et al.,* 1983). It has been shown that ILTV can infect pheasants, pheasant-bantam crosses, and peafowl (Crawshaw *et al.,* 1982).

Turkeys can be experimentally infected with ILTV; however, turkeys might have an age-dependent resistance, since lesions are only seen in younger birds (Winterfield *et al.,* 1968). Pottz *et al.* in 2008 reported a natural infection of ILTV in turkeys. Clinical signs in turkeys were similar to chickens (Portz *et al.,* 2008). Starlings, sparrows, crows, pigeons, ducks, and guinea

fowls were resistant to ILTV infection (Crawshaw *et al*., 1982; Hayles *et al.,* 1976; Seddon *et al.,* 1936). Chicken and turkey embryonating eggs can readily propagate ILTV, but guinea fowl and pigeon eggs could not (Jorden, 1966).

Embryonating chicken eggs and several avian cell cultures can be used to propagate ILTV. In chicken embryos, ILTV forms plaques on the chorioallantoic membrane (CAM). The plaques can be observed 48 hours after infection, and embryos might die in 2-12 days post infection. Strains of ILTV showed different plaque size and morphology on the CAM (Burnet *et al.,* 1934; Brandly, 1937; Srinivasan *et al.,* 1977; Hughes *et al.,* 1988).

The ILTV can be propagated in primary cell cultures, such as chicken embryo liver (CEL), chicken embryo kidney (CEK), and chicken kidney (CK) cell cultures (Chang *et al.,* 1973; Hughes and Jones, 1988). The sensitivity of ILTV isolation and propagation vary depending on the type of cell cultures. CEL was the most sensitive for isolation, followed by CK. The CEK and chicken embryo lung cells were less sensitive (Hughes and Jones, 1988). Chang *et al.* 1977 reported that ILTV can be propagated in chicken leukocytes from buffy coat cells (Chang *et al.,* 1977). Macrophages from bone marrow or spleens were susceptible to ILTV (Bulow *et al.,* 1983). Chicken embryo fibroblasts (CEF), Vero cells, and quail cells were not satisfactory for primary isolation of ILTV. Lymphocytes, thymocytes, and activated T cells were not sensitive to ILTV infection (Hughes and Jones, 1988; Schnitzlein *et al.,* 1994). ILTV replicated in an avian liver cell line, LHM, which are chicken liver tumor cells induced by chemicals. For ILTV to multiply in LHM cells, it must be adapted. Therefore, this cell line is not used for diagnosis (Schnitzlein *et al.,* 1994).

Viral cytopathic effect (CPE) can be observed in cell culture 4-6 hours PI. The CPE contains swelling of cells, chromatin displacement, and rounding of the nucleoli. The characteristic CPE is syncytia, which forms multinucleated giant cells. Intranuclear inclusion bodies could be observed at 12 hours PI. (Reynolds *et al.,* 1968; Guy and Garcia, 2008).

2.6.2 Transmission

Natural transmission of ILTV is through the upper respiratory and ocular routes. Sources of ILTV are clinically affected chickens, latent infected carrier chickens, contaminated dust, litter, and fomites. Egg transmission of the virus has not been verified (Bagust *et al.,* 2000; Guy and Garcia, 2008). Other possible sources of transmission included dog, crows, and cats (Kingsbury *et al.,* 1958). One study showed that wind-borne transmission was critical for ILTV spread (Johnson *et al.,* 2005). After infection, ILTV replicates in the epithelium of the larynx and trachea. Viral particles are present in tracheal tissues and are secreted for 6-8 days PI. The virus may remain in the trachea at 10 days PI (Bagust *et al.,* 1986; Hitchner *et al.,* 1977; Williams *et al.,* 1992).

2.6.3 Latent infection of ILTV

The ILTV can establish latent infections. The virus can be re-isolated from tracheal swabs 7 weeks PI, or two months PI in tracheal samples (Bagust, 1986; Adair *et al.,* 1985). The trigeminal ganglion is the target for latency of ILTV. Four to seven days after ILTV infection by intratracheal route (IT), 40% of infected chickens showed that the virus migrated to trigeminal ganglion, which is nerves system for sensory in face (Bagust, 1986). Fifteen months after vaccination, the latent ILTV in the trigeminal ganglion was reactivated. In mature laying chickens challenged with virulent ILTV, DNA was detected in the trigeminal ganglion by PCR

at 31, 46, and 61 days PI (Williams *et al.,* 1992). When birds were stressed, such as the onset of lay or re-housing, ILTV can re-activate and spread to susceptible birds (Hughes *et al.,* 1989).

2.6.4 Clinical signs

Clinical signs can be observed 6-12 days PI. Experimental challenge with IT results in a 2-4 day incubation period (Kernohan, 1931; Jordan, 1963). There are two clinical forms of ILT infection (severe and mild). Clinical signs of the severe form include dyspnea and bloody mucus. This form can cause 90%-100% morbidity with mortality ranging from 5% to 70% and average mortality being 10-20% (Bagust *et al.,* 2000; Guy and Garcia, 2008).

Clinical signs of the mild form include depression, reduced egg production and weight gain, conjunctivitis, swelling of the infraorbital sinuses (almond shaped eyes), and nasal discharge. Morbidity for the mild form is about 5% and mortality 0.1-2%. Generally, it takes 10 to 14 days for recovery, but with some strains the clinical signs may extend for 1-4 weeks (Hinshaw *et al.,* 1931).

2.6.5 Gross and microscopic lesions

Gross lesions are observed in the larynx and trachea. With the severe form, the mucosa of the respiratory tract shows inflammation and necrosis with hemorrhage. A characteristic feature is intranuclear inclusion bodies in epithelial cells, which are observed about 3 days PI. These cells have a condensed nucleus surrounded by a halo and margining of chromatin. Inclusion bodies are generally present for a few days at the early stage of infection before epithelial cells die. Epithelial cell hyperplasia induces multinucleated cells (syncytia), lymphocytes, histiocytes, and plasma cells, which migrate to the lamina propria. Lesions are followed by desquamation of

necrotic epithelium and loss of mucous glands. At this time, bloody mucus in the trachea is observed (Nair and Gough, 2008; Guy and Garcia, 2008).

2.7 The detection and identification of ILTV

Laboratory diagnosis is required for ILT, because other diseases cause similar clinical signs and lesions, such as infectious bronchitis (IB), Newcastle disease (ND), avian influenza (AI), infectious coryza, and Mycoplasmosis. ILTV infection can be confirmed using several methods, including virus isolation, DNA detection, and serologic tests. For ILTV isolation, the CAM inoculation of 9-to-12-day-old embryos and primary cell culture are used. Samples from the trachea, conjunctiva, larynx, and lung of clinically affected birds are collected and inoculated on the CAM (Hughes *et al.,* 1988). Plaques can be observed 2 days PI; however, they usually develop on the CAM 5-7 days PI. Plaque size and morphology on the CAM can be used to differentiate the virulence of strains. The CEL and CK cell cultures are suitable for ILTV isolation. Multinucleated giant cells may be observed 24 hours PI (Guy and Garcia 2008).

Traditional antigen detection uses ILTV polyclonal or monoclonal antibodies to bind ILTV antigen from clinical samples. Viral antigen was detected using direct or indirect fluorescent antibodies (FA) in the tracheal smear or tracheal tissues (Goodwin *et al.,* 1991). A more sensitive method using immunoperoxidase (IP) labeled monoclonal antibodies can be used as immunoprobes to detect ILTV in tracheal smears. This IP method detected ILTV on the second day PI (Guy *et al.,* 1992). Agar gel immunodiffusion (AGID) uses hyperimmune serum against ILTV to detect antigen in tracheal samples and it can differentiate ILT from the diphtheritic form of fowlpox. However, the sensitivity was lower than other methods (Jorden *et al.,* 1962). Antigen capture enzyme-linked immunosorbent assay (AC-ELISA) uses ILTV monoclonal antibodies for

antigen detection. The AC-ELISA was faster and more accurate than AGIP or FA (York and Fahey, 1988).

ILT DNA detection methods have developed rapidly in recent years. These methods can identify ILTV quickly, accurately, and are highly sensitive. Molecular techniques for ILTV detection include cloned DNA probes for dot-blot hybridization (Nagy, 1992), PCR (Abbas *et al.,* 1996; Chang *et al.,* 1997; Clavijo *et al.,* 1997; Alexander *et al.,* 1998), nested PCR (Humberd *et al.,* 2002), real-time PCR (Creelan *et al.,* 2006), multiplex PCR (Pang *et al.,* 2002), *in situ* hybridization (Nielsen *et al.,* 1998), and PCR followed by restriction fragment length polymorphism (RFLP) (Chang *et al.,* 1997; Kirkpatrick *et al.,* 2006; Oldoni and Garcia, 2007; Oldoni *et al.,* 2008). Comparing the conventional methods of ILTV detection with PCR has reported that PCR was more sensitive than virus isolation in cell culture and electron microscopy. PCR also detected ILTV in the samples, which was contaminated with other pathogens (Williams *et al.,* 1994).

2.8 Loop-mediated isothermal amplification (LAMP)

The loop-mediated isothermal amplification (LAMP) is a novel DNA amplification method used in disease diagnosis. The reaction can be processed at a constant temperature by DNA polymerase, and its rapid and simple features can provide rapid diagnosis. The LAMP method uses four primers that recognize six regions on the target DNA sequence, therefore, the specificity is high. The LAMP method can amplify a few copies of DNA in an hour with just a few reagents. Furthermore, this method can amplify both DNA and RNA. When it is used to detect RNA, the avian myeloblastosis virus (AMV) reverse transcriptase is added to the reaction for the reverse transcription step, followed by the DNA amplification. This method is easy to

perform with less expensive reagents and common laboratory equipment (Notomi *et al.,* 2000). In 2002, Nagamine *et al.* improved this method by adding two additional primers, named loop primers. The loop primers hybridized to the stem-loops structures reducing reaction time to less than half of the original LAMP method (Nagamine *et al.,* 2002).

LAMP method uses *Bst* DNA polymerase, which has strand displacement activity, and a set of two inner primers and two outer primers, which recognize six regions of target sequences. The inner primers are called the forward inner primer (FIP) and the backward inner primer (BIP), and each contains two sequence regions that recognize sense and antisense sequences of target DNA. The Fig 2.2 shows the arrangement of the LAMP primer system. The FIP contains the sequence of F1c, a TTTT space, and F2. The BIP contains the sequence complementary to B1 (B1c), a TTTT space, and B2. Two outer primers are B3 and F3. The reaction adds *Bst* DNA polymerase and continues for 60 minutes (Notomi *et al.,* 2000).

When the reaction is initiated, the inner primer FIP attaches to F2c in the target DNA and synthesizes a complement strand. Then, the outer primer F3 hybridizes to F3c in the target DNA and initiates strand displacement DNA synthesis. The DNA strand is elongated from FIP is replaced and released. The released single-strand DNA forms a loop structure at its 3’ end. This released single-stranded DNA serves as a template for another inner primer. The BIP attaching and DNA elongation is followed by a new BIP primed DNA displacement using an outer primer, B3, primed DNA, which leads to a “dumb-ball” form DNA. This “dumb-ball” form DNA uses self-structure as the template. The self-primed DNA synthesis is initiated from the 3’ end F1 region, and the elongation starts from FIP annealing to the single strand of the F2c region in the loop structure. Newly synthesized DNA primed by the 3’ end F1 region form a loop at the other end. Another self-priming DNA is synthesized from the B1 end of BIP, and a complementary

DNA of origin stem-loop DNA is released. The BIP anneals to these two structures and starts elongation and recycling. Products are a mixture of stem-loop DNA with different length and cauliflower-like structures with several loops formed by annealing between alternately inverted repeats of the target within the same strand (Notomi *et al.,* 2000; Nagamine *et al.,* 2002; Tomita *et al.,* 2008).

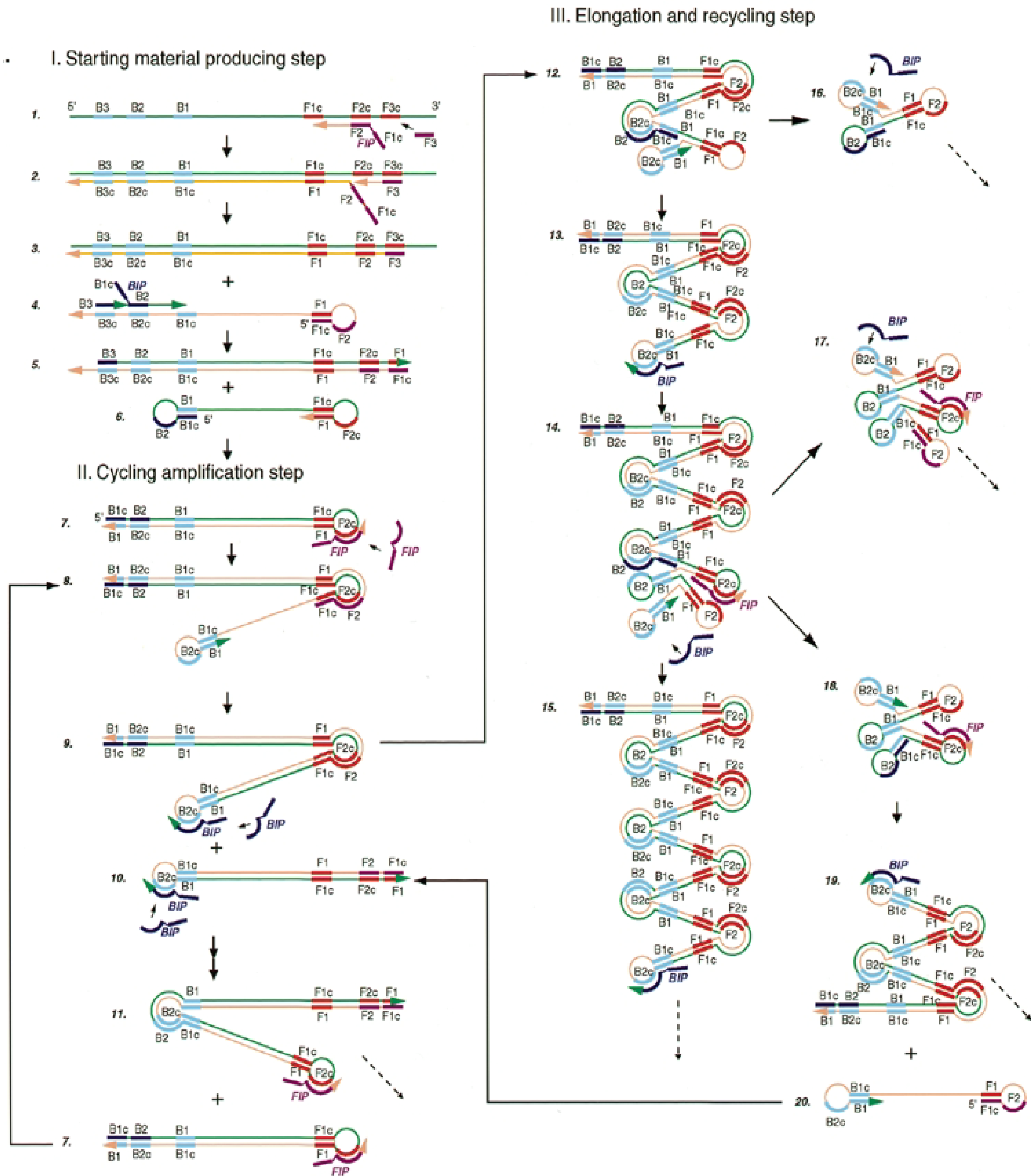

Fig 2.2: The principles of LAMP DNA amplification and primer arrangement (Adopt from Notomi *et al.,* 2000).

In the initial step, the annealing of the four primers to the target DNA is critical. Thus, melting temperatures (Tm) of the inner primers, F2 and B2 in FIP and BIP, must fit the optimal temperature of *Bst* DNA polymerase activation, which is between 60-65℃. To optimize loop formation, the F1c and B1c are in higher concentration than F2 and B2. For ensuring DNA synthesis from inner primers earlier than that of the outer primers the Tm values of outer primers, F3 and B3, are lower than the inner primers. Moreover, the concentrations of inner primers are 4-10 times higher than the outer primers. The loop primers were designed to increase the reaction speed by binding to the target DNA between F1-F2 and B1-B2 regions. To optimize primer efficiency, the GC content of primers is between 40-60%. The distance between 5'ends of F2 and B2 should be 120-180 bp, and the distance between F3 and F2 as well as B2 and B3 should be 0-20 bp. The distance for loop regions (5'of F2 to 3'of F1 and 5'of B2 to 3'of B1) should be 40-60 bp. The optimal target DNA should be less than 200 bp. If the target DNA is more than 500 bp, amplification efficacy is inadequate. For primers stability, the free energy of the following end should be less than -4kcal/mol: 3'end of F2/B2, F3/B3, LF/LB, and the 5'end of F1c/B1c. Primer designation of LAMP is complicated because of its complex primer system. Computer programs can aid in the LAMP primer designation (Notomi *et al.,* 2000; Parida *et al.,* 2008).

The magnesium ion (Mg^{2+}) is an important co-factor for DNA polymerase activity by influencing the primer template annealing temperature, fidelity, specificity, and yield. In the LAMP method, ion concentrations affect results of the DNA amplification method. Magnesium pyrophosphate ions are a by-product produced from the deoxyribonucleotide triphosphates (dNTPs). Large amounts of magnesium pyrophosphate are produced after the LAMP reaction and produce white precipitate in the reaction tube. However, the white precipitate in the reaction

tube is too weak to be visually observed. Turbidity can be measured by spectrophotometer at 400 nm and the detection limit reaches 4μg DNA in a 25 μl tube. If the turbidity is measured every 6 sec during LAMP reaction, a real-time LAMP reaction as well as real-time PCR can be set up and the concentration of target DNA calculated (Mori *et al.,* 2001; Mori *et al.,* 2004; Parida *et al.,* 2008).

There are fluorescent dyes to monitor LAMP reaction products, such as SYBR Green I, ethidium bromide, calcein, etc. These dyes bind to DNA and illuminate visual fluorescent signals with UV light irradiation. The calcein in the reaction mixture combines with manganous ion (Mn^{2+}) at the beginning to remain quenched. When the amplification reaction proceeds, Mn^{2+} and calcein are separated and pyrophosphate ion ($P_2O_7^{4-}$) is produced, which can emit fluorescence by UV irradiation. After the LAMP reaction is finished, free calcein combines with Mg^{2+} in the mixture and the fluorescence emission is strengthened (Notomi *et al.,* 2000; Tomita *et al.,* 2008).

2.9 Polymerase chain reaction

The polymerase chain reaction (PCR) is commonly used in molecular biology for genetic amplification, cloning, DNA sequencing, and diagnosis. The PCR based on Khorana's idea was first proved by Dr. Mullis, who used the Klenow fragment of *E coli* DNA polymerase I to synthesize oligonucleotides *in vitro* (Saiki *et al.*, 1985). Not until the discovery of *Taq* DNA polymerase did PCR efficiency increase. The *Taq* DNA polymerase is a themostable DNA-dependent DNA polymerase, which was discovered from the thermophilic archea *Thermus aquaticus*, which lives in hot springs. This DNA polymerase is stable at high temperature, therefore, the PCR denaturation, annealing, and extension steps can be continuously performed in one tube (Erlich *et al.*, 1991).

Molecular techniques were derived from PCR including reverse transcriptase (RT)-PCR, hot start PCR, multiplex PCR, nested PCR, etc. RT-PCR uses reverse transcriptase, as with avian myeloblastosis virus (AMV) or Moloney murine leukemia virus (M-MLV) reverse transcriptase, to synthesize DNA from RNA templates. Using RT-PCR, the RNA is converted to a complementary DNA (cDNA) strand, followed with PCR steps to amplify cDNAs. Multiplex PCR uses more than one pair of primers in a PCR tube. It can simultaneously amplify several DNA segments from different DNA targets. Primers should have similar melting temperatures, so they cannot form primer dimers. Hot start PCR begins when the reaction reaches the denaturation temperature for inhibiting non-specific priming and primer dimers (Erlich *et al.,* 1991). The DNA polymerase for hot start PCR is blocked by monoclonal antibodies to inhibit its activities during low temperature. When the temperature increases, the antibody dissociates from DNA polymerase and is inactivated at the denaturation temperature. Nested PCR uses two rounds of PCR reactions to increase the sensitivity and specificity of target DNA detection. The second primers are located inside the first PCR amplicon sequence.

2.10 Real-time polymerase chain reaction

Real-time PCR, also called quantitative PCR, is more sensitive for detection of target DNA quantity when compared with traditional PCR. The mechanism of real-time PCR follows the principles of traditional PCR except for the amplificated product detection step, which requires the use of fluorescent reporters. After receiving the fluorescent signals, the computer calculates the amount of DNA present at each cycle of PCR and can quantify the starting amount of a specific DNA sequence.

With real-time PCR, the computer collects the fluorescent signals and the fluorescent signal intensity changes. The ΔRn can be calculated using the equation: $\Delta Rn = (Rn^{+})-(Rn^{-})$ (Heid *et*

al., 1996). Rn^+ is the fluorescence emission intensity ratio at any given time; Rn^- represents the fluorescence emission intensity ratio of prior PCR amplification in the same tube. The amplification plot consists of the ΔRn at y-axis and cycle number at x-axis. During the early stage of PCR amplification, the ΔRn remains under the baseline. As the PCR proceeded, the fluorescent signal intensity increases. The cycle threshold (C_T) or crossing point (C_P) value represents the cycle number at which the fluorescent signal reaches the threshold or baseline and is detectable with a computer. When a standard curve is produced, DNA samples are serial diluted and subjected to real-time PCR. The C_T value of each cycle and the logarithmic value of the input DNA concentration consist of a linear plot with a negative slope, which is the standard curve of the real-time PCR reaction (Heid *et al.,* 1996). Therefore, the relationship between C_T value and the concentration of target DNA are used for absolute quantification. The C_T value is defined by 10 standard deviations from the mean baseline fluorescent signals between the first and 15th cycles (Jung *et al.,* 2000; Mackay et al., 2002).

To determine the equation of standard curve and the absolute quantification calculation, the basic equation of real-time PCR amplification is used:

$$N_C = N_0 \times (E+1)^C$$

The C is the cycle number, E represents the efficiency of PCR amplification, N_0 is the initial copy numbers of the template, and N_C is the copy numbers at C cycles. By rearranging this equation, the copy number of the initial template is obtained:

$$N_0 = \frac{N_C}{(E+1)^C}$$

When the determined copy number from a given C_T value is known, the initial copy number of the template is determinated using the equation:

$$N_0 = \frac{N_{CT}}{(E+1)^{CT}}$$

Absolute quantification is calculated from the standard curve, which consists of the C_T values compared to the copy number of the target DNA. The regression equation of the standard curve is calculated with the following equation:

$$\log (N_0) = \log (N_{CT}) - \log \left[(E+1)^{CT} \right]$$

$$\log (N_0) = - \log (E+1) \times C_T + \log (N_{CT})$$

This standard curve equation corresponds with a line equation y = ax + b. The slope is −log (E+1) and the intercept is log (N_{CT}) (Rutledge and Cote, 2003). When the template nucleic acids are serial diluted, the slop of the standard curve equation 'a' is a negative value and the meaning of 'a' is how many cycles the reaction requires to increase one order of DNA concentration. Therefore, the '-1/a' is the increase in the amount of amplified DNA during one reaction cycle. To define this increase, the '-1/a' is an exponent of 10. Thus, the efficiency E is $10^{-1/a}$ (Rebrikov and Trofimov, 2006).

To maintain sensitivity and specificity, the primer and probe designation are critical. In general, the amplicon site is between 100-200 bp. Compared with traditional PCR, a smaller amplicon can improve the efficiency by shortening the denaturation and primer annealing time. Real-time PCR primers are usually 15-30 bps and the G + C content is about 30-80% with the melting temperature (Tm) between 58-60℃. To avoid non-specific PCR products, the primers should lack repeat identical nucleotides. In addition, the total number of G and Cs in the last five nucleotides of 3' end should not be more than two. Real-time PCR probes are 20-40 in length with the G + C content about 40-60%. The Tm of the probes should be 5-10℃ higher than the

primers. The probe should avoid hybridization or overlapping with primers. There are several programs, which design primers and probes, that can avoid hairpins or complementary between primers.

Real-time PCR instantaneously reports DNA amplification. This is accomplished by the fluorescent dyes interaction with the PCR reactions. There are two fluorescence reporter systems: specific fluorescent probes and non-specific DNA labels. The most common fluorescent probes are hydrolysis probes and hybridization probes, however, the non-specific DNA labels include SYBR Green I, YO-PRO-1, etc.

Hydrolysis probe, also called TaqMan probe, is based on the 5' exonuclease activity of *Taq* or *Tth* polymerase. During the extension step of PCR, the *Taq* DNA polymerase cleaves the non-extendible probes from target DNA sequence. The structure of hydrolysis probes are sequence specific double labeled fluorogenic nucleotides. One fluorescent dye is a reporter at the 5' end of probe and the other is a quencher fluorescent dye, which can absorb the emission spectrum from the reporter. When the quencher and reporter are in close proximity, they are attached to the same PCR amplicon and the quencher absorbs the signal from the reporter. At the PCR extension stage, the 5' to 3' nuclease activity of *Taq* DNA polymerase degrades the hydrolysis probe, and the reporter and quencher are separated, allowing the reporter's fluorescence signal emission. The probe hydrolysis increases fluorescent signals and allows its detection.

Following the improvement of fluorescent technique, there are several quencher fluorophores, which include TAMRA, DABCYL, and BHQ, and the reporters are FAM, VIC, NED, etc (Heid *et al.,* 1996). Compared with DNA-binding dye system, hydrolysis probes provide greater specificity, because only sequence-specific amplificons probes will hybridize, and only the probes remains hybridized to its complementary sequence. This allows the polymerase to cleave

the probes. The conditions for the PCR extension step should fit the Tm of the probes, which is approximated 70°C for probe binding. The TaqMan system uses combined annealing and extension steps of about 60-62°C to ensure that the probe remains bound to its target during the primer extension step.

The hybridization system uses two single labeled probes to improve specificity. The probe at the 5' region of amplicon carries a fluorescein donor at its 3' end. The other probe at the 3' region of amplicon has a fluorophore acceptor at its 5' end. This probe must block its 3' end by phosphorylation to prevent extension during PCR processing. The donor emission spectrum overlaps with the emission spectrum of the acceptor. When they are hybridized to their complementary sequences at a head-to-tail structure, they are close enough (about five nucleotides in length) to transfer the energy from donor to acceptor, so that the fluorescence signal can be emitted from the acceptor. Because the wavelengths of two emission spectra are different, they can be separately detected. When more DNAs are synthesized, the intensity of the fluorescence signals, emitted by the acceptor are measured, and the fluorescence signal of donor serves as a background. Comparing the background and acceptor spectrum estimates the amount of DNA synthesized (Wittwer *et al.,* 1997).

Molecular beacons are a kind of hybridization probe, which forms a stem-loop structure by complementary sequences at its 5' and 3'ends. It is a double labeled probe with a fluorescent marker attached to one end and the other end is a non-fluorescent quencher. In free forms, molecular beacons form a hairpin structure and the stem maintains the arms in close proximity, so the quencher can inhibit the fluorescence signal emission from the other end. When the molecular beacons anneal to their complementary target DNA sequences, the hairpin structures change its conformation and separate its 5' and 3' ends. This probe and target DNA hybrid is

more stable than the hairpin structure (Bonnet *et al.,* 1999). Thus, the quencher can no longer inhibit the fluorescence and the signals are used to calculate the concentration of target DNA. The target DNA and the molecular beacon sequences should be matched exactly, because the hairpin structure is more stable than that of linear probes. It does not tolerate even one nucleotide mismatch between target DNA and hairpin probe sequences. Thus, molecular beacons provide highly specific detection and are used in the investigation of single nucleotide polymorphisms (SNPs) (Tyagi *et al.,* 1998).

The DNA-binding dye is a non-specific method to detect target DNA. It is cheaper and easier to perform than probe hybridization systems. The most popular DNA-binding dye is SYBR green I. The SYBR green I is an asymmetric cyanine dye, which has two aromatic groups containing nitrogen. When they are free in solution, the vibration around both aromatic groups transmit the energy from electronic excitation to heat and release the heat to the surrounding solvent. Thus, unbound dye exhibits little fluorescence in the solution. However, when they bind to double stranded DNA minor groove, the rotation of the aromatic components is restricted. The dye emits more fluorescence signals during PCR processing (Nygren *et al.,* 1998).

Because the DNA-binding dyes are non-specific, the binding of primer dimers and non-specific amplification products can be problematic. This can be solved by analysis of the melting curve of the amplicon. When the temperature above the Tm of amplicon is increased, the amplicon is denatured. A characteristic melting peak at the Tm of the amplicon will separate from primer dimmers that melt at lower temperatures. Another disadvantage of SYBER Green I is that more than one molecule will bind to single double stranded DNA. A longer amplicon will emit a stronger fluorescence signal than a short amplicon.

2.11 ILTV strain differentiation

It is difficult to identify different strains of ILTV by serological methods, because the ILTVs have close immunodominant domains (Shibley *et al.,* 1962). The most common and effective molecular method for ILTV differentiation is PCR followed by restriction fragment length polymorphism (RFLP). PCR-RFLP analysis of single or multiple viral genome regions can differentiate strains from various geographic areas and vaccine from wild (field) strains. (Leib *et al.*, 1986; Keeler *et al.*, 1993; Chang *et al.,* 1997; Oldoni *et al.,* 2007 and 2008; Neff *et al.,* 2008; Oldoni *et al.,* 2009). Restriction endonuclease analysis of ILTV DNA can differentiate vaccine strains from wild type strains (Guy *et al.,* 1989). Moreover, PCR-RFLP analysis of partial ICP4 gene, gC gene, and TK gene can distinguish field strains from vaccines. However, some virulent isolates could not be separated from vaccine strains (Chang *et al.,* 1997). Han *et al.* (2001) analyzed multiple genes with PCR-RFLP combined with DNA sequence analysis of gG gene and TK gene to differentiate vaccine and non-vaccine strains. Researchers demonstrated that multiple gene PCR-RFLP was more reliable to differentiate vaccines from field strains (Kirkpatrick *et al.,* 2006).

Oldoni and Garcia (2007) investigated ILTV isolates from commercial poultry that were collected between 1988 and 2005 using multiple gene PCR-RFLP analysis (ORFB-TK, ICP4, UL47/gG, and gM/ UL9). They were able to separate ILTVs into nine genetic groups. Group I and II comprised the USDA reference strain and tissue culture origin (TCO) vaccine strains. Group IV isolates were identical to chicken egg origin (CEO) vaccine strains, whereas group V isolates, which had one PCR-RFLP pattern different from the CEO vaccine strains are CEO-related isolates. Group III, VI, VII, VIII, and IX were field ILTV strains with genomic types

different from CEO and TCO vaccines. In that report, most of ILTV positive poultry isolates were related to vaccine strains (Oldoni & Garica, 2007). Oldoni *et al.* in 2008 investigated 46 ILTV field isolates collected in the U.S. from 2006 to 2007. After multiple gene PCR-RFLP analysis, most isolates (63%) were closely related to vaccine strains (group III, IV, and V) (Oldoni *et al.,* 2008). According to these reports, most ILTV field isolates in the U.S. were derived from vaccines.

In Europe, 104 field isolates were collected during 35 years from eight different countries. These virus isolates were analysed with PCR-RFLP targeting the TK gene and it was shown that they separate into 3 genetic groups. It was also showed that 98 of these field isolates had the same RFLP patterns as vaccine strains (Neff *et al.,* 2008). In Australia, PCR-RFLP analyzed ILTV gG, TK, ICP4, ICP18.5, and ORFB-TK genes in 20 strains. These isolates could be discriminated into five genetic groups. Some isolates could not be separated from vaccine strains (Kirkpatrick *et al.,* 2006).

2.12 Immunology and vaccination

ILT vaccination induces partial protection in one week against challenge at 3-4 days post infection (PI). Humoral immunity is not the major immune response against ILTV in chickens. Research verified the importance of cell-mediated immunity (CMI) in the infection of ILTV resistance. An experiment was designed in which chickens were bursectomized with cyclophsophamide and surgical methods to block the humoral immune responses. Vaccinated bursectomized chickens developed CMI responses against virulent ILTV challenges (Fahey *et al.,* 1983). It confirmed that CMI was more important than humoral immunity. Furthermore, local

CMI responses in the trachea produced protection from ILTV challenge in bursectomized chickens. Mucosal antibodies were not essential for resistance to challenge (Fahey *et al.,* 1990).

Vaccination is effective to prevent ILTV infection. However, ILT vaccine viruses can create latent infected carrier chickens. These latent carriers are a source for spread of virus to non-vaccinated flocks. Therefore, it is recommended that ILT vaccines be used only in areas where ILT is endemic. The most currently used ILT vaccine strains are attenuated modified-live TCOs or CEOs viruses. Compared with protection afforded by TCO and CEO vaccines, there was no significant difference in the immunity of chickens at 10 weeks PI. However, when chickens over 20 weeks of age were vaccinated, the CEO vaccines induced better protection than TCO vaccines (Andreason *et al.,* 1989). Methods for vaccine administration are eye drop, drinking water, and aerosol spray. The drinking water route poses some problems in that chickens might not receive enough virus at the target organ (nasal epithelial cells) and drinking water quality varies between poultry houses. Thus, these birds may fail to develop protective immunity and may have rolling (continual) reactions (Robertson *et al.,* 1981). On the other hand, with spray route, some chickens may develop severe reactions, because excess dosages of small droplets can penetrate deep into the respiratory tract (Clark *et al.,* 1980).

There are several disadvantages when using modified-live ILT vaccines. They include: insufficient attenuation, latent infected carriers, and the spreading of virus to non-vaccinated flocks. Reports have shown that modified-live vaccines increase their virulence by bird-to-bird passage (Guy *et al.,* 1991; Kotiw *et al.,* 1995). Kotiw *et al.* (1995) serially passaged modified-live ILT vaccines *in vivo* for 35 generations. After the 6th passage, this vaccine strain showed severe clinical signs in challenged chickens. Furthermore, restriction endonuclease analysis of

the viral genomes between original and final passage showed no differences between isolates (Kotiw Kotiw *et al.,* 1995). CEO vaccines have the tendency to increase in virulence more than TCO vaccines, when passed in chickens (Guy *et al.,* 1991). Investigations of ILTV isolates collected from around the world were analyzed by PCR-RFLP. They revealed that current wild virulent isolates were closely related to vaccine strains. This implies that field isolates originated from vaccine strains after back passage in chickens (Leib *et al.,* 1986; Keeler *et al.,* 1993; Chang *et al.*, 1997; Oldoni *et al.,* 2008; Neff *et al.,* 2008; Oldoni *et al.,* 2009).

Recent molecular advances have developed recombinant vaccines. Researchers have developed other vaccines using inserted partial ILTV genes into fowlpox and HVT modified genomes. A recombinant fowlpox vaccine, which contained ILTV gB gene, was shown to induce protection against virulent strains and the protection was similar to attenuated-live vaccines (Tong *et al.,* 2001). Another recombinant fowlpox virus, which contains ILTV gB and UL 32 genes, was shown to provide protection against virulent strain challenge (Davison *et al.,* 2006). Two licensed commercial recombinant ILT vaccines are used around the world. One is produced by the CEVA (Biomune Company, Lenexa KS), which uses fowl poxvirus as a vector with an insertion of ILTV gene. The other is produced by Intervet (Intervet Inc. Millsboro, DE), in which ILTV genes are cloned into a herpesvirus of turkey (HVT). They have shown some efficacy in the field.

Several studies tried to develop new ILT vaccine candidates by gene deletion. Some ILTVs, with deleted virulent viral genes, retained their ability to induce immune responses without producing clinical signs. Recombinant virus with deleted gJ, TK, and, UL0 genes readily showed attenuation, and could be used for vaccine production (Okamura *et al.,* 1994; Schnitlzlein *et al.,* 1995; Veits *et al.,* 2003b). The gG-deficient ILTV administered by either eye-drop or drinking-

water routes induce adequate immunity against challenge. Therefore, it may have a use for large-scale vaccination (Devlin *et al.,* 2008). There were also ILTV non-essential genes, which were deleted to test their ability as vaccines. The ILT mutants, such as deleted five unique open reading frames (ORF A-E), removed gN and gM, and the green fluorescent protein was inserted into the UL50 gene deleted region, could be used as recombinant ILT vaccines (Fuchs *et al.,* 1999; Fuchs *et al.,* 2005b; Fuchs *et al.,* 2000). ILT recombinant vaccines used ILTV as a viral vector to contain foreign viral genes. One ILTV vaccine contains H5 and H7 genes of highly pathogenic AIVs. This recombinant ILTV may be used as bivalent vaccine against ILT and pathogenic AIV (Veits *et al.,* 2003b).

2.13 Prevention of ILTV infection using chicken house management

Field isolates and vaccine viruses can establish latent infected carriers. Thus, it is important to avoid contact between vaccinated or recovered field virus infected birds with non-vaccinated chickens. It is also critical to remove contaminated fomites for prevention and control of ILTV infection. To control ILTV outbreaks, improved biosecurity and management practices are necessary. Biosecurity includes protocols and procedures to prevent pathogens from infecting and transmitting disease by humans, insects, wild birds, or other animals (Kingsbury *et al.,* 1958). A study found that heating litter at 38°C for 24 hours, using commercial litter treatments, and in-house composting for 5 days reduced this virus below isolation detection levels (Giambrone *et al.*, 2008). Patnayak *et al.* in 2008 tested 9 commercial disinfectants and 3 hand sanitizers for their efficacy in inactivating avian metapneumovirus, AIV, and NDV. Viruses were deposited on disks and concentrations of disinfectants added to the disks. After 1, 3, 5, and 10 minutes, they were used to infect cell cultures for re-isolation of the viruses. Under manufacturer's direction,

phenolic compounds reduced concentrations of all three viruses by 99.9%. However, some quaternary ammonium compounds were less effective than other disinfectants. The 3 hand sanitizers containing phenolic compounds and gluteraldehyde (Aero, GermEX, and Purell) inactivated the viruses in one minute (Patnayak *et al.,* 2008).

Biofilms are an adherent matrix, which are produced and enclosed by bacterial populations. Biofilm formation consists of several steps: initial attachment, microcolony and extracellular polymeric substances (EPS) production, followed by maturation (Davey and O'Toole, 2000). Natural ecosystems are generally low in available nutrients and biofilm formation is important for bacteria survival (Mittelman, 1998). For adhesion processes, the bacterial cell surface facilitates attachment. Cell surface properties of bacteria such as flagella, pili, adhesion protein, capsule, and surface charge affect attachment (Kumar and Anand, 1998). Microorganisms adhere to solid surfaces in the solid–liquid interphase and form microcolonies and produce EPS, which can form biofilms. Biofilms, containing various bacteria, are more resistant to disinfectants and antibiotics than planktonic bacteria (Costerton, 1999). They increase the contamination rate in medical equipment, potable water distribution systems, and in the food processing industry (Jessen and Lammert, 2003; van der Wende *et al.,* 1989; Reid, 1999). Chemical and physiological features of biofilm provide resistance to antimicrobial agents, such as antibiotics, disinfectants, or germicides, which can rapidly inactivate planktonic microorganisms (Donlan and Costerton, 2002).

Biofilms in water lines of chicken houses contain different microorganisms. Therefore, more than one pathogen can attach in the biofilm structures and be protected from environmental damage. Because biofilms are formed inside the water lines, they can be re-established in 2-3 days in which the biofilm bacteria and structures are not totally removed. When biofilms are

established in water lines, the microorganisms harbored in the biofilms can slowly release into the water and be consumed by susceptible hosts. According to Zimmer *et al.* in 2003, water lines of broiler houses were a source for *Campylobacter jejuni* (Zimmer *et al.,* 2003). Trachoo and Frankin in 2002 used 4 chemical sanitizers to treat water lines in chicken houses and surveyed the survival of *Campylobacter jejuni* in the biofilm. They found that *Campylobacter jejuni* in biofilm was susceptible to all the tested sanitizers. However, the bacteria in the water lines were not completely inactivated when the sanitizer treatment was not adequately performed (Trachoo and Frank, 2002). Furthermore, an investigation used 3 disinfectants, glutaraldehyde (50% vol/ vol), formaldehyde (37% vol/ vol), and hydroxide peroxide (35% vol/ vol), to evaluate their ability to remove *Salmonella* in biofilms of water lines in chicken houses. However, these disinfectants used at the manufacturer's recommended concentration were inadequate for *Salmonella* elimination regardless of the serotype and the disinfectant contact time (Marin *et al.,* 2009).

2.14 Disease transmission by darkling beetles and rodents

Lesser mealworms or their adult darkling beetles, *Alphitobius diaperinus*, inhabit the litter of farms and are abundant in poultry litter (Pfeiffer *et al.,* 1980; Rueda *et al.,* 1997). Lesser mealworms are omnivorous scavengers that feed on feces, chicken feed, broken eggs, and chicken carcasses (Pfeiffer *et al.,* 1980; Axtell *et al.,* 1990; Rueda *et al.,* 1997). Beetles are eaten by chickens, wild birds, and rodents. It is impossible to totally remove them from poultry houses and they can transmit pathogens (Watson *et al.,* 2000; McAllister *et al.,* 1994; McAllister *et al.,* 1995).

Researchers analyzed chicken and turkey farms and showed that *Salmonella* spp. and *Campylobacter* spp. could be isolated from darkling beetles and their larvae. This showed that

darkling beetles were a possible reservoir for bacterial pathogens (Desping *et al.,* 1994; Skov *et al.,* 2004; Templeton *et al.,* 2006; Hazeleger *et al.,* 2008). In a laboratory experiment, darkling beetles harbored *Salmonella* spp. and *Campylobacter* spp. When chickens were fed *Salmonella* spp. and *Campylobacter* spp. contaminated beetles, the bacteria could be re-isolated for several weeks from the birds (Roche *et al.,* 2009; Desping *et al.,* 1994).

Darkling beetles on chicken and turkey farms serve as vectors for viral diseases. Healthy chickens fed homogenized beetles collected from an IBDV infected farm and developed clinical signs associated with IBD (McAllister *et al.,* 1995). When darkling beetles were fed chicken feed mixed with IBDV, Reovirus, fowlpox virus, or NDV, the viruses could be re-isolated from the beetles for a long time period. In laboratory tests, IBDV survived in the beetles for at least 56 days, reovirus in beetles for at least 9 days, and fowlpox virus and NDV for at lease 7 days (De Las Casas *et al.,* 1973; De Las Casas *et al.,* 1976; McAllister *et al.,* 1995). Turkey poults were fed beetle homogenates, which were infected with turkey coronavirus (TCV) and TCV contaminated materials. Turkeys showed clinical signs associated with TCV and some mortality. They concluded that darkling beetles transmitted TCV (Watson *et al.,* 2000). These studies confirmed that darkling beetles can transmit viruses to poultry.

Rodents are common in farms. Rodents eat feed, increase the feed costs, destroy curtains, and insulation, as well as other structures in chicken houses. They can break and eat eggs, and kill young chicks. They can transmit diseases, which make disease control complicated. Rodents spread diseases by contaminating feed with their urine and feces. Mice are important sources for *S. enteritidis* transmission in chickens (Davies and Wray, 1995; Garber *et al.,* 2003). A single mouse dropping can contain more than 10^5 colony-forming units of *S. enteritidis* (Henzler and Optiz, 1992). Rodents can spread bacteria and other infectious organisms in chicken, such as

Pasturella, *E. coli*, *Mycoplasma*, *Eimeria*, helminths, and other parasites both inside and outside the houses (Koshimizu *et al.,* 1993; Webster and MacDonald, 1995; Stojcevic *et al.,* 2004; Seong *et al.,* 1995).

Rodents are carriers and reservoirs of viruses. They can transmit IBDV and AIV. In IBDV infected farms, antibodies against IBDV could be detected in rat tissues by AGP. Mice carried IBDV in their intestines, liver, and spleen, which can be spread to other mice and chickens (Okoye and Uche, 1986; Park *et al.,* 2010). Rodents transmit several zoonotic diseases, such as rabies, Hantavirus, plague, Rickettsia, etc (Hinson *et al.,* 2004; Heyman *et al*., 2004; Levy, 1999; Smith *et al.,* 1968; Webster and MacDonald, 1995). Therefore, rodent control is critical for disease prevention in poultry and humans.

CHAPTER III

STUDY I: DEVELOPMENT AND COMPARISION OF A TAQMAN® REAL-TIME PCR AND LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (LAMP) ASSAY FOR THE DETECTION OF INFECTIOUS LARYNGOTRACHEITIS VIRUS

3.1 Introduction

ILT is an acute respiratory disease of chickens. It occurs world-wide and causes significant economic losses to the poultry industry (Guy and Garcia, 2008). ILTV belongs to the family *Herpesviridae*, subfamily *Alphaherpesviridae* (McGeoch *et al.,* 2000; Thuree and Keeler, 2006).

Standard procedures for ILTV diagnosis include clinical signs, gross and microscopic lesions, and virus isolation. The histopathologic lesions associated with ILTV infection include intranuclear inclusion bodies in tracheal epithelial cells (Nair *et al.,* 2008; Guy *et al.,* 2008). The immunoperoxidase (IP) and FA tests with labeled monoclonal antibodies are used to detect ILTV in tracheal smears (Guy *et al.,* 1992). The AC-ELISA and ELISA can be used for ILTV antigen and antibody detection (York *et al.,* 1988; Chang *et al.*, 2002). ILTV can be isolated in embryonic eggs inoculated via the CAM route. Primary chicken cells, such as chicken embryo liver (CEL), chicken embryo kidney (CEK), and chicken kidney (CK) are also used for ILTV isolation (Srinivasan *et al.,* 1977; Hughes *et al.,* 1988). However, viral isolation and

identification requires several days and is labor intensive. Therefore, developing rapid and less expensive detection methods is necessary for improved ILT diagnosis.

PCR provides a quick, accurate, and highly sensitive method for ILTV DNA detection. Different gene segments have been used in PCR assays to detect ILTV (Abbas *et al.,* 1996; Clavijo *et al.,* 1997; Alexander *et al.,* 1998; Vogtlin *et al.,* 1999). Nested PCR has been used to detect ILTV DNA in formalin-fixed and paraffin-embedded respiratory tissues of clinically infected birds. Nested PCR can also be used to detect ILTV DNA in flocks showing subclinical or clinical infection (Humberd *et al.,* 2002; Chacon *et al.,* 2008).

Multiplex PCR, which uses several pairs of specific primers in one reaction tube, can simultaneously detect ILTV from avian influenza virus (AIV), NDV, infectious bronchitis virus (IBV), and *Mycoplasma spp.* Infected samples (Pang *et al.,* 2002; Rashid *et al.,* 2009). Quantitative PCR (qPCR) provides a quantitative and qualitative method to detect ILTV DNA. ILTV detection by qPCR is more sensitive than traditional methods, such as histological, fluorescent antibody, and electron microscopy tests (Crespo *et al.,* 2007). The SYBR Green I based qPCR has been reported to detect 140 molecules/ µl of ILTV DNA (Creelan *et al.,* 2006). Callison *et al.* in 2007 reported a real-time PCR using the ILTV gC gene detection. The sensitivity was 100 copies/ reaction. In that report, a standard curve using the ILTV gC gene from 10^6 to 10^2 copies was examined. Although the real-time PCR was stable and reproducible, the standard curve could only be applied in that viral concentration range (Callison *et al.,* 2007).

LAMP assay provides a rapid and low-cost method for pathogen detection. It is has been used for viral and bacterial nucleic acid detection of AIV (Imai *et al.,* 2007), foot-and-mouth disease virus (Dukes *et al.,* 2006), pseudorabies virus (En *et al.,* 2008), *Salmonella* (Ueda *et al.,*

2009), and *Campylobacter* (Yamazaki *et al.,* 2009). LAMP assay uses *Bst* DNA polymerase, which has strand displacement activity, and a set of two inner, two outer, and two loop primers to recognize eight regions of target sequences. The LAMP method amplifies DNA at temperatures between 60 and 65°C in 60 minutes (Notomi *et al.,* 2000; Nagamine *et al.,* 2002; Tomita *et al.,* 2008). To our knowledge the LAMP assay has not been use for the diagnosis of ILTV.

In this study, we report on the development of a TaqMan® probe based real-time PCR to detect and quantity ILTV DNA. We also developed a LAMP assay for ILTV DNA detection and compared its sensitivity and specificity with qPCR.

3.2 Materials and methods

Viral strains

Five commonly used commercial ILT vaccines were used: four CEO vaccines—AviPro® LT (Lohmann Animal Health Inc., Winslow, ME), LT Blen® (Merial Select Inc., Gainesville, GA), Laryngo-Vac® (Fort Dodge Animal Health, Overland Park, KS), Trachivax® (Schering-Plough Animal Health Corp., Kenilworth, NJ), and a TCO vaccine—LT-IVAX® (Schering-Plough Animal Health Corp., Kenilworth, NJ). In addition, for the purpose of evaluating specificity the following non-ILTV vaccines were used as controls: *Mycoplasma gallisepticum* (MG) vaccine—MycoVac-L® (Intervet Inc., Millsboro, DE), fowl pox vaccine—Chicken –N-Pox™ TC (Fort Dodge Animal Health, Overland Park, KS), and MD serotype 3 vaccine—MD-Vac® CFL (Fort Dodge Animal Health, Overland Park, KS). Only MD serotype 3 vaccine was used for the brevity of the study.

Viral DNA extraction

Total DNA from ILTV vaccines and non-ILTV avian pathogens was extracted using Qiagen DNeasy® Blood & Tissue Kit (Qiagen, Valencia, CA, USA). Briefly, 200µl of each sample were mixed with 20µl proteinase K and 200µl of Buffer AL. The mixture was mixed and incubated at 56°C for 10 min. After mixing, 200µl of 100% ethanol was added and vortexed. The mixture was transferred into the DNeasy Mini spin column and centrifuged at 6000 x g for 1 min and the flow-through discarded. The column was placed in a 2 ml tube and 500µl of Buffer AW1 added. The tube was centrifuged at 6000 x g for 1 min, and again the flow-through was discarded. The column was transferred into a 2 ml tube and 500µl of Buffer AW2 was added. The tube was centrifuged at 20,000 x g for 3 min to remove excess reagents from the column membrane. Finally, the DNA was eluted in 100µl of Buffer AE by centrifuging at 6000 x g for 1 min and stored at -20°C.

Construction of standard DNA

To construct a plasmid containing the ILTV ICP4 gene, a 942 bp segment of LT Blen® ILTV partial ICP4 gene was amplified by PCR using the following primers: ICP4-F: 5'-CGCAGAGGACCAGCAAAGACCG-3'; ICP4-R: 5'-GAAGCAGACGCCGCCGTAGGAT-3'. For PCR, 50 µl of reaction was set up as follows: 5 µl of 10x PCR buffer, 5 µl of 25 mM $MgCl_2$, 1µl of 10 mM dNTP each, 1 µl of 100 µM ICP4-F, 1 µl of 100 µM ICP4-R, 0.25µl of Taq DNA polymerase (5 U/µl; AmpliTaq® DNA polymerase, Applied BioSystems, Foster City, CA), 28.75µl of water, and 5 µl of sample DNA. PCR steps were subjected to a 94°C initial denaturation for 2 min and 35 cycles of 94°C denaturation for 30 seconds, 55°C annealing for 30 seconds, and 72°C extension for 1 min, followed by 72°C final extension for 5 min. PCR was

conducted in GeneAmp® PCR System 9700 (Applied BioSystems, Foster City, CA). The PCR products were detected with 2% agarose gel electrophoresis.

The PCR products were purified with the Wizard® PCR preps DNA purification system (Promega, Madison, WI). The cDNA was cloned into the pT7Blue-3 vector. A blunt-end cloning kit (Novagen, Darmstadt, Germany), was used according to the manufacturer's instructions. The plasmids were transformed into NovaBlue® Singles™ competent cells (Novagen, Darmstadt, Germany). Several clones were selected and the plasmid DNA extracted using Wizard® Plus SV kit (Promega, Madison, WI), according to the manufacturer's directions. Clones containing the proper insert were verified by DNA sequencing. The concentrations of cloned plasmids were measured by the NanoDrop® ND-100UV-Vis Spectrophotometer (Wilmington, DE).

Copy number of ILT ICP4 cloned DNA was calculated with the following formula (Ke et al., 2006):

$$\text{ICP4 DNA (copies/ µl)} = \frac{6.022\times10^{23}\left(\frac{molecules}{mole}\right)\times concentration\left(\frac{g}{\mu l}\right)}{Weight\left(\frac{g}{mole}\right)}$$

Weight in Daltons (g/mole) = (bp size of plasmid + insert) × (330 Da × 2 nucleotide/ bp)

Real-time, conventional PCR, and LAMP assays

Real-time PCR amplification of a partial ILTV ICP4 gene was performed in a LightCycler® (Roche, Applied Science, Indianapolis, IN) with 20 µl in volume. For real-time PCR assay, each reaction contained 10µl of 2X master mix (QuantiTect® Probe PCR kit, Qiagen, Valencia, CA), 1 µl of 10 µM each primer (0.5 µM), 0.5 µl of 4 µM probe (0.1 µM), 2.5 µl of water, and 5µl of

DNA template. The real-time PCR program was 95°C initial activation for 15 min and 40 cycles for 95°C denaturation at 0 second and a 60°C combined annealing and extension step for 60 seconds.

The PCR was performed in the GeneAmp® PCR System 9700 (Applied BioSystems, Foster City, CA) using the same primers as for real-time PCR described above. Fifty µl of PCR reagents in a tube containing 5 µl of 10X PCR buffer, 5 µl of 25 mM $MgCl_2$, 1µl of 10 mM dNTP each, 1 µl of each primer in 100µM, 0.25µl of Taq DNA polymerase (5 U/µl; AmpliTaq® DNA polymerase, Applied BioSystems, Foster City, CA), 28.75µl of water, and 5 µl of sample DNA. The PCR steps were subjected to a 94°C initial denaturation for 2 min and 35 cycles of 94°C denaturation for 30 sec, 50°C annealing for 30 sec, and 72°C extension for 30 sec, followed by 72°C final extension for 5 min. The PCR products were detected using 2% agarose gel electrophoresis.

The LAMP assay was performed in 25 µl, which contained 1X ThermolPol buffer (New England Biolabs Inc., Beverly, MA). Each dNTP was used at a concentration of 1.2 mM, inner primers to a final concentration of 1.6 µM, outer primers to a concentration of 0.2 µM, loop primers to a concentration of 0.4 µM, 1.0 M of betaine (Sigma, Saint Louis, MO), 1 µl of 8U *Bst* DNA polymerase (Large Fragment; New England Biolabs Inc., Beverly, MA), 4 mM of $MgSO_4$, and 5 µl of DNA template. To optimize the reaction, 60°C, 63°C, and 65°C temperatures were tested. Reaction times of 15, 25, 35, 45, 50, and 60 min were also examined to optimize the LAMP. The reaction was stopped at 95°C for 3 min to terminate the enzyme activity. After LAMP reaction, DNA products were verified by 2% agarose gel electrophoresis, stained with ethidium bromide, and visualized on a UV transilluminator (Fotodyne® Inc., Hartland, WI).

During DNA amplification (PCR or LAMP), the betaine increased the yield and specificity. Betaine also reduced secondary structure in GC-rich regions and eliminated base pair composition dependence of DNA melting (Rees *et al.,* 1993; Henke *et al.,* 1997).

Designing primers and probe

Primers and probe for real-time PCR and primers for LAMP were designed in the ICP4F/R amplicon. The partial ICP4 gene sequences of the following ILTV strains: CEO vaccine (Genbank accession number EU104900), TCO vaccine (accession number EU104908), ILTV assembled total genome sequence (accession number NC_006623), and 2 ILTV vaccines, AviPro® LT and LT Blen®, were sequenced at Auburn University Genomics & Sequencing Laboratory. They were aligned with AlignX® from vector NTI sequence analysis and data management software v10.3 (Invitrogen Co., Carlsbad, CA). Real-time PCR primers and probe, which generated a 125 bp product, were selected from the conserved regions of the ICP4 gene (Fig 3.1 and Table 3.1). The target DNA sequence was searched using the BLAST service in the Genbank database. Results indicated that the real-time PCR product was located in the ILTV ICP4 gene. Primers and probes were produced by Integrated DNA Technologies®, Inc. (Coralville, CA).

The LAMP primers were designed using the conserved region of ILTV ICP4 gene in the ICP4F/R amplicon as determined by the Primer Explorer V4 software (Eiken Chemical Co., Ltd, Japan). To produce the end stability, the primer selection followed the rule that the free energy (ΔG) of 3'end of F2/B2, F3/B3, LF/LB, and the 5'end of F1c/B1c should be -4kcal/mol or less. One set of primers was chosen. This primer set comprising two outer, two inner, and two loop primers recognized eight distinct regions in the target sequence. The forward inner primer (FIP)

and the backward inner primer (BIP) each had two distinct sequences corresponding to the sense and anti-sense sequence. The DNA strands synthesized from the outer primers (F3 and B3), displaced the primary strands. The forward loop primer (LF) recognized the complementary strand corresponding to the region between F1 and F2, and the reverse loop primer (LB) annealed to the complementary strand corresponding to the region between B1 and B2 (Fig 3.2 and Table 3.2).

Specificity, detection limit, and reproducibility of real-time PCR and LAMP assays

To determine the sensitivity and detection limit for real-time PCR and LAMP, serial 10-fold dilutions of plasmid DNA from 10^0-10^9 copies/ µl were analyzed. The real-time PCR assay was repeated four times. Standard curves indicating the linear relationships between the threshold crossing points (Cp) and the logarithms of initial ILTV ICP4 gene count were constructed. Serial dilutions were repeated in the same run to evaluate intra-experiment reproducibility.

To determine specificity, five ILTV strains, non-template negative controls, MG, fowl pox, and MD vaccines were tested. The detection limit and reproducibility of the real-time PCR assays were determined by four independent runs using 10-fold serial dilutions (10^0-10^9) of the ICP4 gene plasmids as template. A standard curve and equation were generated. Copy number of the ILTV template per amplification reaction was estimated using the standard curve equation. Serial dilutions of the ICP4 plasmids from 6, 15, 30, 60, 6×10^2, 6×10^3, and 6×10^4 copies/ µl determined the sensitivity of the LAMP assay.

Viral titration and ILTV genome copy number conversion

To determine the conversion between viral titer and ILTV DNA copy number, ILTV vaccine stock was titrated in SPF chicken embryonated eggs and the copy number was checked with real-time PCR. For viral titration, 50% embryonic infective dosage (EID_{50}) of the LT Blen® vaccine was initiated using 9 serial 10-fold dilutions of viral stock made in PBS. Four 9-day-old SPF embryonic eggs were used per viral concentration and each egg was inoculated with 200 µl of viral dilution via the CAM route. After 7 days the CAMs were checked for ILTV associated pock formation. The viral titer was calculated using the Reed-Muench formula (Reed and Muench, 1938). The EID_{50} titrations were repeated three times and the ILTV genome copy number was detected with the real-time PCR.

Viral concentration was detected in one dose of LT Blen® vaccine. The vaccine virus was diluted using the manufacture's protocol to 4 doses and the ILTV genome copy number checked with real-time PCR. The tests were repeated three times.

3.3 Results

Specificity of real-time PCR and LAMP assays

Both assays were positive for only ILTV DNAs. No fluorescent signal or positive gel patterns were detected with the non-template control and non-ILTV vaccines (Figs 3.9 and 3.10). As expected the LAMP assay produced stem-loop DNA structures with a different length of inverted repeats and cauliflower-like structures of target sequence. When the LAMP products

were analyzed with agarose gel electrophoresis, they typically showed many bands with different sizes and a smeared DNA between these bands at each loading well.

Sensitivity, reproducibility, and detection limit

Four repeats of qPCR tests generated standard curves, which had an average intercept of 38.28 ± 0.63 and an average slope of -3.14 ± 0.06 (Figs 3 and 4, Table 4). The standard curves had a significant correlation between Cp value and copy number with the square of the sample correlation coefficient (R^2) above 0.99 and the average efficiency was 2.063 ± 0.048. The standard deviation of Cp value was low, which indicated excellent reproducibility (Table 3.4). The real-time PCR maintained linearity at 10 copies/ µl and the standard deviation (SD) and standard error (SE) were stable and low (Table 3.3). One of four repeated real-time PCR tests at 1 copy/ µl was negative, and the Cp values of the other three repeats were above 35. The LightCycler® could not estimate the Cp value when the Cp value was between 35 and 40 for a 40-cycle real-time PCR. Quantification limits were determined at 10 copies/ µl. Samples with the Cp value≤ 35 were considered positive for ILTV DNA (Table 3.3). The sensitivity of conventional PCR, using the same primer set as real-time PCR, was 10^3 copies/ µl (Fig 3.6). Therefore, real-time PCR was about 100 times more sensitive than conventional PCR. The LAMP assay detected ILTV DNA templates at 60 copies/ µl (Fig 3.7). The sensitivity of real-time PCR was 6-fold higher than the LAMP.

Correlation between virus genome count and virus titer

One EID_{50} was equal to (2.1 ± 1.3) × 10^2 ILTV genomic copies, and one dose of ILTV vaccine was equal to (6.9 ± 0.85) × 10^5 copies (Tables 5A and 5B).

Optimizing the LAMP assay conditions

The LAMP reaction created many DNA bands of different sizes in the target sequence region. The amplification with LAMP assay showed a ladder-like pattern upon agarose electrophoresis. The optimal temperature for *Bst* DNA polymerase was between 60-65°C. DNA products using the LAMP assay at 65°C were brighter and clearer than at other temperatures (Fig 3.7). Thus, 65°C was selected as the optimal temperature for this ILTV LAMP assay. Reaction time also affected the LAMP efficiency. The LAMP products were observed with bright bands after the LAMP reaction was performed above 45 min (Fig 3.8).

3.4 Discussion

A TaqMan® probe based real-time PCR for quantitative and qualitative detection of ILTV genome was developed. Although real-time PCR assay is highly sensitive, the thermal cycler and reagents are expensive that routine diagnosis by real-time PCR is a heavy load for basic laboratories. Thus, a more economical method, the LAMP assay, was developed for detecting ILTV DNA. The LAMP assay is more economical and faster than real-timer PCR. Moreover, the sensitivity of LAMP assay was higher than conventional PCR. There is no previous report to compare both methods; therefore, we evaluated the sensitivity and specificity of them.

The sensitivity of this real-time PCR was 10 copies/ μl of ILTV DNA. It was highly repeatable. According to ANOVA statistics analysis, Ct values at the same genomic copy number of multiple repeats were almost identical ($p = 0.9948 > 0.05$). The variations of slopes (-3.14 ± 0.06) and intercepts (38.28 ± 0.63) of regression equations were minimal, implying that the real-time PCR assay was highly repeatable.

Since the real-time PCR can determine the concentration of nucleic acids in samples by estimating using the standard curve, the viral titer can be accurately determined. Titers of one dose of vaccine and viral concentration in EID_{50} were compared with the copy number of ILTV genome by real-time PCR. Results indicated that one EID_{50} of LT Blen® vaccine was equivalent to $(2.1 \pm 1.3) \times 10^2$ ILT genomic copies. One dose of LT Blen® vaccine was equivalent to $(6.9 \pm 0.85) \times 10^5$ copies.

In the current study, the LAMP assay was highly specific and sensitive. Although the sensitivity of the ILTV LAMP assay (60 copies/ µl) was slightly lower than qPCR (10 copies/ µl), it was about 16 times more sensitive than conventional PCR (1000 copies/ µl).

The LAMP assay was simple, rapid, and economical. The reaction was performed at 65°C for less than one hour and did not require a thermal cycler. In addition, the reaction time was faster than qPCR. Reagents and equipment for LAMP assay were less expensive than qPCR and could be easily adopted for most laboratories. The sensitivity of LAMP assay was less affected by contaminating components, feces, feed, and blood, which can be contained in clinical samples, than for PCR. Therefore, the DNA purification steps can be omitted (Kaneko *et al.,* 2007).

The detection limit of real-time PCR and LAMP assays was higher than that of other methods. Nested PCR can detect ILTV DNA in formalin-fixed and paraffin-embedded tissues at 50 fg/ ml (Humberd et al., 2002). A report set up a nested PCR to detect the ILTV gE gene and the sensitivity reached 62.5 fg/ ml. It was concluded that the sensitivity of nested PCR was similar to real-time PCR (Chacon *et al.,* 2008). In the current study, the sensitivity of conventional PCR reached 10^3 copies/ µl using the same primers as for real-time PCR. However, the real-time PCR (10 copies/ µl) and LAMP (60 copies/ µl) were more sensitive than conventional PCR.

In the current study, the real-time PCR, the standard curve of ILTV ICP4 gene was stable from 10^9 to 10^1 copies. Therefore, this real-time PCR was more suitable to estimate the ILTV genome copy number of unknown viral concentration samples with a wider range than the previous real-time PCR assays. Furthermore, the current real-time PCR estimated the viral genome copies in one dose of commercial vaccine and one EID_{50}. This conversion provided a quick and more accurate method to predict the viral titer in a sample than traditional methods. Creelan *et al.,* (2006) used real-time PCR to detect ILTV ICP4 gene fragment with SYBR-Green I chemistry and the sensitivity was 140 copies/ µl (Creelan *et al.,* 2006).

The current real-time PCR using a fragment of the ICP4 gene was the most sensitive detection method reported so far. Because the binding of SYBR-Green I dye to dsDNA molecules is non-specific, the SYBR-Green I based PCR can be affected by the formation of primer-dimmers and sample concentration. In addition, multiple SYBR-Green I dye molecules can bind to the same dsDNA fragment. These disadvantages have limited the application of SYBR-Green I based real-time PCR for use in DNA quantitative detection. Although the nested PCR had similar sensitivity as the real-time PCR and LAMP assays, it requires two rounds of PCR cycles. Therefore, it is more time consuming and the possibility of nucleic acid contamination is higher than for real-time PCR and LAMP assays. Since the ILTV LAMP detection method used six primers, which recognized eight distinct regions of the ILT ICP4 gene, the specificity of LAMP was high. However, in this study, the selection region of the LAMP primer designation was limited to the same region as the real-time PCR. This could affect the sensitivity of the LAMP assay. Designing LAMP primers from different genes or different regions of the ICP4 gene could increase its sensitivity.

In summary, the real-time PCR and LAMP assays were specific, sensitive, and reproducible for ILTV detection. Although the sensitivity of LAMP was lower than that of real-time PCR, it is faster, has a lower cost, and does not require a temperature cycler or expensive real-time PCR equipment. This was the first report comparing these two methods for ILTV DNA detection.

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Table 3.1.

Real-time PCR primers and TaqMan probe sequences

Primers and probe (5'-3')	Length	Position [c]
ICP4 qPCR-F： CCCCACCCAGTAGAGGAC	18	143906-143923
ICP4 qPCR-R： CGAGATACACGGAAGCTGATTT	22	144010-144031
ICP4 Probe： FAM [A]-CAGTCTTTGGTCGATGACCCGC-TAMRA [B]	23	143949-143971

A. FAM, 6-carboxyfluorescein;

B. TAMRA, 6-carboxytetramethylrhodamine.

C. The position numbers of the primers and probe were obtained from GenBank accession #NC_006623.

Table 3.2.

Details of LAMP primer sequences for ILTV LAMP assay

Primer name	Primers sequence (5'-3')	Type	Length
ICP4- F3	CTGGAGAATGTCCCGATGTC	Forward outer	20
ICP4-B3:	TGGGGACGGGATAATAGCT	Reverse outer	19
ICP4-FIP (F1c+F2)	ACCGACCCGTCTGTACCGTCCTAGAGCCACTCTGGCGAG	Forward inner	39
ICP4-BIP (B1c+B2)	ATGTACTCTCACGAGCGTTGGCCTGGAACAAAAACGCGAGC	Reverse inner	41
ICP4-LF:	CGTTTCGACCCACTCCCT	Forward loop	18
ICP4-LB	GTCGACCTCCATAGTTCCGA	Reverse loop	20

Table 3.3.

Intra-experimental reproducibility of real-time PCR assay

Copy number	Mean Cp (cycles) [A]	SD[B]	SE[C]
10^9	10.26	±0.45	±0.22
10^8	13.26	±0.56	±0.27
10^7	16.24	±1.02	±0.51
10^6	18.97	±0.88	±0.44
10^5	22.66	±0.85	±0.42
10^4	25.67	±0.82	±0.41
10^3	28.99	±0.76	±0.38
10^2	32.40	±0.50	±0.25
10^1	34.90	±0.12	±0.06
10^0	>35	-[D]	-[D]

A. Average Cp value from four independent runs.

B. Standard deviations of Cp values.

C. Standard error of the mean Cp values.

D. One of four repeats was negative and the Cp values of other 3 repeats were higher than 35.

Table 3.4.

Statistics for standard curve and regression equations for real-time PCR [A]

	Repeat 1	Repeat 2	Repeat 3	Repeat 4	Mean	SD[C]
Intercept	38.49	38.31	37.41	38.90	38.28	±0.62
Slope	-3.202	-3.135	-3.064	-3.146	-3.137	±0.056
E	2.054	2.003	2.118	2.080	2.063	±0.048
R^2 [D]	0.9947	0.9974	0.9951	0.9960		

A. Cp= slope × log (genome number) + intercept

B. E=Efficiency= $10^{-1/slope}$

C: SD= Standard deviation

D: R^2=Correlation coefficient

Table 3.5A.

Correlation between ILTV viral genomic copy number and EID_{50}

	Titration1	Titration 2	Titration 3	Mean	SD^A
EID_{50}/ml	8.9×10^5	8.0×10^5	5.0×10^5		
Real-time PCR (copies/ml)	3.2×10^8	9.8×10^7	7.6×10^7		
Conversion factor (copies/EID_{50})	3.6×10^2	1.2×10^2	1.5×10^2	2.1×10^2	$\pm 1.3 \times 10^2$

1 $EID_{50} \approx (2.1 \pm 1.3) \times 10^2$ copies

A: SD= Standard deviation

Table 3.5B.

Correlation between vaccine dosage and ILTV viral genomic copy number

	4 doses of ILTV vaccine				
Real-time PCR (copies/4 doses)	2.4×10^6	3.1×10^6	2.8×10^6	Mean	SD^A
Conversion factor (copies/dose)	6.0×10^5	7.7×10^5	7.0×10^5	6.9×10^5	$\pm 0.85 \times 10^5$

1 dose $\approx (6.9 \pm 0.85) \times 10^5$ copies

A: SD= Standard deviation

```
                                ICP4 qPCR-F
ILT-vac-M : CCAGAAGCTATTATCCCGTCCCCACCCAGTAGAGGACAAGTATATCATGG 50
ILT-vac-V : CCAGAAGCTATTATCCCGTCCCCACCCAGTAGAGGACAAGTATATCATGG
CEO_ICP4  : CCAGAAGCTATTATCCCGTCCCCACCCAGTAGAGGACAAGTATATCATGG
TCO-ICP4  : CCAGAAGCTATTATCCCGTCCCCACCCAGTAGAGGACAAGTATATCATGG
Consensus : CCAGAAGCTATTATCCCGTCCCCACCCAGTAGAGGACAAGTATATCATGG

                           ICP4 probe
ILT-vac-M : AAGTGGCTATAGACAGTCTTTGGTCGATGACCCGCAGGTTCGGGAGGCCG 100
ILT-vac-V : AAGTGGCTATAGACAGTCTTTGGTCGATGACCCGCAGGTTCGGGAGGCCG
CEO_ICP4  : AAGTGGCTATAGACAGTCTTTGGTCGATGACCCGCAGGTTCGGGAGGCCG
TCO-ICP4  : AAGTGGCTATAGACAGTCTTTGGTCGATGACCCGCAGGTTCGGGAGGCCG
Consensus : AAGTGGCTATAGACAGTCTTTGGTCGATGACCCGCAGGTTCGGGAGGCCG

                                      ICP4 qPCR-R
ILT-vac-M : CGGCCGAATTCCATGCCAATCCGAAATCAGCTTCCGTGTATCTCGAAGAG 150
ILT-vac-V : CGGCCGAATTCCATGCCAATCCGAAATCAGCTTCCGTGTATCTCGAAGAG
CEO_ICP4  : CGGCCGAATTCCATGCCAATCCGAAATCAGCTTCCGTGTATCTCGAAGAG
TCO-ICP4  : CGGCCGAATTCCATGCCAATCCGAAATCAGCTTCCGTGTATCTCGAAGAG
Consensus : CGGCCGAATTCCATGCCAATCCGAAATCAGCTTCCGTGTATCTCGAAGAG
```

Fig 3.1. Multiple alignment analysis of particle ILTV ICP4 gene and positions of the real-time PCR primers and TaqMan probe.

```
ILT-vac-M : ACCTGGAGAATGTCCCGATGTCGCGCTAGAGCCACTCTGGCGAGTTTTGAGGGAGTGGGTCGAAACGGACGGTACAGACGGGTCGGTTCAGTCAGTAACCGCGCCT
ILT-vac-V : ACCTGGAGAATGTCCCGATGTCGCGCTAGAGCCACTCTGGCGAGTTTTGAGGGAGTGGGTCGAAACGGACGGTACAGACGGGTCGGTTCAGTCAGTAACCGCGCCT
CEO_ICP4  : ACCTGGAGAATGTCCCGATGTCGCGCTAGAGCCACTCTGGCGAGTTTTGAGGGAGTGGGTCGAAACGGACGGTACAGACGGGTCGGTTCAGTCAGTAACCGCGCCT
TCO-ICP4  : ACCTGGAGAATGTCCCGATGTCGCGCTAGAGCCACTCTGGCGAGTTTTGAGGGAGTGGGTCGAAACGGACGGTACAGACGGGTCGGTTCAGTCAGTAACCGCGCCT
Consensus : ACCTGGAGAATGTCCCGATGTCGCGCTAGAGCCACTCTGGCGAGTTTTGAGGGAGTGGGTCGAAACGGACGGTACAGACGGGTCGGTTCAGTCAGTAACCGCGCCT
               F3                 F2                      LF          F1c

ILT-vac-M : ACGGAGCTCCCAGACCTAGGCCGCGGCATTACCCTGAATGTACTCTCACGAGCGTTGGCCGTCGACCTCCATAGTTCCGAGGCTCGCGTTTTTGTTCCAGAAGCTA
ILT-vac-V : ACGGAGCTCCCAGACCTAGGCCGCGGCATTACCCTGAATGTACTCTCGCGAGCGTTGGCCGTCGACCTCCATAGTTCCGAGGCTCGCGTTTTTGTTCCAGAAGCTA
CEO_ICP4  : ACGGAGCTCCCAGACCTAGGCCGCGGCATTACCCTGAATGTACTCTCACGAGCGTTGGCCGTCGACCTCCATAGTTCCGAGGCTCGCGTTTTTGTTCCAGAAGCTA
TCO-ICP4  : ACGGAGCTCCCAGACCTAGGCCGCGGCATTACCCTGAATGTACTCTCACGAGCGTTGGCCGTCGACCTCCATAGTTCCGAGGCTCGCGTTTTTGTTCCAGAAGCTA
Consensus : ACGGAGCTCCCAGACCTAGGCCGCGGCATTACCCTGAATGTACTCTCaCGAGCGTTGGCCGTCGACCTCCATAGTTCCGAGGCTCGCGTTTTTGTTCCAGAAGCTA
                                                B1C                 LB              B2

ILT-vac-M : TTATCCCGTCCCCACCCAGTAGAGGACAAGTATATCATGGAAGTGGCTATAGACAGTCTTTGGTCGATGACCCGCAGGTTCGGGAGGCCGCGGCCGAATTCCATGC
ILT-vac-V : TTATCCCGTCCCCACCCAGTAGAGGACAAGTATATCATGGAAGTGGCTATAGACAGTCTTTGGTCGATGACCCGCAGGTTCGGGAGGCCGCGGCCGAATTCCATGC
CEO_ICP4  : TTATCCCGTCCCCACCCAGTAGAGGACAAGTATATCATGGAAGTGGCTATAGACAGTCTTTGGTCGATGACCCGCAGGTTCGGGAGGCCGCGGCCGAATTCCATGC
TCO-ICP4  : TTATCCCGTCCCCACCCAGTAGAGGACAAGTATATCATGGAAGTGGCTATAGACAGTCTTTGGTCGATGACCCGCAGGTTCGGGAGGCCGCGGCCGAATTCCATGC
Consensus : TTATCCCGTCCCCACCCAGTAGAGGACAAGTATATCATGGAAGTGGCTATAGACAGTCTTTGGTCGATGACCCGCAGGTTCGGGAGGCCGCGGCCGAATTCCATGC
             B3c
```

Fig 3.2. Partial ILT ICP4 gene sequences used to design LAMP primers.

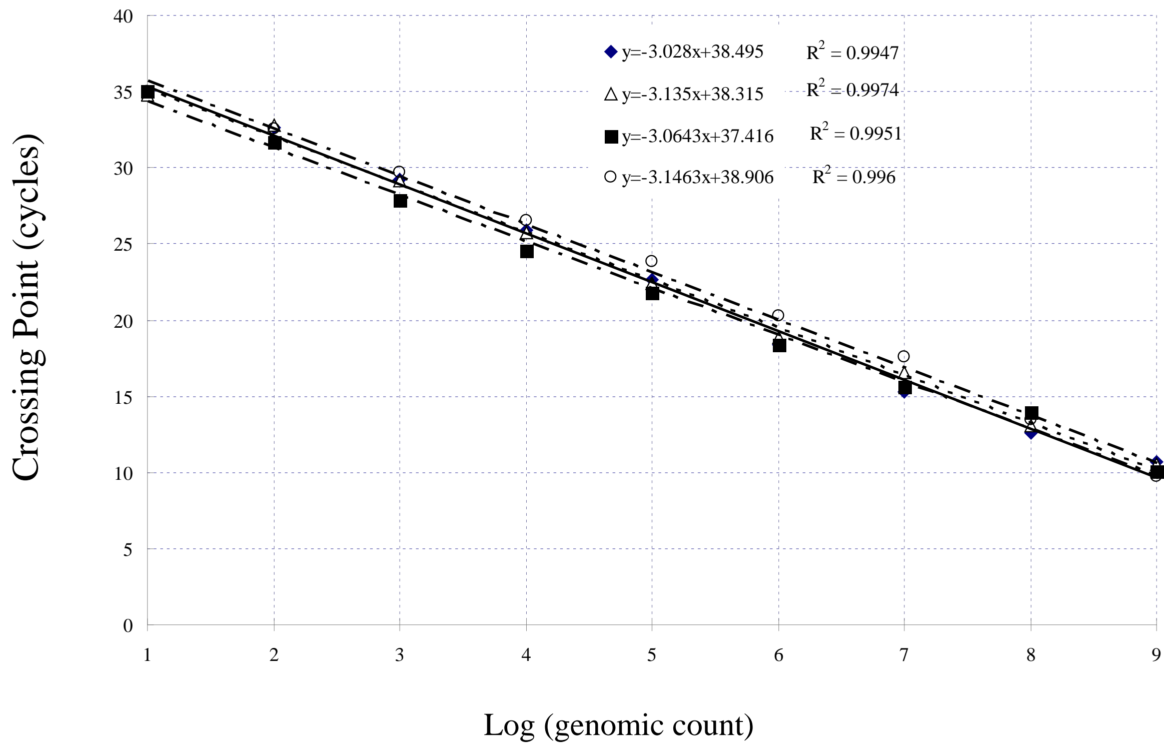

Fig 3.3. Standard curve (four repeats) of the TaqMan real-time PCR assay and regression equations. The slopes and intercepts were not significant different ($p > 0.05$).

x=Log (genomic count)

y=Crossing point

R^2=Correlation coefficient

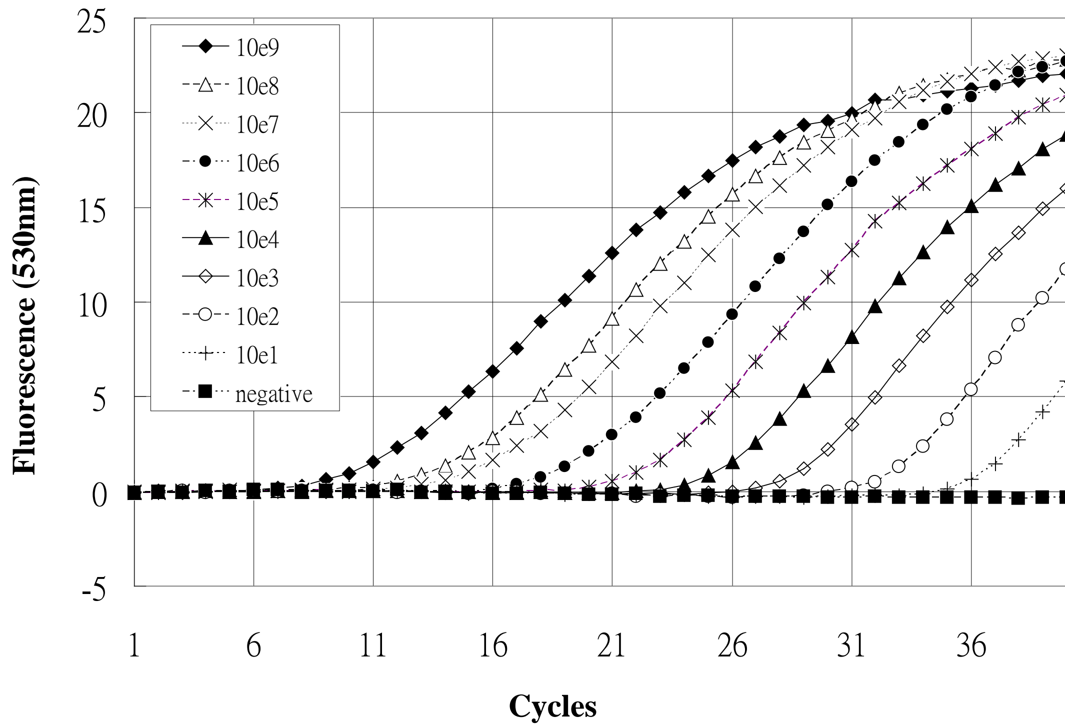

Fig 3.4. Amplification curves of serial 10-fold dilutions of cloned ILTV ICP4 genome. Legend indicates the number of copies of ICP4 DNA template.

e= exponent.

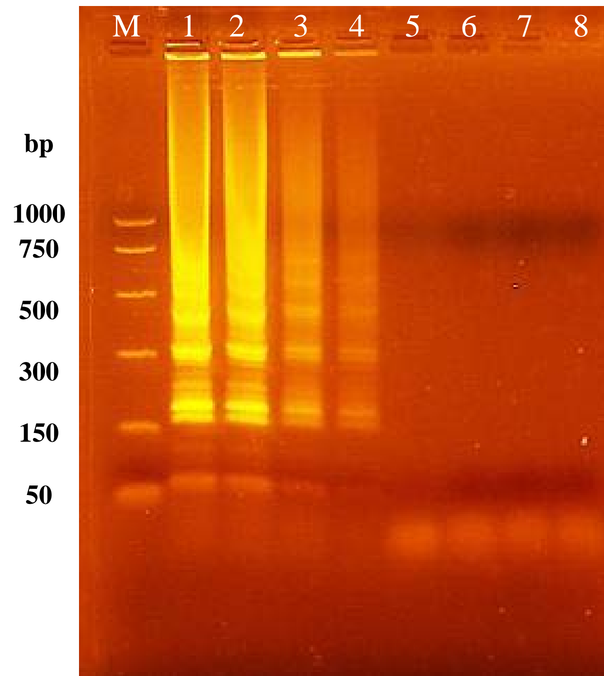

Fig 3.5. Sensitivity of LAMP for ILTV detection. Electrophoresis photo of serial 10-fold dilutions of ILTV ICP4 gene subjected to LAMP assay. The ILTV positive products in wells showed many bands with different sizes and a smeared DNA between these bands. The bands smaller than 50 bp were primer dimmers. M: DNA marker. 1: 6×10^4 ; 2: 6×10^3; 3: 6×10^2; 4: 60; 5: 30; 6: 15; 7: 6 copies/ µl; 8: negative control.

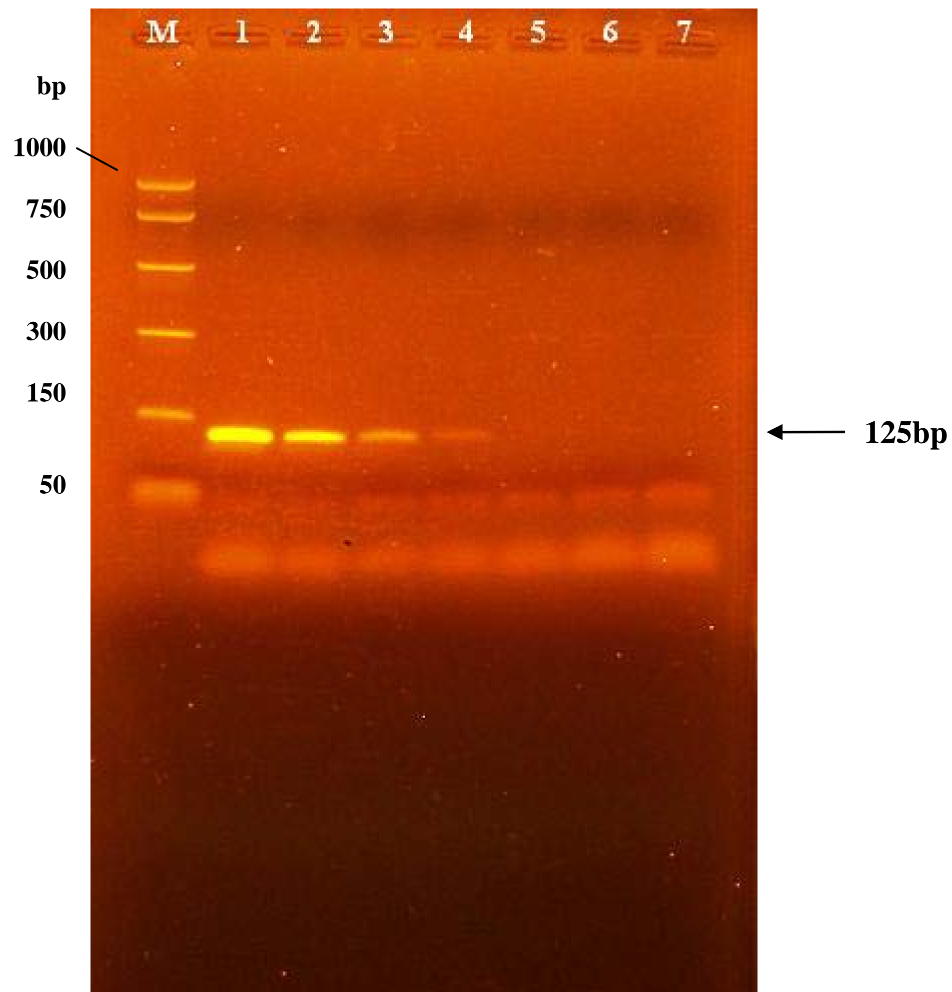

Fig 3.6. Photo of conventional PCR products. Serially dilution of ILTV ICP4 gene clones on 2% agarose gel indicated 125 bp target amplicon. M: marker; 1: 10^6 ; 2:10^5;3: 10^4; 4: 10^3; 5:10^2; 6: 10 copies/µl; 7: negative control.

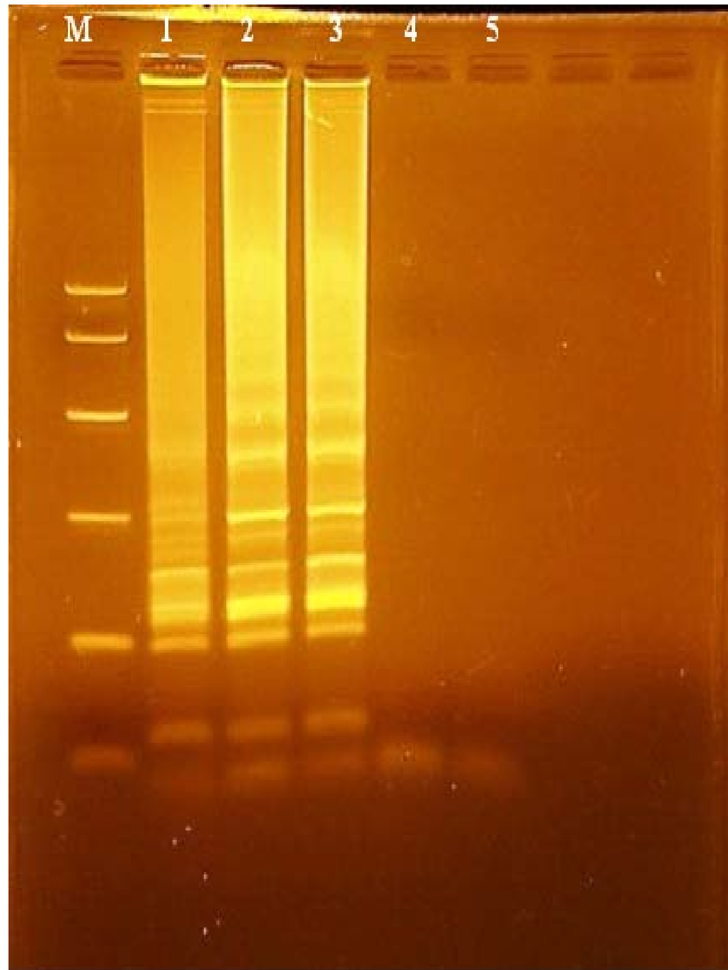

Fig 3.7. The optimal reaction temperature for LAMP reaction. 2% agarose gel electrophoresis demonstrating different reaction temperatures. M: marker: 1: 60℃; 2: 63℃; 3: 65℃. 4 and 5: negative controls.

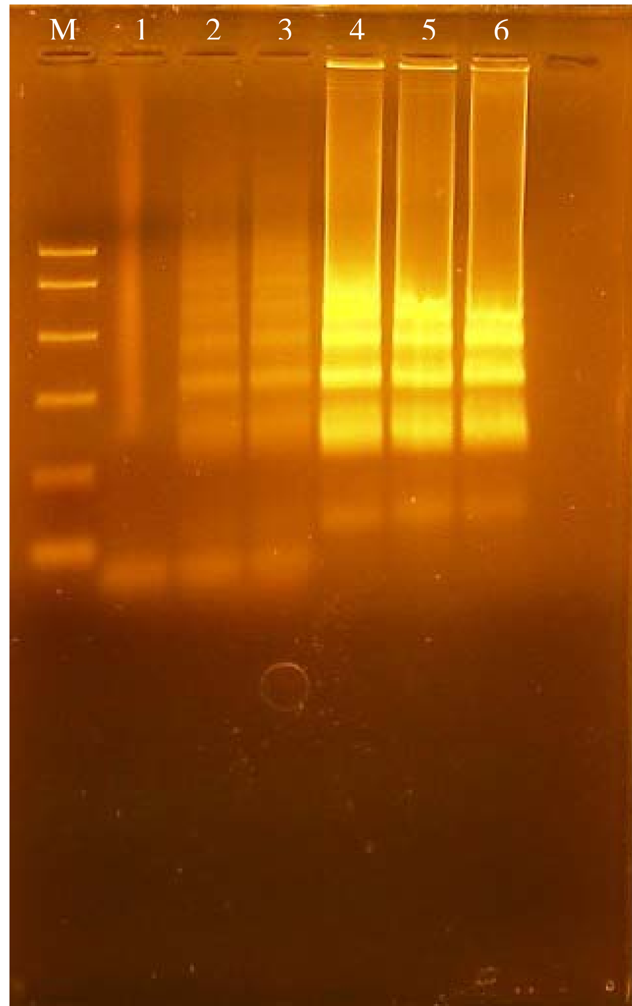

Fig 3.8. The effect of reaction time on the LAMP reaction. 2% agarose gel electrophoresis of LAMP reaction performed with different reaction time periods. M: DNA marker; 1: 15 min; 2: 25 min; 3: 35 min; 4: 45 min; 5: 50 min; 6: 60 min.

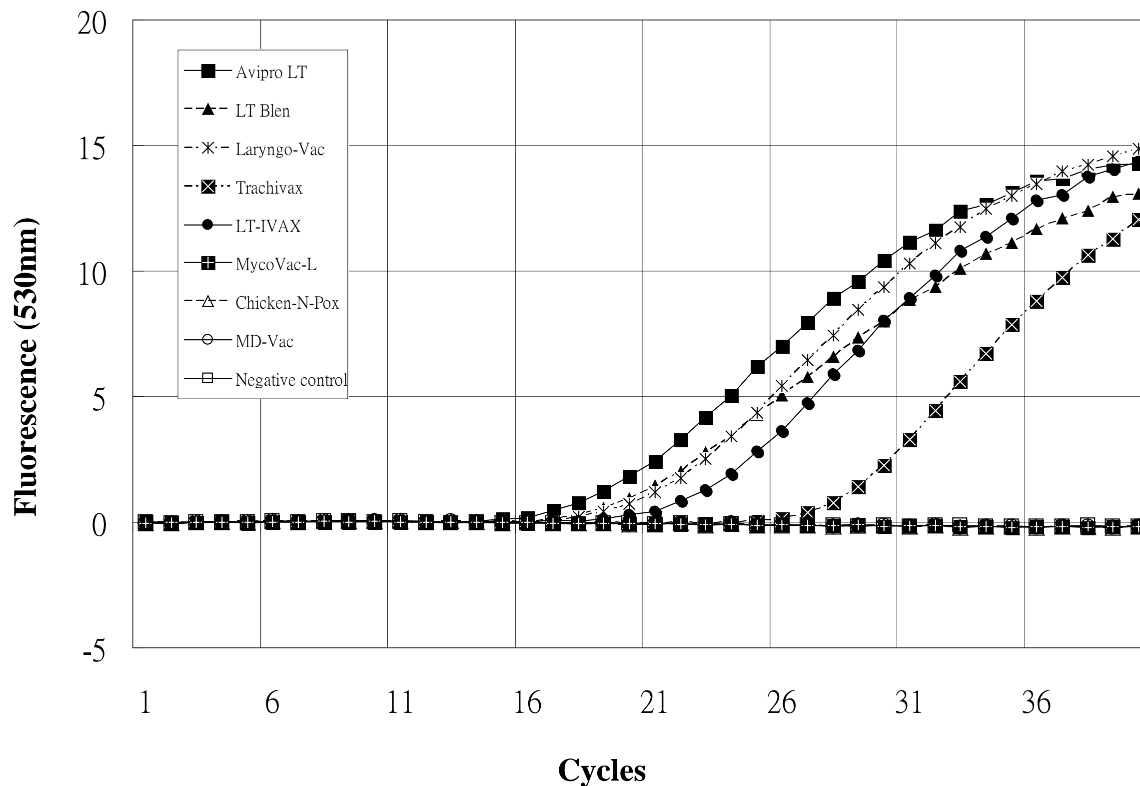

Fig 3.9. Specificity test of real-time PCR using four CEO vaccines: AviPro® LT, LT Blen®, Laryngo-Vac®, and Trachvax® and one TCO vaccine: LT-IVAX®, and non-ILTV DNA from MG vaccine (MycoVac-L®), fowl pox (Chicken-N-Pox™), and MD vaccine (MD-Vac®). The concentrations of ILT vaccine DNAs were different. There were no fluorescent signals from MG, flowpox virus, MDV vaccine DNAs and negative control.

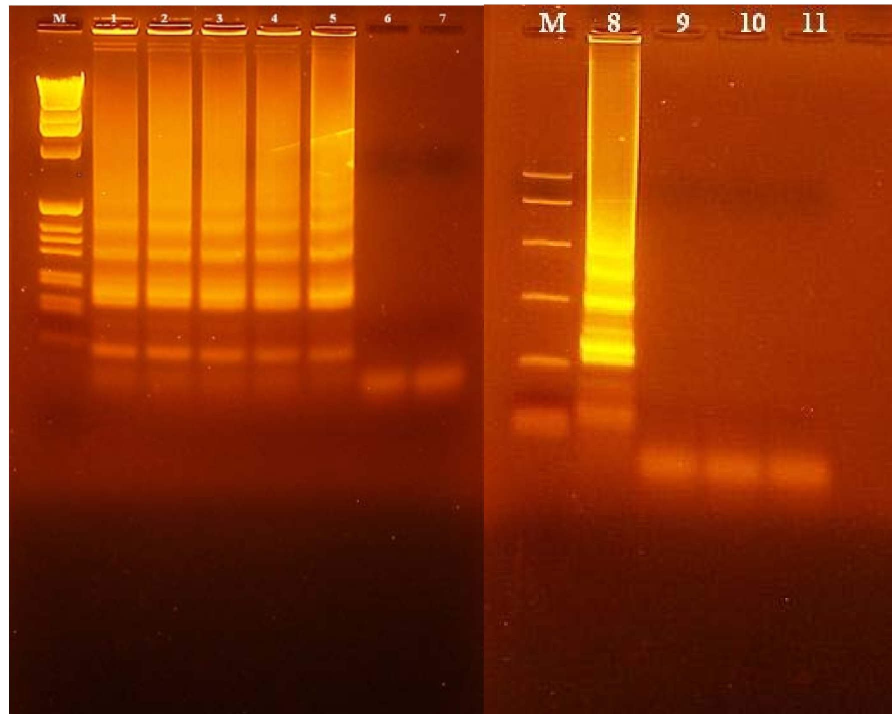

Fig 3.10. Specificity of LAMP assay. M: DNA marker; 1: AviPro® LT; 2: LT Blen®; 3: Laryngo-Vac®; 4: Trachivax®; 5: LT-IVAX®; 6: MycoVac-L®; 7: Negative control; 8: LT Blen®; 9: Chicken –N-Pox™ TC; 10: MD-Vac® CFL; 11: Negative control.

CHAPTER IV

STUDY II: DETECTION OF INFECTIOUS LARYNGOTRACHEITIS VIRUS IN POULTRY WATER LINES USING REAL-TIME PCR AND VIRUS ISOLATION AND EFFECTIVENESS OF CHEMICAL SANITIZERS IN REMOVING VIRAL CONTAMINATION

4.1 Introduction

Transmission of ILTV occurs via respiratory and ocular routes. Oral cavity is a route of infection, because the nasal epithelium cells can come in contact with the virus during ingestion. Mechanical transmission of ILTV occurs via contaminated equipment, human, and animals in chicken houses. Litter contaminated with ILTV can be disseminated via the wind (Kingsbury *et al.,* 1958; Johnson *et al.,* 2005). Although ILTV is heat sensitive, it can survive in deep litter and carcasses up to several months (Jordan, 1966). Therefore, rigorous biosecurity, sanitation, rapid detection, and vaccination are critical for ILT control.

Biofilm is a layer of microorganisms and their by-products can adhere to surfaces and form a matrix, which is a polysaccharide based (Costerton *et al.,* 1995). Organisms, which make up biofilms, can be homologous or heterogonous and may be symbiotic. Biofilms can attach to surfaces such as plastic, glass, metal, wood, and food. Biofilms can protect microorganisms from environmental, physical, and chemical exposure. Biofilms in water lines are difficult to

remove. Microorganisms can slough from the biofilms and be released into the water allowing spread to susceptible animals and humans (Costerton, 1999; Donlan and Costerton, 2002; Midelet and Carpentier, 2002). Biofilm may reduce the water line diameter and prevent distribution of vitamins, minerals, medication, and vaccines in the water.

In poultry farms, *Campylobacter jejuni* can be isolated from birds and the drinking water (Zimmer *et al.,* 2003). Strains of *Campylobacter jejuni* and *Pseudomonas spp*. can be isolated from water systems of chicken houses. In laboratory tests, bacteria can attach to pre-existed biofilm on the polyvinyl chloride (PVC) surface and cannot be completely inactivated by sanitizers (Trachoo *et al.,* 2002a and 2002b). *Escherichia coli* O157:H7 produced biofilms on glass and stainless steel, and the bacteria were difficult to remove when present on food surfaces (Silagyi *et al.,* 2009). *Salmonella* from commercial poultry farms cannot be eliminated in biofilms using water line disinfectants (Marin *et al.,* 2009). Biofilms in poultry houses allowed multiplication of pathogenic and opportunistic pathogens, such as *E. coli*, *Mycobacterium*, *Campylobacter*, *Salmonella*, and *Pseudomonas* (Engel *et al.,* 1980; Zimmer *et al.,* 2003; Gradel *et al.,* 2004). Virus transmission via drinking water systems is an important source for dissemination. Poliovirus can attach to biofilms in the water system and be recovered from the water flow (Quignon *et al.,* 1997). Leung *et al.,* (2007) reported that H9N2 avian influenza virus (AIV) was isolated from drinking water in chicken farms and the isolation rate in drinking water reached 7% of 2503 water samples (Leung *et al.,* 2007). In a laboratory experiment, H5N1 AIV infected ducks released virus to drinking water and the virus was transmitted to uninfected ducks via the drinking water (Sturm-Ramirez *et al.,* 2004).

In prior studies, the efficacy of water line sanitizers for poultry farms was tested to remove pathogens from biofilms. However, some routinely used water line sanitizers were not satisfied

after treated. Citric acid is commonly used for water treatment. It can quickly decrease the pH of water. However, citric acid is not an antimicrobial chemical, because it cannot completely kill bacteria, fungi, and algae (Hancock *et al.,* 2007). However, it can remove mineral deposits in water system. Sodium hypochlorite is widely used for water disinfection. It dissociates $HOCl^-$ and OCl^-, which can penetrate the cells of microorganisms. Sodium hypochlorite eliminates free microorganisms, but cannot remove bacteria in biofilm. Moreover, high concentrations of sodium hypochlorite will damage the water line whereas at low concentration the bactericidal efficiency is not satisfactory (Hancock *et al.,* 2007). Hydrogen peroxide sanitizer is a weak acid and non-corrosive oxidizer. Concentrations of hydrogen peroxide sanitizers, commonly used for water disinfection, range from 16 to 34%. The 50% H_2O_2 products are used in removing biofilm from water systems between flocks (Carpentier and Cefr, 1993; Clark *et al.,* 2009). Commercial sodium hydrogen sulfate is used to remove water line scale and sanitize by reducing the water pH. It penetrates the biofilm and dissolves the scale.

Drinking water was suspected to transmit ILTV in the field; however, it was not proven. Drinking water is a common method to administer ILT vaccines. However, routinely used disinfectants might not completely inactivate vaccine virus in water lines. Furthermore, ILT vaccine virus can revert to high virulence via bird-to-bird transmission (Guy *et al.,* 1991; Kotiw and May, 1995). Vaccine residues in drinking water might provide a source for bird-to-bird transmission and an increase in viral virulence.

In the current study, a commercial CEO produced ILTV vaccine was placed in water lines of isolation units. SPF chickens were placed in these units. Swab samples from water lines and

chicken trachea were examined for ILTV DNA by real-time PCR and ILTV viral isolation. Four sanitizers were used to determine their efficiency in removing ILTV from biofilms.

4.2 Materials and methods

ILTV preparation and water line inoculation

ILT produced in CEO -- LT Blen® (Merial Select Inc., Gainesville, GA) was added to a water bottle and diluted to 1,000 doses. Virus dilution was followed according to the manufacturer's directions. The bottle was connected to the water system and 7 g of skim milk powder for every liter of tap water was added to the water system as a buffer to protect vaccine virus. The water line was 3 m in length, 2.54 cm inside diameter and biofilm has built in it. The vaccine was maintained in the bottle and water lines for 72 hours. Negative controls were in a different unit and received only tap water.

Experiment design 1

ILTV detection in water lines

Since other pathogens were found to attach to biofilm, we wanted to verify that ILTV could attach to biofilm in the water system and be transmitted to chickens. A pre-test was done without sanitizers. After ILTV vaccine was in the water systems, the bottle and water lines were flushed three times with 7.56 liters of tap water. Three 3-week-old specific pathogen free (SPF) broiler chickens were placed in the Horsfall-Bauer isolation units, which were housed in the biocontainment facility of the Department of Poultry Science. Feed and water were provided *ad libitum*. This pre-test was simultaneously repeated 3 times in the different units.

Swabs from the trachea and nipple drinkers were collected on the 1st, 5th, and 21st day after bird placement. On the 21st day, all birds were killed, and trachea and nipple drinker swabs collected. Each swab was immersed in 2 ml brain heart infusion broth (BHIB) media, which contained 1% (w/v) streptomycin and 0.6% (w/v) penicillin-G to eliminate bacterial contamination. Swabs were kept on ice during transportation from the biocontainment facility to the laboratory. DNA extracted from swabs, drinkers and, tracheae were detected by real-time PCR. All birds used in this study were reared according to the University's Institutional Animal Care and Use Committee (IACUC) standards.

Experiment 2

Viral Disinfection procedures

Drinkers and bottles connected to the water lines were inoculated with 1,000 doses of the LT Blen® vaccine virus and washed with tap water as described in experiment 1. Four water sanitizers were added into the water system of separate units: the first sanitizer was 12.5% sodium hypochlorite, which was diluted to 5.25% for stock solution, and then, 4 ounces of stock solution was added to tap water per gallon that was equal to a final concentration of 0.19 ml/L. The second sanitizer was citric acid. Sixty four ounces of citric acid were added to 1 gallon of water for a stock solution. One ounce of stock citric acid was added to 1 gallon of water, which produced a final concentration of 3.05 ml/L. Sodium hypochlorite and citric acid concentrations were according to the study of Watkins *et al.* (Watkins *et al.,* 2004). These chemicals are routinely used in farms for water line sanitation, because they are less expensive than other sanitizers. The following 2 commercial sanitizers, designed for removing biofilms, were administered following the manufacturer's directions. Sodium hydrogen sulfate (pH water

treatment, PWT®, Jones-Hamilton Co., Walbridge, OH) was added 16 oz/gal of tap water for a stock solution, and then, 1 ounce of stock solution was added to 1 gallon of tap water that was equal to a final concentration of 0.309 ml/L. 3.84 ounces of 50 % hydrogen peroxide (Proxy-Clean®, Kanters Special Products USA, St. Paul, MN) was added into per gallon of tap water, which was equal to a final concentration of 30.03 ml/L. The positive control unit received only vaccine and was washed with water. The negative control unit did not receive vaccine virus nor sanitizer. Sanitizers were maintained in the water systems for 24 hours. At that time, water lines were flushed with tap water for 3 minutes. Four 3-week-old chickens were placed in each unit, and swabs from biofilms in the water lines, tracheae, and nipple drinkers were collected on the 7th and 14th days after the chickens were placed in the units. On the 21st day, chickens were killed and the tracheae, biofilm, and nipple drinker swabs were collected. Swabs were subjected to virus isolation in SPF embryos followed by ILTV DNA detection with real-time PCR.

Swab and trachea sample preparation

Swabs from water lines, drinkers, and trachea were stirred in 2 ml of brain heart infusion broth (BHIB) to release viral particles. Collection tubes containing released material were centrifuged at 2,000 x g for 10 min to precipitate large particles. The tracheae were placed in sterile disposable centrifuge tubes containing 5 ml of sterile phosphate buffered saline (PBS), which contained 1% (w/v) streptomycin and 0.6% (w/v) penicillin-G and then homogenized. Samples were centrifuged at 2,000 x g for 10 min. Supernatants from swabs were transferred to 2 ml plastic tubes and stored at -80˚C.

Virus isolation

Samples were inoculated into 9-to-11-day-old embryos via CAM route. Eggs were incubated at 37°C and candled daily for 7 days. During incubation, eggs with dead embryos were chilled at 4°C. After 7 days of incubation, eggs with live embryos were chilled at 4°C overnight. CAMs were examined for plaques and thickening and harvested and homogenized in PBS according to Senne (Senne, 2008). If no plaques were observed, homogenized CAMs were re-passaged in embryos as before. A total of 3 consecutive passages were performed for the negative samples. CAMs with plaques processed for viral identification by real-time PCR.

Viral DNA extraction

Total DNA from swabs, tracheae, and CAMs were extracted using the Qiagen DNeasy® Blood & Tissue Kit (Qiagen, Valencia, CA, USA). Briefly, 200µl of sample suspension were mixed with 20µl proteinase K and 200µl of Buffer AL. Mixtures were mixed and incubated at 56°C for 10 min. Two hundred µl of 100% ethanol were added and vortexed. The mixtures were transferred into the DNeasy Mini spin column and centrifuged at 6000 x g for 1 min and the flow-through discarded. The columns were placed in a 2 ml collection tube, and 500µl of Buffer AW1 was added. The tubes were centrifuged at 6000 x g for 1 min, and the flow-through discarded. The columns were transferred into a new 2 ml collection tube, and 500µl of Buffer AW2 was added. The tubes were centrifuged at 20,000 x g for 3 min to remove excess reagents from the column membrane. Finally, the DNA was eluted in 100µl Buffer AE by centrifuging at 6000 x g for 1 min and stored at -20°C.

Real-time PCR

Real-time PCR primer and a TaqMan® probe sequences are listed in Table 4.1. The real-time PCR targeted the conserved region of ILTV ICP4 gene. Real-time PCR was performed with a LightCycler® (Roach, Applied Science, Indianapolis, IN) using 20 µl in volume, containing 5µl of DNA samples obtained from swabs, tracheae, and CAMs. For real-time PCR assay, each reaction contained 10µl of 2X master mix (QuantiTect® Probe PCR kit, Qiagen, Valencia, CA), 1 µl of 10 µM each primer (0.5 µM), 0.5 µl of 4 µM probe (0.1 µM), 2.5 µl of water, and 5µl of DNA template. The real-time PCR was subjected to 95°C initial activation for 15 minutes, and 40 cycles for 95°C denaturation 0 second and 60°C combined annealing and extension for 60 seconds.

Statistical analysis

Real-time PCR data were subjected to Chi-square tests to differentiate ILTV DNA positive and negative rates between treatment groups.

4.3 Results

Experiment 1

The ILTV DNA detection showed that one swab from nipple drinkers was positive for ILTV on the 1st day. On the 5th and 21st days, samples from nipple drinkers were positive. Results indicated that the water lines maintained ILTV in the biofilms and released virus in the water lines for several weeks. One of the trachea swabs, which was collected from the 1st day, was positive for ILTV DNA. On the 5th and 21st days, the ILTV DNA positive rates, from swab and

homogenized trachea, were about 77% and 55% respectively. The ILTV DNA positive rate for swabs, collected from the 5th and 21st days, were significantly different from the negative controls ($p<0.05$) (Table 4.2). Highest ILTV DNA concentration was on the 5th day, when tracheal samples reached approximately 1071 copies and the drinker samples reached about 427 copies.

Experiment 2

Efficacy of sanitizer treatment

After sanitizer treatment, swabs collected from the trachea on the 1st day of bird placement were negative for ILTV DNA. However, swabs from nipple drinkers in the citric acid treated group were ILTV DNA positive using real-time PCR detection at this time. However, on the 7th, 14th and 21st days, positive rates for ILTV DNA in the citric acid and sodium hypochlorite treated units were significantly different from the negative controls ($p<0.05$). The drinkers from these two groups contained ILTV DNA. However, the sodium hydrogen sulfate treated group had one of four tracheal swabs, which were positive for ILTV DNA on the 14th and 21st days. Swabs from drinkers treated with this chemical were negative for ILTV DNA. In the hydrogen peroxide treated group, only one of four swabs from the trachea was positive for ILTV DNA. The drinker and biofilm samples were negative for viral DNA on the 7th and 14th days. However, on the 21st day, the tracheal and biofilm samples were positive for ILTV DNA (Table 4.3).

Virus isolation

Positive controls, which were not treated with sanitizers, contained ILTV in the biofilms and drinkers from the 7th to 21st days. Tracheae also contained ILTV on the 14th and 21st days. The

ILTV could be isolated in the biofilm and tracheae, which were treated with sodium hypochlorite and citric acid, on the 14th and 21st days. Swabs from the drinkers treated with citric acid contained ILTV on the 7th day. However, ILTV could not be isolated from the biofilm, drinkers, and tracheal samples in the sodium hydrogen sulfate and hydrogen peroxide treated groups after three embryo passages (Table 4.4).

4.4 Discussion

In the current study, ILTV DNA and live virus were detected in the biofilm of water lines 72 hours after being exposed and was spread to susceptible birds for at least 3 weeks. The ILTV was re-isolated from the ILTV-attached biofilms and chickens, which consumed the water from ILTV-contaminated water systems.

The present study showed that ILTV was isolated in chicken embryos from water lines treated with sodium hypochlorite and citric acid and was transmitted to chickens from 14 to 21 days after the water systems were treated. Citric acid and sodium hypochlorite could not penetrate or remove the biofilm and ILTV in the water lines. In the sodium hydrogen sulfate and hydrogen peroxide treated groups, ILTV was not re-isolated from the water lines or tracheal samples. However, some water and trachea swab samples were positive for ILTV DNA by real-time PCR. Either live virus concentrations were too low to be detected in embryos or the viruses were inactivated with sodium hydrogen sulfate and hydrogen peroxide. However, the DNA was still amplified *in vitro*.

The TaqMan® probe based real-time PCR readily detected ILTV DNA in water and tracheal samples containing biofilm. ILTV vaccine attached to the biofilm of the drinking water was

released though the water. Water lines in chicken houses may be an important source for re-infection of newly placed flocks with pathogenic organisms. ILTV vaccine that is administered in the water may remain in the system as shown herein. Citric acid and sodium hypochlorite were not adequate for removal of ILTV vaccine. In contrast, sodium hydrogen sulfate and hydrogen peroxide removed ILTV. However, poultry farmers may need more than one treatment with these sanitizers for complete removal of biofilm and inactivation of microorganisms in the drinking water system.

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Table 4.1

Real-time PCR primers and TaqMan probe sequences

Primers and probe(5’-3’)	Length	Position [c]
ICP4 qPCR-F： CCCCACCCAGTAGAGGAC	18	143906-143923
ICP4 qPCR-R： CGAGATACACGGAAGCTGATTT	22	144010-144031
ICP4 Probe： FAM [A]-CAGTCTTTGGTCGATGACCCGC-TAMRA [B]	23	143949-143971

A. FAM, 6-carboxyfluorescein.

B. TAMRA, 6-carboxytetramethylrhodamine.

C. The position numbers of the primers and probe were obtained from GenBank accession #NC_006623.

Table 4.2

Rates for ILTV DNA detection in the pre-test by real-time PCR.

Treatment	Samples	1st Day	5th Day	21st Day
Positive [D]	Drinkers	1/3[A]	3/3	3/3
	Group 1 trachea swabs	0/3	3/3	1/3
	Group 2 trachea swabs	0/3	2/3	3/3
	Group 3 trachea swabs	1/3	2/3	1/3
	Sum of positive	1/9	7/9[C]	5/9[C]
Negative control [E]	Drinker	-[B]	-	-
	Trachea swabs	0/3	0/3	0/3

On the 1st and 5th days after chicken placement, swabs were collected from the trachea. On the 21st day, chickens were killed and drinker and homogenized tracheal samples were subjected to real-time PCR.

A. Positive sample number over total number of samples.

B. The sample was negative with real-time PCR.

C. ILTV DNA positive rates within the same column were significantly different from the negative controls by Chi-square test ($p<0.05$).

D. Water lines received vaccine.

E. Water lines received no vaccine

Table 4.3

ILTV DNA detection by real-time PCR after four sanitizer treatments

Sanitizer	Sample	1st Day	7th Day	14th Day	21st Day
Sodium hypochlorite	Trachea	0/4 [A]	3/4[B]	3/4[B]	2/4
	Drinker	- [C]	+[D]	+	+
	Biofilm	N.D. [E]	N.D.	N.D.	+
Citric acid	Trachea	0/4	2/4	3/4[B]	2/4
	Drinker	+	+	+	+
	Biofilm	N.D.	N.D.	N.D.	+
Sodium hydrogen sulfate	Trachea	0/4	0/4	1/4	1/4
	Drinker	-	-	-	-
	Biofilm	0/2	1/2	0/2	1/2
Hydrogen peroxide	Trachea	0/4	1/4	1/4	3/4[B]
	Drinker	-	-	-	-
	Biofilm	0/2	0/2	0/2	1/2
Positive control [F]	Trachea	2/4	3/4[B]	3/4[B]	2/4
	Drinker	+	+	+	+
	Biofilm	2/2[B]	2/2[B]	2/2[B]	2/2[B]
Negative control[G]	Trachea	0/4	0/4	0/4	0/4
	Drinker	-	-	-	-
	Biofilm	0/2	0/2	0/2	0/2

A. Positive sample number over total number of samples.

B. The ILTV DNA positive rates within the same column were significantly different from the negative controls by Chi-square test ($p<0.05$).

C. The sample was negative with real-time PCR.

D. The sample was negative with real-time PCR.

E. Not done.

F. Water lines received vaccine, but no sanitizer.

G. Water lines received no vaccine or sanitizer.

Table 4.4

Viral isolation from the trachea, drinker, and biofilm samples in SPF embryonating eggs after sanitizer treatments

Sanitizer	Sample	1st Day	7th Day	14th Day	21st Day
Sodium hypochlorite	Trachea	- [A]	-	+ [B]	+
	Drinker	-	-	+	+
	Biofilm	N.D. [C]	N.D.	N.D.	+
Citric acid	Trachea	-	-	+	+
	Drinker	-	+	+	+
	Biofilm	N.D.	N.D.	N.D.	+
Sodium hydrogen sulfate	Trachea	-	-	-	-
	Drinker	-	-	-	-
	Biofilm	-	-	-	-
Hydrogen peroxide	Trachea	-	-	-	-
	Drinker	-	-	-	-
	Biofilm	-	-	-	-
Positive control [D]	Trachea	-	-	+	+
	Drinker	-	+	+	+
	Biofilm	+	+	+	+
Negative Control [E]	Trachea	-	-	-	-
	Drinker	-	-	-	-
	Biofilm	-	-	-	-

A. Negative for virus isolation.

B. Positive for virus isolation.

C. Not done.

D. Water lines received vaccine, but no sanitizer.

E. Water lines received no vaccine or sanitizer.

CHAPTER V

STUDY III: DETECTION OF INFECTIOUS LARYNGOTRACHEITIS VIRUS FROM DARKLING BEETLES AND ITS IMMATURE STAGE (LESSER MEALWORM) AND A RODENT BY REAL-TIME PCR AND VIRUS ISOLATION

5.1 Introduction

ILTV causes significantly economic losses. The clinical signs of ILT are observed 6-12 days post infection (PI); however, the incubation period is shorter by experimental intra-tracheal infection (Kernohan, 1931; Jordan, 1963). Clinical characteristics of ILT include nasal discharge, conjunctivitis, reduced egg production and shell quality, decreased weight gain, and increased condemnation rate. Severe form of ILT causes bloody mucus in trachea and increased mortality (Bagust *et al.,* 2000; Guy *et al.,* 2008).

ILTV can establish latent infections in chickens. The virus may escape to the chicken nervous systems and the birds become carriers. When birds are stressed, ILTV can re-activate and be transmitted to susceptible birds (Williams *et al.,* 1992). Affected birds, contaminated dust, litter, and fomites are sources for ILTV transmission. Control of ILTV requires rigorous biosecurity. ILTV in a contaminated house can be controlled by in-house litter composting, poultry litter treatments, and heating the house to 100°F for 24 hours (Giambrone *et al.*, 2008).

The lesser mealworm (*Alphitobius diaperinus* Panzer) and its adult, the darkling beetle, is a cosmopolitan insect in poultry facilities. They, as well as rodents, are omnivorous scavengers that consume feed, water, poultry carcasses, and feces. They are nocturnal and live in compacted earth and litter and can destroy poultry building insulation and other structures. They are difficult to eliminate (Axtell *et al.,* 1990; Rueda *et al.,* 1997; Skov *et al.*, 2004). Farms can have economical losses associated with darkling beetles and rodents, which include house damage, pest control expenses, and decreased feed conversion. Birds may consume beetles rather than feed.

Darkling beetles and rodents can transmit avian pathogens. Rodents spread colibacillosis, pasteurellosis, mycoplasmosis, and infectious bursal disease (IBD) (Garber *et al.,* 2003; Koshimizu *et al.,* 1993; Webster and MacDonald, 1995; Stojcevic *et al.,* 2004; Seong *et al.,* 1995; Okoye and Uche, 1986; Park *et al.,* 2010). Rodents can spread protozoa and helminths. *Salmonella*, *E. coli*, *Campylobacter*, and fungi have been isolated from larvae and adult beetles from turkey farms (De las Casas *et al.,* 1972). Skov *et al.* in 2004 reported that the darkling beetle was a reservoir for *Salmonella enterica* and *Camplyobacter spp*. (Skov *et al.,* 2004). In a laboratory experiment, 10-day-old chicks received one larva or adult beetle contaminated with *Salmonella* by gavage, and bacteria were re-isolated at 48 hours from the birds (McAllister *et al.,* 1994). Darkling beetles are a vector for bacterial pathogens on poultry farms (Skov *et al.,* 2004; Templeton *et al.,* 2006; Hazeleger *et al.,* 2008). Darkling beetles transmitted avian diseases, such as avian leucosis, MDV, fowl pox, avian reovirus, IBD, Newcastle disease virus (NDV), fungi, *Eimeria*, and bacterial pathogens (De Las Casas *et al.,* 1973, 1976; Goodwin, 1996; McAllister *et al.,* 1995; Eidson *et al.,* 1996). Darkling beetles harbored turkey enterovirus and rotavirus (Despins and Axtell, 1994). McAllister *et al.* (1995) fed beetles containing IBDV and beetle

surfaces were treated with H_2O_2. The IBDV was recovered from these beetles 14 days after they ingested the virus. Virus was isolated from the intestinal tracts of the lesser mealworms 10 days of incubation. Snedeker *et al.* (1967) reported that chickens fed beetle homogenates, which came from an IBDV infected farm, developed IBD associated clinical signs and that beetles carried IBDV for at least 56 days. These studies indicated that beetles serve not only as fomites carrying the viruses on their outside surfaces, but also harbor viruses internally.

In the current study, beetles and their larvae were collected from two commercial broiler farms, which were diagnosed with a mild vaccinal ILTV outbreak. Beetles and larvae from the farms and a rodent from one of the ILTV positive farms were investigated for ILTV with real-time PCR and viral isolation. Darkling beetles and larvae from the infected houses contained live ILTV and the rodent had ILTV DNA in its lung. Since ILTV is endemic, it is important to determine carriers and sources for transmission.

5.2 Materials and methods

Darkling beetles and larvae

Darkling beetles and their larvae were collected from two poultry farms determined to have vaccinal ILTV outbreaks by the Alabama State Veterinary Laboratory in Auburn, Alabama. Beetles and their larvae were randomly collected from 3 locations in the chicken houses (front, middle, and back). A rat was trapped and killed in one of the ILTV positive houses on an ILTV infected farm on the 17th day after the outbreaks were confirmed. Beetles and larvae in ILTV non-infected houses of both farms were also collected for testing. The beetles and larvae were

killed by placing them into a clean plastic bag and storing these bags at -80˚C. The rat was killed by cervical dislocation and the organs were stored at -80˚C.

Sample preparation

Darkling beetles and larvae were separated into two groups. In one group, the insect surfaces were treated with 10% hydrogen peroxide to remove surface microbes, and the other group was not. The cleaning surface procedure was prepared as previously described by McAllister *et al.* (1995). Eighty darkling beetles and larvae were placed in 30 ml of 10% hydrogen peroxide and agitated to ensure complete coverage for 1 minute. This was done to inactivate the microorganisms on the surface of the insects. The insects were then moved to a container having sterile water and rinsed for 30 seconds. The water rinse was repeated three times and the insects were placed into a 50-ml sterile disposable centrifuge tube and suspended in 10 ml of sterile phosphate buffered saline (PBS), which contained penicillin (2000 unit/ ml), streptomycin (2 mg/ ml), gentamicin (50 ug/ ml), and mycostatin (1000 unit/ ml) to prevent bacteria and fungal contamination. Insects were homogenized and centrifuged at 2,000 xg for 10 min and the pellet discarded. Supernatants were transferred to 2 ml plastic tubes and stored at -80˚C. The darkling beetles and larvae of the second group were not treated with 10% hydrogen peroxide and were processed as described previously.

The lung, kidney, and liver of the rat were excised aseptically. The organs were placed in sterile disposable centrifuge tubes containing 2 ml of sterile PBS, which contained penicillin (2000 unit/ ml), streptomycin (2 mg/ ml), gentamicin (50 ug/ ml), and mycostatin (1000 unit/ ml), and homogenized. The homogenized organs were processed as previously described. Organ supernatants were transferred to 2 ml plastic tubes and stored at -80˚C.

DNA extraction

Total DNA from homogenized rat organs, beetles, and larvae were extracted using Qiagen DNeasy® Blood & Tissue Kit (Qiagen, Valencia, California, USA) according to the manufacturer's directions. Briefly, 200µl of sample suspensions were mixed with 20µl proteinase K and 200µl of Buffer AL. The mixtures were mixed and incubated at 56°C for 10 min and then 200µl of 100% ethanol was added and vortexed. The mixtures were transferred into the DNeasy Mini spin column and centrifuged at 6000 x g for 1 min and the flow-through discarded. The columns were placed in a 2 ml collection tube, and 500µl of Buffer AW1 added. Tubes were centrifuged at 6000 x g for 1 min, and the flow-through discarded again. The columns were transferred into 2 ml collection tubes and 500µl of Buffer AW2 added. The tubes were centrifuged at 20,000 x g for 3 min to remove excess reagents from the column membrane. Finally, the DNA was eluted in 100µl of Buffer AE by centrifuging at 6000 x g for 1 min and stored at -20°C.

Real-time PCR

A real-time PCR was developed with specific primers and a TaqMan® probe (Table5.1) for ILTV detection. The real-time PCR targeted the conserved region of ILTV ICP4 gene and was performed in a LightCycler® (Roach, Applied Science, Indianapolis, IN) with a 20 µl reaction volume, containing 5µl DNA samples from homogenized beetles, larvae, rat organs and CAMs. Each reaction contained 10µl of 2X master mix (QuantiTect® Probe PCR kit, Qiagen, Valencia, CA), 1 µl of 10 µM each primer (0.5 µM), 0.5 µl of 4 µM probe (0.1 µM), 2.5 µl of water, and 5µl of DNA template. The real-time PCR program was as follows: 95°C initial activation for 15

minutes, and 40 cycles for 95°C denaturation at 0 second and combined annealing and extension at 60°C for 60 seconds.

Virus isolation

The 0.2 ml homogenized samples of beetles, larvae, and rat organs obtained from ILTV infected and non-infected houses were inoculated into 9-to-11-day-old specific pathogen free (SPF) chicken embryos via CAM route. Eggs were incubated at 37°C and candled daily for 7 days. During incubating, eggs with dead embryos were chilled at 4°C. After 7 days of incubation, eggs with live embryos were chilling at 4°C overnight. The CAMs were examined for the presence of plaques and thickening. The CAMs were harvested and homologized in PBS. If no plaques were observed, CAMs were harvested and prepared for re-passage in SPF embryos. Three consecutive passages were performed for the ILTV negative samples. The CAMs with plaque formations were processed by real-time PCR.

Identification of ILTV

PCR followed by restriction fragment length polymorphism (RFLP) determined the genomic type of the virus and determined if the virus was of vaccine origin. The PCR-RFLP test was performed following the method described by Chang *et al.* (Chang *et al.,* 1997). The 4.9 kb ILTV ICP4 gene PCR products were amplified from total DNAs of a TCO (LT-IVAX® , Schering-Plough Animal Health Corp., Kenilworth, NJ) and 2 CEO (LT Blen® Merial Select Inc., Gainesville, GA and Trachivax® Schering-Plough Animal Health Corp., Kenilworth, NJ) vaccines, beetles, and rat lung with the primers. PCR was performed using the Expand High Fidelity PCR System kit (Roach, Applied Science, Indianapolis, IN). Fifty µl for PCR were

processed as follows: 5 µl of 10X Expand High Fidelity Buffer with 15 mM $MgCl_2$, 1 µl of 10 mM dNTP each, 1 µl of 100 µM each primer, 0.75 µl of Expand High Fidelity enzyme mix, 33.25 µl of water, and 5 µl of sample DNA. The PCR was subjected to 35 cycles of 94°C for 1 min, 57°C for 1.5 min, and 68°C for 4.5 min followed by a final extension at 68°C for 10 min. PCR was conducted in GeneAmp® PCR System 9700 (Applied BioSystems, Foster City, CA). Three enzymes, *Hae*III, *Hinp*1I, and *Msp*I were used to digest the PCR products. This was performed by taking 16µl of the PCR products and adding this with 2 µl (10 U/ µl) of each restriction enzyme (New England Biolabs Inc., Beverly, MA) and 2 µl of 10X restriction enzyme buffer in each tube. The test was performed at 37°C for 1 hour. Digested DNA bands were analyzed in 2% agarose gel electrophoresis.

Statistical analysis

Data were subjected to Chi-square tests to differentiate ILTV DNA positive and negative rates among the treatment groups.

5.3 Results

The ILTV DNA remained on the surface of darkling beetles for at least 42 days after the ILT farm outbreak (Tables 5.2 and 5.3). Although the ILTV DNA was detected in beetle samples from the infected house of farm 1 before hydrogen peroxide (H_2O_2) treatment, the ILTV DNA and virus were not detected after H_2O_2 treatment. The ILTV DNA was positive in beetle samples from the infected house of farm 2 before and after H_2O_2 treatment. Before H_2O_2 treatment, live ILTV was isolated from the farm 2 beetle samples on the 13 and 42 days after the outbreak; however, after H_2O_2 treatment, live ILTV from beetle samples was detected only in samples

collected on the 13th day after the outbreak (Table 5.4). The ILTV DNA was detected only from the lung of the rat. However, live ILTV could not be isolated from the lung. Beetle samples from negative houses of both farms were negative for ILTV by real-time PCR and virus isolation.

The ICP4 gene digested with *Hae* III indicated that the beetle and rat lung samples had the same patterns as vaccine viruses (Fig 5.1). Digestion of the ICP4 region with the *Msp* I generated 2 different patterns. As compared with TCO vaccine, the beetle and lung samples lacked approximately 200 and 550 bp fragment patterns as seen in CEO vaccines (Fig 5.2). Digestion with *Hinp1*I produced 2 patterns. The beetle and lung samples had the same patterns as the CEO vaccine and lacked a 350 bp fragment, which was present in the TCO vaccine (Fig 5.3). The RFLP test indicated that the ILTVs from these infected farms were related to CEO vaccines.

5.4 Discussion

After H_2O_2 treatment, live ILTV was isolated from the internal parts of the beetles for 13 days after the ILTV outbreaks on the farms 1 although at a lower rate (Tables 5.3 and 5.4). Without H_2O_2 treatment, the virus was isolated from both the inside and outside of the insects for 42 days. Beetles, larvae, and rats may play a role for ILTV transmission and spread to other chicken houses by carrying the virus inside and outside their bodies.

ILTV can maintain its infectivity for a long period at low temperature. The virus can survive up to 100 days in tracheal exudates and chicken carcasses at 13-23°C (Jordan, 1966). In our investigation, darkling beetles in ILTV contaminated farms can harbored the virus for several days, which makes the disease difficult to eliminate even after disinfection. The ILTV can

maintain its infectivity in darkling beetle populations and not be inactivated by chemical disinfectants. Despins and Axtell (1995) reported that when 2- to 3-day-old chicks were given a choice between starter feed and lesser mealworms, one chick consumed an average of 389 lesser mealworms per day. The present investigation indicated that ILTV transmission with lesser mealworms and darkling beetles may occur. Kingsbury and Jungherr (1957) believed that ILTV could be transmitted between chicken flocks indirectly by human, wild birds, dogs, rats, clothes, shoes, and equipment (Kingsbury and Jungherr, 1957). In the current study, darkling beetles and lesser mealworms carried ILTV whereas the rat only had ILTV DNA in the lung. This was the first report of ILTV DNA detection in a rodent.

Some beetles and a rat lung were positive for ILTV DNA by real-time PCR and typed by PCR-RFLP, but the virus was not isolated. The reason may be due to a low titer or incomplete viral particles in the rat lung. If more rats from ILTV positive farms were investigated, a significant determination of the role of rats in ILTV transmission could be determined. Moreover, ILTV could be inactivated by several environmental factors. However, the beetles and rat lung contained adequate viral DNA to be detected by real-time PCR. Beetles from ILTV non-infected houses were not positive for ILTV DNA or live virus.

Although ILTVs are homologous, isolates can be separated by RFLP. The test can differentiate vaccine and field ILTV isolates (Leib *et al.*, 1986; Keeler *et al.*, 1993; Chang *et al.,* 1997; Oldoni *et al.,* 2008). The ICP4 gene digested with restriction enzymes *Hae* III, *Msp* I, and *Hinp1*I was able to distinguish between vaccine and non-vaccine isolates in Taiwan (Chang *et al.,* 1997), Northland (Graham *et al.,* 2000), U.K. (Creelan *et al.,* 2006), and the U.S. (Oldoni *et al.,* 2007 and 2008). U.S. ILTV isolates examined by multiple gene PCR-RFLP could be separated into 9 genomic groups. It was shown that some field strains were separated from vaccine strains

(Oldoni *et al.,* 2007). In the current study, ILTV DNA from beetles and a rat lung were tested with PCR-RFLP at partial ICP4 gene region. Results showed that the ILTVs from the infected farms were related to CEO vaccines.

The beetle's exoskeleton provides refuge for adhering pathogens. Joints, wings, and shell serve as protective covers for bacteria and viruses. Moreover, the insect body structure may shield pathogens from sanitizers during poultry house cleaning (Chapman, 1982). Thus, as the insects circulate in the house, the pathogens can spread. Crippen and Sheffield (2006) compared disinfectants and procedures for examining their ability to remove bacteria from the lesser mealworm surface. They found that combined 95% ethanol evaporation with H_2O_2 or H_2O_2 with peracetic acid removed 100% of the bacteria on the insect surface. A 10% H_2O_2 solution could remove more than 90% of the bacteria on the beetle surface. In the present investigation, before H_2O_2 treatment, ILTV DNA and live virus were detected in beetle homogenates from infected houses after the disease occurred. Therefore, live ILTV may attach to the surfaces of darkling beetles and their larvae.

In summary, this study provided evidence that lesser mealworms, their adult beetles, and rats contained ILTV and might serve as a source for ILTV transmission. Data showed that ILTV could survive in the bodies of beetles for at least 13 days after the disease occurred. In addition, the beetles carried the virus up to 42 days inside and outside their bodies. Since beetles can be killed by external temperatures below 6°C (Renault *et al.,* 1999), it would be interesting to know how long the virus may be viable in dead beetles. A similar study with more rodents would also be informative. This research showed that improved beetle and rodent control should be a major part of disease preventive on farms, especially those with persistence disease problems.

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Table 5.1

Real-time PCR primers and TaqMan probe sequences

Primers and probe (5’-3’)	Length	Position [c]
ICP4 qPCR-F： CCCCACCCAGTAGAGGAC	18	143906-143923
ICP4 qPCR-R： CGAGATACACGGAAGCTGATTT	22	144010-144031
ICP4 Probe： FAM [A]-CAGTCTTTGGTCGATGACCCGC-TAMRA[B]	23	143949-143971

A. FAM, 6-carboxyfluorescein.

B. TAMRA, 6-carboxytetramethylrhodamine.

C. The position numbers of the primers and probe were obtained from GeneBank accession #NC_006623.

Table 5.2

The ILTV DNA detection rate from beetles and larvae before H_2O_2 treatment using real-time PCR.

	Days after outbreak	ILT positive house [A]	ILT negative house
Farm 1	17	1/3	0/3
	53	0/3	0/3
	103	0/3	0/3
Farm 2	13	3/3[B]	0/3
	42	2/3[B]	0/3
	90	0/3	0/3

A. Number positive over total number of samples

B. The beetle sample positive rates for ILTV DNA in farm 2 of ILTV positive and negative houses were significantly different by Chi-square test ($p<0.05$).

Table 5.3

The ILTV DNA positive rates from beetles and larvae samples after H_2O_2 treatment using real-time PCR.

	Days after outbreak	ILT positive house [A]	ILT negative house
Farm 1	17	0/3	0/3
	53	0/3	0/3
	103	0/3	0/3
Farm 2	13	1/3	0/3
	42	0/3	0/3
	90	0/3	0/3

A. Number positive over total number of samples

Table 5.4

ILTV isolation from beetle samples in SPF embryos.

	Days after outbreak	Before H_2O_2 treated	After H_2O_2 treated
Farm 1	17	-[A]	-
	53	-	-
	103	-	-
Farm 2	13	+[B]	+
	42	+	-
	90	-	-

A. Negative for virus isolation.

B. Positive for virus isolation.

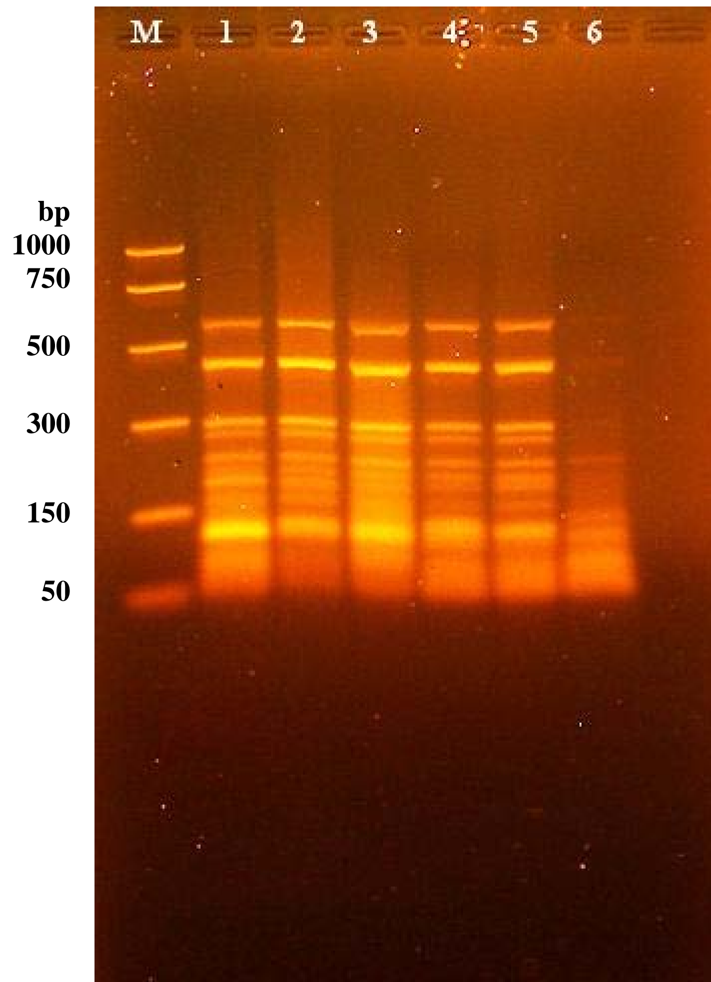

Fig 5.1. *Hae* III digestion of 4.9 kb ICP4 gene region. M: DNA marker; 1: TCO (LT-IVAX®) vaccine; 2: CEO (Trachivax®) vaccine; 3: CEO (LT Blen®) vaccine; 4: ILTV DNA from farm 1 beetles; 5: ILTV DNA from farm 2 beetles; 6: ILTV DNA from the rat lung. After *Hae* III digestion, the ILTV DNA from the beetles and rat lung produced the same patterns as TCO and CEO vaccines.

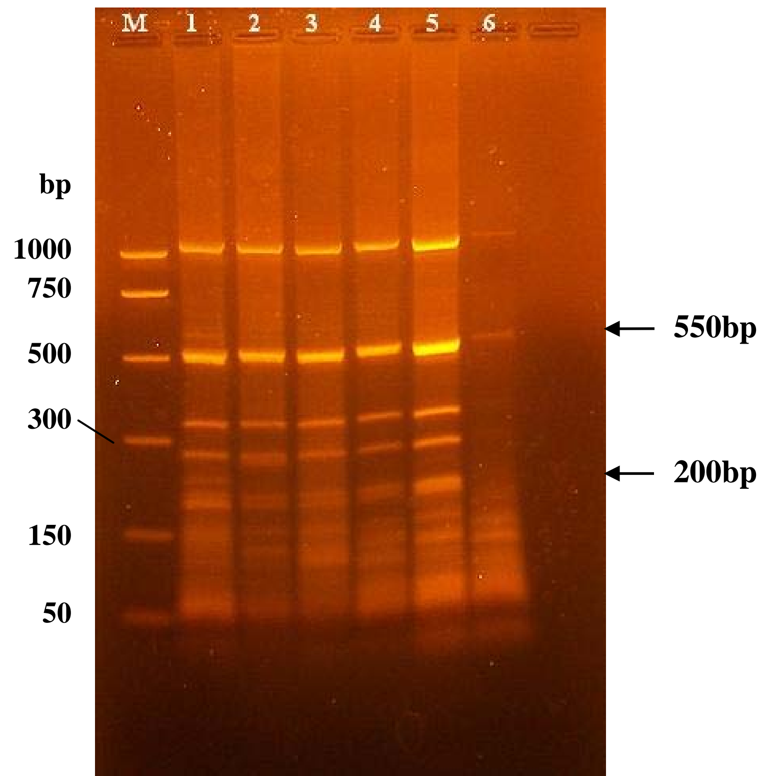

Fig 5.2. *Msp* I digestion of 4.9 kb ICP4 gene region. M: DNA marker; 1: TCO (LT-IVAX®) vaccine; 2: CEO (Trachivax®) vaccine; 3: CEO (LT Blen®) vaccine; 4: ILTV DNA from farm 1 beetles; 5: ILTV DNA from farm 2 beetles; 6: ILTV DNA from the rat lung. After *MSP* I digestion, the TCO vaccine had additional 550 bp and 200 bp bands, which were not evident in CEO vaccines, beetle, and rat lung ILTV DNA samples.

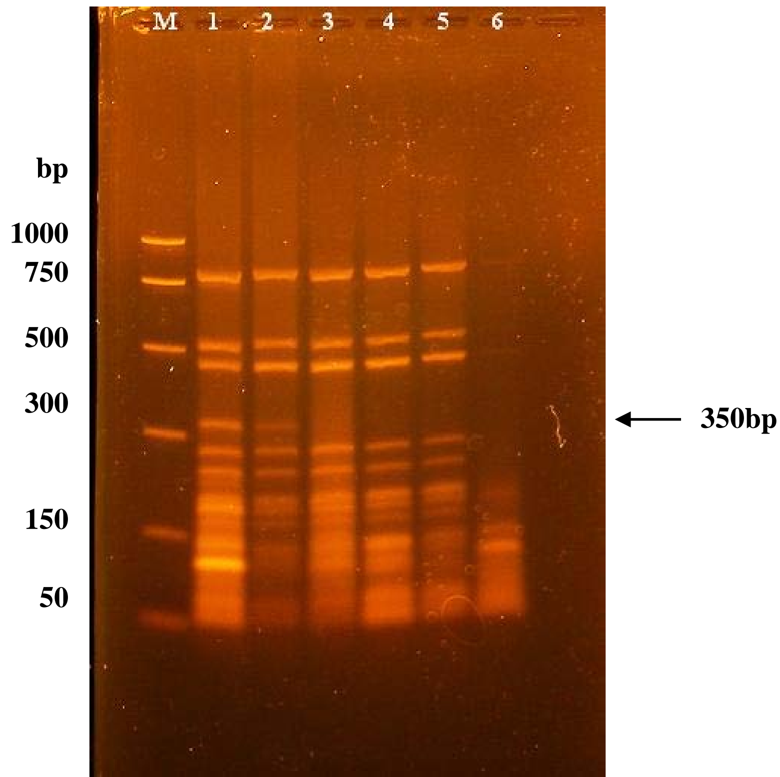

Fig 5.3. *Hinp1*I digestion of 4.9 kb ICP4 gene region. M: DNA marker; 1: TCO (LT-IVAX®) vaccine; 2: CEO (Trachivax®) vaccine; 3: CEO (LT Blen®) vaccine; 4: ILTV DNA from farm 1 beetles; 5: ILTV DNA from farm 2 beetles; 6: ILTV DNA from the rat lung. After *Hinp1* I digestion, the TCO vaccine DNA had an additional 350 bp band, which was not in LT Blen® vaccine, beetle, and rat lung ILTV DNA samples.

CONCLUSION

ILTV causes an acute and chronic respiratory disease in chickens. It can result in significant economic loss to the commercial poultry industry. The virus is not easy to control on farms, because it is highly contagious, can cause latent infections, and has several transmission sources. Therefore, detection, prevention, and control are important.

Current ILTV diagnosis relies on histopathologic tests for intranuclear inclusion bodies in the respiratory organs, virus isolation (VI), serologic tests, and PCR. However, these methods are low sensitive and time-consuming. Serological tests such as ELISA, agar gel immunodiffusion (AGID), and FA test are less sensitive and cannot separate vaccine from field type viruses. Therefore, highly specific and sensitive methods for ILTV detection and characterization are needed.

A TaqMan® probe-base real-time PCR and a novel nucleic acid amplification method—loop-mediated isothermal amplification assay (LAMP) to detect ILTVs were developed and compared. The primers and probe for real-time PCR and LAMP assay were designed from the conserved region of the ILTV ICP4 gene. Both real-time PCR and LAMP assays were highly specific. They detected five ILT vaccine viruses without cross reaction with other pathogens. For sensitivity, a partial ILTV ICP4 gene was cloned and the standard DNA constructed. The sensitivity of real-time PCR was 10 copies/ µl, and was highly reproducible. Repeats of the real-time PCR tests

curves were nearly identical with a small variation of slopes (-3.14 ± 0.06) and intercept (38.28 ± 0.63) of the regression equation.

The sensitivity of the LAMP assay was 60 copies/ µl. Although the sensitivity was slightly less than that of real-time PCR, this assay is more sensitive than traditional PCR. Moreover, the LAMP assay was simpler, more rapid, and less expensive than real-time PCR. The ILTV LAMP could be performed at 65°C for 45 minutes without a thermal cycler. Therefore, the LAMP assay offers another choice for ILTV detection.

Water systems on chicken farms are an important source for bacteria and virus transmission. Several pathogens have been shown to reside in biofilms in water lines. These pathogens may be released from the biofilms into the drinking water and infect birds. The organic matter in the water lines of chicken houses such as feces, dusts, feed, and nutritional and antimicrobial additives provide an ideal environment for biofilm development. Once developed a biofilm is difficult to remove. In addition, live vaccines are often given in the drinking water and may attach and remain viable in biofilms within the lines for a long time periods.

An ILTV vaccine was used as a model for attaching and absorbing to water line biofilms. Real-time PCR and virus isolation detected ILTV in the water lines. Live ILTV was isolated from the biofilms, drinkers, and chickens, which drank from the water lines.

The ability of 4 sanitizers (sodium hypochlorite, citric acid, sodium hydrogen sulfate, and hydrogen peroxide) to remove or inactivate ILTV in the biofilms of water lines was tested. Neither sodium hypochlorite nor citric acid inactivated ILTV from the drinkers. Although real-time PCR detected ILTV DNA from water samples, sodium hydrogen sulfate and hydrogen

peroxide treatment inactivated ILTV in the drinkers. This work showed that water lines on farms could be a source for ILTV transmission. Furthermore, less expensive and commonly used sanitizers such as chlorine bleach and citric acid may not remove biofilms and inactivate ILTV after one use.

Darkling beetles, their larvae (*Alphitobius diaperinus*), and rodents are commonly found in chicken houses and are difficult to eradicate. Avian pathogens from sick or dead birds can be harbored on beetle surfaces or inside their bodies. Rodent organs can also harbor bacteria and contaminate poultry facilities. Several investigations indicated that several different bacteria and viruses can survive inside or outside darkling beetles, their larvae, and rodents for weeks to months.

Darkling beetles, their larvae, from ILTV infected and non-infected chicken farms and a rat from an infected farm were collected. ILTV DNA was detected in the insects with real-time PCR for 42 days after the disease occurred on the farms. ILTV DNA was detected in the rat lung using real-time PCR. Live ILTV was isolated from inside and outside of the beetles and their larvae. Treatment with H_2O_2 was able to remove the virus from the external surface of the beetles. This was the first study, which showed that darkling beetles, their larvae, and a rodent can carry ILTV and may transmit it to susceptible chickens. For effective ILTV control, darkling beetle, their larvae, and rodent numbers need to be reduced.

The significance of this dissertation was that it developed improved detection methods for ILTV, showed that several disinfectants, designed for biofilm removal, could remove and inactive ILTV in the water and that beetles, their larvae, rodents, and drinking water are potential sources of live ILTV for re-infection of newly placed chickens.

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