IDENTIFICATION AND CHARACTERIZATION OF NOVEL LIVE ATTENUATED VACCINE STRAINS OF INFECTIOUS LARYNGOTRACHEITIS

by

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A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Animal Science

Fall 2013

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ACKNOWLEDGEMENTS

I would like to thank Dr. Keeler for providing me with the opportunity to receive my master's degree and allowing me to hone in on my skills in wet bench work. I would like thank Dr. Brannick at the UD Lasher Poultry Diagnostic Laboratory for histopathologic analysis and guidance along the way. I would also like to thank Cindy Boegtter for technical/procedural help and continued support throughout my research project. Finally I would like to thank my family for always believing in me.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Infectious laryngotracheitis (ILT) is an acute respiratory disease of chickens that can result in decreased egg production and increased mortality. A tissue culture origin vaccine (TCO), several chicken embryo origin vaccines (CEO), and three recombinant vaccines are currently used to control the disease. However, TCO vaccines may provide incomplete coverage, CEO vaccines are moderately pathogenic, and recombinant vaccines take several weeks to induce immunity. The objective of this study is to identify a live attenuated strain of ILTV that demonstrates both reduced pathogenicity and virulence through two approaches. Two ILTV field isolates (88-627 and 11-11349) were identified after an analysis of clinical submissions to the University of Delaware. One isolate (88-627) was chosen for further characterization. This isolate did not prove to be a potential vaccine candidate as it exhibited similar levels of pathogenicity to currently used CEO vaccines. In the second approach attempts were made to attenuate a CEO vaccine strain of ILTV through serial passage in tissue culture. After 20 passages in chicken embryo liver cells (CEL), strain UDCEOD1/CEL20 was evaluated in birds. UDCEOD1/CEL20 exhibited reduced levels of pathogenicity, and was able to elicit a protective immune response. The UDCEOD1/CEL20 genome was sequenced and found to be 153,694 nucleotides in length. Two single nucleotide polymorphisms (SNPs) were identified. One SNP resulted in a silent mutation in the glycoprotein J gene. The other SNP is located within both copies of the ILTV ICP4 gene. This SNP results in a

threonine to alanine mutation that may be responsible for the observed decrease in virulence.

Chapter 1

INTRODUCTION

1.1 Infectious Laryngotracheitis (ILT)

Poultry meat makes up a significant proportion of meat production worldwide. According to the United States Department of Agriculture (USDA), the United States produced 8.61 billion broilers and profited a total of $30.47 billion [1]. Infectious larygnotracheitis (ILT) threatens the livelihood of the poultry industry because of economic losses associated with mortality, decreased growth rate and egg production. The disease is primarily found in the United States, Europe, Australia, New Zealand, China, and Southeast Asia [2], [3], [4], [5], [6], [7].

ILT is an acute upper respiratory disease initially confused with other respiratory diseases such as infectious bronchitis [8]. The disease was initially defined by May and Tittsler in 1925 [9]. Classically, ILT is commonly found in chickens, although peafowl and pheasants can also contract the disease [10]. Ducks are refractory to the disease, but can still become carriers [11]. Experimentally, turkeys can be infected [12], but quail and guinea fowl show no susceptibility [13].

The incubation period for ILT ranges from 7-12 days, and the duration of the disease is 10-14 days [8]. The major means of direct transmission is from infected birds. Birds in the incubation period of the disease, or who have already been through an outbreak of the disease, also pose a threat as carriers [4]. In a series of outbreaks recorded

by Kingsbury et al., [14] other probable means of transmission were traced to rats, dogs and crows, contaminated clothing from people in direct contact with the diseased birds, and poor disposal methods. Beaudette [15] identified contaminated farm equipment such as soiled crates and feedbags as a general means of transmission. Preventative measures for transmission include emergency vaccination, the disinfection of clothing and equipment, the proper disposal of dead diseased birds, and the extermination of rats [14].

There are two epizootic forms of ILT: mild and virulent [16]. In the virulent form of ILT chickens display extreme respiratory distress involving open mouth gasping, violent spasms of coughing and sneezing, convulsions, conjunctivitis, choking, bloody mucus, nasal discharge, decreased appetite, and decreased egg production [2], [8], [9]. Mortality averages 20%, but can reach 75% in some cases [2], [8], [9]. In the mild form of ILT there is little mortality and the clinical symptoms may be intermittent. These symptoms include conjunctivitis, nasal discharge, infraorbital swelling, unthriftiness, shaking of the head, gasping for air, consistent swallowing, and sharp laryngeal sounds [5], [6], [16].

Upon necropsy, lesions are generally found in the upper larynx, and trachea [17]. Typically, the airways have an accumulation of mucus and blood, although the amount varies during the course of the infection [17] and depending on the strain of the virus. Small amounts of mucus with slight hemorrhages are seen and progress to yellow-cheese like mucous plugs in the late stages of infection [17]. The mucus plugs may block the airway and can lead to death by asphyxiation [17]. The nostril and cleft palate may expel yellowish material with slight pressure and small round thin patches of film are often

observed in the mouth. The histopathological lesions characteristic of ILT are primarily found in the conjunctiva, trachea and lung [5], though the sinuses, the palatine cleft, and the oro-cranial portion of the anterior larynx can also exhibit pathology in turkeys [12]. The eyelid and trachea present syncytia formation with or without intranuclear inclusion bodies in the conjunctival and mucosal epithelium, respectively [5]. Also conjunctival and mucosal sloughing occur with an accumulation of fluid consisting primarily of white blood cells, red blood cells, heterophils, and cellular debris [5]. The lungs also exhibit sloughing of the mucosa, as well as syncytia formation and intranuclear inclusion bodies (INIB) [5]. In turkeys, cilia are lost from the parabronchial wall, and necrosis of the bronchial epithelium is observed [12].

1.2 Infectious Laryngotrachetis Virus (ILTV)

The agent of ILT is infectious laryngotracheitis virus (ILTV). ILTV is a herpesvirus, containing a linear double-stranded DNA genome. The morphology of the ILTV particle was first identified by Watrach and Hanson [18] through a negative contrast technique. The International Committee on Taxonomy of Viruses places ILTV under the order of *Herpesvirales*, family *Alloherpesviridae*, subfamily *Alphaherpesvirinae*, genus *Iltovirus*, species *gallid herpesvirus* [19].

The ILTV contains three main structural features: core, capsid, and envelope [18]. The core and capsid both have a hexagonal shape measuring 800 ± 20 Å and 1075 ± 25 Å, respectively [18]. The capsid is comprised of smaller polygonal or hexagonal subunits called capsomeres arranged in equilateral triangles, approximately 105 Å in length or 90-100 Å in diameter, respectively [18], [20]. The virus particle shows a

distinct 5:3:2 rotational symmetry [20]. The envelope diameter varies between 1950-2500 Å. Small projections (50-70 Å long and 25 Å thick) can be seen from the particle surface [18].

The ILTV genome was published in 2006 (Figure 1) [21]. The genome consists of a unique long region (UL) and a unique short region (US). The US region is flanked by two inverted repeats (IR-internal repeat and TR-terminal repeat). The US region can invert and exists in two isomeric forms. The complete ILTV genome is 148,665 bp in length, 113,039 bp corresponding to the UL region (contains 62 genes), 13,232 bp to the US region (contains 9 genes), and 11,202 bp to the IR and TR repeats (contains two copies of three genes: ICP4, US10, and a homolog of the MDV sORF4/3 gene). The ILTV genome has a G+C content of 48.16% and contains 77 predicted open reading frames (ORFs).

1.3 Diagnosis of Infectious Laryngotracheitis

Chickens are diagnosed with ILT through a variety of techniques. Tissue suspected of being infected (trachea, eyelid, lung) are commonly stained with hematoxylin and eosin for histopathogical examination [22]. Diagnosis of ILT is confirmed by identification of herpesviral intranuclear inclusion bodies in the ectodermal epithelium [17], [22]. Erosion/ulceration and increased inflammation of mucosal surfaces are highly suggestive of ILT when INIB are not present [17].

Virus isolation can also be used to confirm a diagnosis of ILT. Virus can be identified by characteristic growth in chicken embryo liver (CEL) and chicken embryo

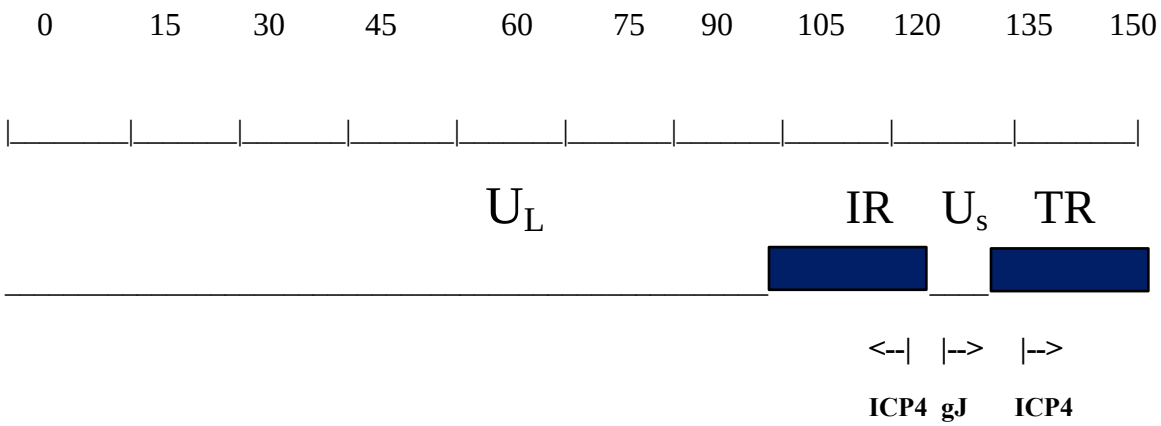


Figure 1. Structure of the ILTV genome. The genome consists of a unique long region (UL) and a unique short region (US). The US region is flanked by two inverted repeats (IR-internal repeat and TR-terminal repeat) [61]. U_L- Unique Long region, U_S-Unique Short region, IR-Internal Repeat, TR-Terminal Repeat, ICP4- Infected Cell Protein 4, gJ-glycoprotein J.

kidney (CK) cells, as well as through the presence of plaques on the chorioallantoic membrane of embryonated eggs [23]. Cytopathic effects in CEL cells and CK cells are commonly seen 2-4 days post-inoculation [24, 25]. Cytopathic effects include granular degeneration of cells, and plaque formation [26]. Upon inoculation of eggs, ILT creates a yellow/white thickening on the CAM called a pock [27]. Pock formation is largely due to proliferative and necrotic changes and the appearance of inflammatory mesodermal changes. Death of the embryo is a common result from interference with respiration and usually occurs 5-6 days after inoculation [28]. Burnet [28] observed that some virulent strains of ILTV exhibited more extensive necrosis, and a larger pock diameter. Gelenczei et al., [27] also documented variation among CAM lesions depending on the number of passages of the virus in tissue culture. Small pin head size pocks surrounded by translucent edema was seen more common in higher passages of the virus.

The polymerase chain reaction (PCR) is another method commonly used for the diagnosis of ILT. The purpose of this method is to identify a portion of the ILTV genome. DNA may be obtained from CAM homogenate, CEL infected cells, or swab samples. Garcia et. al., [29] used specific primers to amplify an 1847-bp fragment corresponding to the ILTV genome [29]. The visualization of this product confirms the presence of the virus.

Although less commonly used, hybridization methods can also be employed to detect the presences of ILTV. Nielsen et al., [30] used an in situ hybridization method to detect ILTV. In this method, labeled DNA corresponding to conserved regions of the ILTV glycoprotein C and thymidine kinase genes were

hybridized to sections of the trachea embedded in paraffin wax. Key et. al., [31] developed a digoxgigenin-labeled DNA probe that was optimized in dot blot hybridization for the detection of ILTV DNA. Using this method, the hybridization of the probe creates the appearance of a dot on a positively charged nylon membrane indicating the detection of ILTV. This method allows for initial detection of disease.

Several serologic tests are also employed for the detection of antibodies to ILTV or ILTV antigens. Enzyme-linked Immunosorbent Assay (ELISA) is commonly used for the detection of antibodies in sera through the binding of ILTV antibody to antigen. The procedure can determine whether a flock has been previously exposed to the ILT disease [32]. A variation of the ELISA was developed by Ohkubo et al., [33] the labeled avidin-biotin enzyme-linked immunosorbent assay (LAB-ELISA) also detects antibodies to ILTV in sera. The LAB-ELISA was able to detect antibodies in chickens 5-23 weeks after vaccination and reduced non-specific reactions. York et al., [34] modified this procedure using a monoclonal antibody derived from mice infected with a strain of ILTV to detect viral antigen from tracheal exudates.

Other methods, such as Indirect Immunofluorescent Assay (IFA), have also been used to detect viral antigen of ILTV. Hitchner et. al., [35] demonstrated the detection of viral antigen through the use of fluorescently labeled antibody (IFA). This technique allows for visualization of virus when fluorescent antibody is applied to infected tissues and observed under ultraviolet light. Since the duration of the disease is short lived, detection must occur when birds are actively presenting clinical signs. IFA confirmed

that ILTV is a respiratory disease by identifying viral antigen in the conjunctiva, nasal turbinate, trachea, esophagus, lung, and air sacs.

1.4 Control of Infectious Laryngotracheitis

In order to reduce the transmission of ILT, property biosecurity measures are critical. Biosecurity is broken down into four major categories [36]. In grower biosecurity, a farmer from an infected farm does not visit other farms, as he could introduce virus from his clothes or the use of shared equipment. In farm biosecurity, transmission is avoided by decreasing the level of incoming pedestrian traffic to the infected farm. In company biosecurity, measures are taken to reduce the transmission of ILT through by reducing service visits and feed deliveries. Trucks can potentially transfer the virus to nearby birds. In area biosecurity, growers are made aware of local farms that are infected.

Since biosecurity measures do not provide complete protection, the poultry industry relies heavily on vaccines to control ILT. Tissue culture origin (TCO) vaccines have been established by sequential passages in CEL or CK cell cultures. Through a variety of different routes of administration, TCO vaccines demonstrate low pathogenicity and good protection. The minimum dose for 100% protection from TCO vaccines ranges from $10^{\wedge 3.0}$ EID50/ml to $10^{\wedge 3.5}$ EID50/ml when administered ocularly [37]. CEO vaccines are propagated on the chorioallantoic membrane (CAM) of embryonated eggs [20]. CEO vaccines induce a high level of protection but may result in severe vaccine reactions. Also, Guy et al., [38] noted that following sequential bird passages, CEO vaccines increased in their virulence. These findings suggested that CEO

vaccine could play a role in active outbreaks of ILT. Restrictions on ILTV vaccinations have been implemented in many states due to this theory that CEO outbreaks stem from CEO vaccines. This phenomenon is often referred to as "vaccinal laryngotracheitis" [36]. Generally, TCO vaccines are considered a safer alternative to CEO vaccines [27].

More recently, recombinant vaccines for ILT have been developed. These vaccines eliminate clinical disease of ILT, while still stimulating the host immune response. For instance, adequate protection against challenge virus was demonstrated with a recombinant fowl pox virus expressing glycoprotein B of ILTV [39]. While production of recombinant vaccines is expensive, it is essential to note that this process overcomes some of the drawbacks of currently used live attenuated ILT vaccines.

Live attenuated ILT vaccines are applied via the ocular route (eye drop), oral (drinking water), or spray [40], [41]. Vaccination via eye drop has become the standard method of inoculation and it provides uniform protection. However, vaccine reactions can involve conjunctivitis of the eye [40]. Spray application may slow the development of immunity and it carries the potential risk of developing an inflammatory reaction due to virus multiplication in the mucosal linings [40].

1.5 Immunity

Immunity is the ability to confer protection from infectious disease. The exact mechanism of immunity,whether through a humoral-mediated response or a cell-mediated response has not been conferred. However, several studies strongly suggest a cell-mediated protective function involving T-lymphocytes, natural killer cells, macrophages and the release of cytokines. Robertson et al., [42] demonstrated that the

level of protection against ILTV maintained even when antibody development is aborted by the combination of surgical bursectomy and cyclophoshamide. Fahey et. al., [43] observed no measurable differences to the susceptibility to ILTV when ILTV antibody-positive serum was transferred into to 2-day-old and 4-week-old chickens. Andreasen et. al., [44] still observed mortality in birds who maintained measurable antibody titers from vaccines before challenge.

In birds, the duration of immunity to ILTV varies significantly by age of bird, and challenge route of inoculation. Hatchlings carry a strong immunity to ILTV from the transmission of maternal antibodies of a vaccinated bird. However, protection against ILTV declines significantly starting at 3 weeks of age [45]. Birds who have previously been vaccinated with ILTV have carried immunity up to 364 days when challenged by intratracheal route and 372 days when by challenged the infraorbital sinus route [46], [47]. Duration of immunity is also suggested to increase when birds are challenged with less virulent strains of ILTV and vaccinated with high titers [48], [49].

1.6 Latency and Carrier State

Latency is the ability of a virus to remain dormant within a infected cell. Latent infections of ILTV is established in the trigeminal ganglion or trachea [50], [51]. Combining flocks and the on-set of lay is suggested to contribute to the activation of the virus from latently-infected birds [52]. Tracheal organ culture (TOC) has been used to identify latently infected birds [51]. TOC has identified latent infections of ILTV, 3-16 months post exposure, and in vaccine strains 2-10 months post-exposure.

Latent infections results is situations where the virus, although present in birds without presenting clinical signs, can still maintain the ability to transmit disease. The carrier state was first noted by Gibbs in 1931 when he identified ILTV from tracheal swabs taken from birds after recovery from the disease [53]. The shedding of virus varies between birds. In extreme cases, ILTV was observed to shed 467 days after the initial infection, while in other cases, viral shedding occurs intermittent for 7-14 weeks after exposure [53], [54].

1.7 Objectives

Aim 1: Identify and characterize potential novel vaccine strains of infectious laryngotracheitis virus.

The purpose of this aim is to identify a field isolate of ILTV that demonstrates both reduced pathogenicity and virulence when compared to currently used CEO vaccines. We will survey field isolates of ILTV submitted to the University of Delaware's Lasher Poultry Diagnostic Laboratory to identify ILTV isolates that may exhibit reduced pathogenicity. Candidate strains will be evaluated for pathogenicity in broiler chickens.

Aim 2: Attenuate a strain of infectious laryngotracheitis virus through serial passage in chicken embryo liver cell culture.

As an alternative to screening field isolates of ILTV, attempts will be made to attenuate a CEO vaccine strain of ILTV through serial passage in tissue culture. After multiple passages in CEL, virus will be evaluated for pathogenicity in broiler chickens.

Chapter 2

MATERIALS AND METHODS

2.1 Selection of ILTV Field Isolates

The University of Delaware's Delmarva Laboratory Information Management System (LIMS) has records of clinical poultry submissions to two poultry health laboratories in Delaware and Maryland. Each entry contains information regarding the age and vaccination status of a submitted sample, clinical signs of disease, flock mortality, and any diagnostic results. Potential virus candidates for pathogenicity testing were identified using three criteria: low mortality (less than 3 deaths/1000), origination from an unvaccinated ILTV flock, and the presence of characteristic herpesvirus inclusion bodies and epithelial syncytia by histologic analysis of the trachea and eyelid.

2.2 Serial Passage of ILTV through Cell Culture

An ILTV CEO vaccine derivative (UDCEOD1) was passaged multiple times in chicken embryo liver (CEL) tissue culture in an attempt to attenuate the virus. After multiple passages, the virus was evaluated for pathogenicity in broiler chickens.

2.2.1 Chicken Embryo Liver (CEL) Tissue Culture

Fifteen-day SPF chicken eggs were used for CEL tissue culture [55]. Eggs were sprayed with 70% ethanol, opened on the blunt end, and the embryos were humanely killed by cervical dislocation. Livers were excised, minced, and placed in a petri dish containing 25 ml of Media 199 on ice. Livers were washed 3 times in 40 ml of 1X phosphate buffer saline (PBS) supplemented with Penicillin and Streptomycin (Pen

Strep). Livers were trypsinized 4 times for 5 minute in 20 ml of warm (37° C) trypsin. The trypsinizaton solution was poured over cheese cloth into a beaker containing 8 ml of chicken serum. The final trypsinized cell suspension was poured into a 50 ml tube and centrifuged for 20 min at 1,200 rpm to pellet the liver cells. The resulting liver cell pellet was then suspended in 60-80 ml of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % Fetal Bovine Serum (FBS). Ten ml of the CEL cell suspension were added to a 100 ml tissue culture dish. The resulting CEL cultures were placed in a 37° C, 5% CO_2 incubator overnight.

2.2.2 Propagation of ILTV on CEL

A strain of ILTV derived in our laboratory from an ILTV CEO vaccine (UDCEOD1) was used to infect CEL cell culture (Cindy Boettger). One ml of UDCEOD1 infected CAM homogenate was used to infect one 100 mm dish of CEL. After 2 hours, unattached virus was aspirated, the plate was washed with 10 ml of 1X PBS supplemented with Pen Strep, and 10 ml of DMEM supplemented with 2 % FBS was added. Infected cells were incubated at 37°C, 5% CO_2 for 48 hrs. Cells were harvested when 85% of the CEL cells were infected demonstrated by cytopathic effects of rounding and clumping. The infected cell monolayer was removed using a cell scraper, creating UDCEOD1/CEL1, and 1 ml aliquots were placed in the -80°C freezer. UDCEOD1/CEL1 was then used to infect CEL cells, creating UDCEOD1/CEL2. A 1 ml aliquot of UDCEOD1/CEL1 was thawed at 37° C and then sonicated twice for 10 seconds in a Sonics Vibra Cell machine at 50% duty before infection. This process was repeated 29 times, creating UDCEOD1/CEL1-29.

2.3 Identification and Characterization of ILTV

Selected field isolates meeting the criteria listed above and virus propagated on CEL were confirmed for the presence of ILTV by the polymerase chain reaction (PCR). PCR was also used to confirm that the field isolates were not contaminated with adenovirus.

2.3.1 Nucleic Acid Isolation

Viral DNA was isolated from 200 µl of infected CEL lysate or 200 µl of infected CAM homogenate [29]. The material was mixed with 100 µl of lysis buffer and 5 µl of proteinase K followed by a 40 min incubation at 37°C. Two phenol isoamyl alcohol and chloroform (25:24:1) extractions were performed (1 volume each). Total DNA was precipitated from the aqueous layer by the addition of 2 volumes of 100% ethanol and 1/10 volume of 3 M sodium acetate (pH 5.2). DNA was precipitated overnight at 20°C and then pelleted at 14,000 rpm (Eppendorf Centrifuge 5417C) for 20 min and washed with 1 ml of 70% ethanol. The concentration and quality of the viral DNA was determined using the Thermo Scientific Nanodrop^{TM} 1000 Spectrophotometer V3.6, measuring 1 µl samples.

2.3.2 Polymerase Chain Reaction and Gel Electrophoresis

The polymerase chain reaction (PCR) was used to amplify and detect ILTV or adenovirus DNA. An 847-bp fragment of the ILTV glycoprotein E (gE) gene was amplified through this method to confirm the presence of ILTV [29]. ILTV DNA amplifications were conducted in a 52 µl reaction volume consisting of 45 µl of PCR supermix, 5 µl of DNA, and 2 µl of LT gE forward/reverse primers (5'-

GGCTGACCAGGATAGTGAAC-3') and (5'-GGTAAGATTTCCCGATTTCTC-3'). The ILTV PCR program was performed in a GeneAmp PCR system 9700 and included a 35 cycle run of 94°C for 15 sec, 50°C for 45 sec, 72°C for 1 min, followed by an extension cycle of 72°C for 3 min [29].

The same method was used to amplify an 897 bp fragment of adenovirus DNA to confirm that selected viral strains were not contaminated with this virus. DNA amplifications were conducted in a 52 µl reaction volume composed of 45 µl of PCR supermix, 5 µl of DNA, and 2 µl of Hexon A/ Hexon B adenovirus primers (Hexon A sequence- 5'-CAARTTCAGRCAGACGGT-3')(Hexon B sequence- 5'-TAGTGATGMCGSGACATCAT-3') [56]. The adenovirus PCR program was performed on the Applied Biosytems 2720 Thermal Cycler. An initial incubation at 95°C for 30 sec was followed by 20 cycles using the following parameters: 95°C for 20 sec, 52°C for 25 sec, 65°C for 45 sec. This was followed by an additional 25 cycles using the following parameters: 95°C for 35 sec, 52°C for 32 sec, 65°C for 1 min, 95°C for 20 sec, 52°C for 25 sec, 65°C for 45 sec, 95°C or 35 sec, 52°C for 32 sec, and 65°C for 1 min.

A 0.8% agarose gel was used to visualize any amplified DNA. The gel contained 5 µl of Red dye (RGB-4103) to visualize DNA fragments under ultra violet light. Seven microliters of loading dye was added to the 52 µl PCR reaction mix. Twenty microliters was loaded into a 0.8% agarose gel and the DNA was electrophoresed for 1.5 hrs at 60 volts. Amplified DNA fragments were sized against an E-Gel 1 kb DNA Plus ladder suitable for linear double-stranded DNA fragments from 100 bp to 12 kb. Positive controls for both ILTV and adenovirus were also included.

2.3.3 DNA Sequencing

Using the Ovation Ultralow Library Systems kit (Nugen Technologies), genomic DNA libraries were prepared from 5 μg of UDCEOD1/CEL20 DNA. The process consists of three major steps: (1) DNA end repair/purification, (2) adaptor ligation/purification, and (3) DNA amplification/purification. In the first step, DNA was fragmented to an average size of 150-200 bases using the Covaris S-series sonication system. The sheared ends were repaired by annealing primers to the 5' ends of the DNA. DNA was repaired by incubating the reaction at 25°C for 30 min, followed by a 70°C incubation for 10 min. DNA was then purified by bead purification in which the transported end repaired DNA was bound to Agencourt RNAClean XP beads. Using a Promega MagnaBot II magnetic separation device, DNA was eluted from the beads. In the second step, specific amplification adaptors were ligated to the fragmented DNA, creating adapter-flanked template for PCR amplification. The bead purification process was repeated again. In the third and final step, primers were added and the DNA fragment libraries were amplified using PCR (cycling program: 72°C-2 min, 5 cycles of (94°C-30 sec, 55°C-30 sec, 72°C-1min), 10 cycles of (94°C-30 min sec, 63°C-30 sec, 72°C-1 min), 72°C-5 min, hold at 10°C). The bead purification process was repeated again as described above

The UDCEOD1/CEL20 DNA sequencing library was sequenced using the Illumina sequence-by-synthesis system at the University of Delaware Sequencing and Genotyping Center in Newark, DE. Sequencing was carried out on Illumina HiSeq2000, running HiSeq Control Software (HCS) V1.4.8. The sequence of UDCEOD1/CEL20 was

determined by alignment to the published ILTV CEO vaccine reference sequence (Genbank accession # JN580313) using Lasergene (DNASTAR, Madison, WI), NCBI Entrez and other web-based tools. Homology searches were performed using the Basic Local Alignment search Tool for nucleotides (BLASTn) on the National Center for Biotechnology Information website (NCBI) [21]. Single-nucleotide polymorphisms (SNPs) were aligned against the ILTV CEO TVAX vaccine sequence (Genbank accession # JN580313) to identify gene correspondence. The Seqbuilder package within Version 2 of Lasergene was used to identify predicted amino acid changes.

2.4 Animal Studies

Selected ILTV field isolates and CEL-passaged strains of ILTV were evaluated for pathogenicity, and virulence through *in vivo* bird studies.

2.4.1 Viral Titer

Using the method outlined by Senne (57), 10-day-old embryonated eggs were inoculated with virus via the chorioallantoic method. A 10-fold dilution of virus was prepared and 0.1 ml was inoculated onto the CAM of each egg, which were placed in a 37° C Jamesway egg incubator. After 7 days, the eggs were sprayed with 70% ethanol, opened on the blunt end, and scored for ILTV lesions. The Reed and Muench method [58] was used to calculate an endpoint dilution. The endpoint dilution defines the highest dilution of virus that will produce an ILTV lesion in 50% of the eggs. The viral titer, defined as the number of infectious units per unit volume, is the negative exponential of the endpoint dilution [58].

2.4.2 Pathogenicity Evaluation

One-day-old chickens, purchased from Moyer Chick's (Quakertown, PA) were housed in filtered air incubator rooms in the University of Delaware's Charles C. Allen Laboratory. Chickens were divided into groups of 8-10 birds in each isolator. All birds had free access to food and water. Caregivers practiced strict biosecurity procedures and observed guidelines set forth the University of Delaware's Agriculture Animal Care and Use Committee (AACUC) (Protocol # (14)-04-27-10Ra).

At 2 or 5 weeks of age, birds were inoculated by the intrachoanal route with 100 µl of $10^{\wedge 3.5}$ EID_{50} of the USDA virus, 88-627, UDCEOD1, or UDCEOD1/CEL20 virus. Other groups were inoculated with either the tissue culture origin vaccine (LT-IVAX, Schering-Plough (Millsbouro, DE)) or chicken embryo origin vaccine (Trachivax, Schering-Plough (Millsbouro, DE)). Prior to administration, the manufacturer's recommended dose of 30 µl was brought up to 100 µl in vaccine diluent (DMEM + Pen Step + 1% FBS). Control birds were inoculated with 100 µl of vaccine diluent.

Birds were scored for clinical signs from day 2-10 post-inoculation using the Shaffer [59] scoring system, outlined in Table 1. The total clinical score (∑ of individual bird scores) and mean clinical scores were calculated daily per group, and compared among groups. JMP software was used to analyze mean daily scores by a one-way analysis of variance (ANOVA). Statistical significance was determined at the 5% level ($P \leq 0.05$) and followed by the Tukey's HSD test.

From 2-10 days post-inoculation, birds were sacrificed and necropsied and samples of tracheas and eyelids were placed in 10%. neutral buffered formalin. Tissues

Table 1. ILT scoring system. Criteria for observational scoring of clinical signs in broiler chickens intrachoanally exposed to ILTV.

Score #	Observational findings
0	No clinical signs
1	Minimal clear exudate upon application of pressure to nares
2	Light unilateral nasal exudate upon application of pressure to nares or mild conjunctivitis
3	Moderate visible unilateral nasal exudate or moderate conjunctivitis
4	Extreme bilateral nasal exudate and conjunctivitis
5	Death

were embedded in paraffin, sectioned into 4 μm specimens, and stained with Hematoxylin & Eosin. Tissues were evaluated by Dr. Brannick at the University of Delaware and analyzed for characteristic herpesviral inclusion bodies and epithelial syncytia.

Chapter 3

RESULTS

3.1 Identification of Potential ILTV Vaccine Candidates

A total of 68 ILTV field isolates were identified from the University of Delaware's Delmarva Laboratory Information Management System (LIMS) from the years of 2010-2012 and 26 from the Keeler laboratory collection. Of these, 56 were identified as potential vaccine candidates, based on their low reported mortality (less than 3 deaths/1000), their origination from an unvaccinated ILTV flock, and the presence of characteristic herpesviral inclusion bodies and epithelial syncytia by histologic analysis. Twelve of the 56 potential candidates had been characterized by growth in eggs, ILTV PCR, or adenovirus PCR, as shown in Table 2. Ten of the twelve field isolates failed screening. Four were positive for adenovirus, five did not grow in eggs (no virus), and one did not contain ILTV. Two field isolates (88-627 and 11-11349) were identified for evaluation *in vivo*.

3.2 Pathogenicity Testing of Field Isolate 88-627

Field isolate 88-627 was evaluated for pathogenicity in broiler chickens. Eight broiler chickens were inoculated with $10^{\wedge 3.5}$ EID_{50} of 88-627 by the intrachoanal route (100 µl). Groups of sixteen birds were separately inoculated with the IVAX vaccine (TCO), TVAX vaccine (CEO), or mock infected (control). Chickens were scored 2-10 days PI. Clinical signs (nasal discharge, conjunctivitis, and open mouth breathing) of ILT

Table 2. Identification of potential ILTV vaccine candidates. From 56 potential candidates, 12 field isolates were selected for further analysis by PCR and growth in eggs.(-), no growth in eggs or a negative PCR result. (+), positive growth in eggs or a positive PCR result. (ND), not determined.

Accession #	Death/1000	Growth in Eggs	ILTV PCR	Adenovirus PCR
88-627	2/1000	+	+	-
11-1641	1/1000	ND	+	+
11-9494	1.5/1000	-	ND	ND
11-10408	1/1000	-	ND	ND
11-10465	1/1000	-	ND	ND
11-11349	1/1000	ND	+	-
11-11380	0.5/1000	-	ND	ND
11-11897	1.5/1000	ND	+	+
11-12457	3/1000	ND	-	-
11-12945	2/1000	-	ND	ND
12-8650	2.25/1000	ND	+	+
12-11979	2.25/1000	ND	+	+

were observed in all groups except the control group. Total susceptibility (number of sick birds/total number of birds per group) was observed to be 75% (88-627), 70% (TVAX), 60% (IVAX) and 0% (control). Figure 2 depicts the daily values of susceptible birds corresponding to 88-627, the IVAX vaccine, the TVAX vaccine, and control. For the IVAX and TVAX vaccine, susceptibility to ILTV peaked at 60% and 80% by day 4 PI, respectively. 88-627 reached 75% susceptibility 3-5 days PI, and peaked at 87.5% on day 7 PI. Although no statistical differences ($P \leq 0.05$) were found between all inoculation groups, the IVAX vaccine was observed to be less pathogenic. 88-627 was comparable to the TVAX vaccine in pathogenicity. Figure 3 depicts the mean daily score of clinical signs. Statistical differences ($P \leq 0.05$) were found for field isolate 88-627 on days 7 and 8 PI when analyzed against all other inoculation groups. This indicated an increase level of sickness as compared to the IVAX and TVAX vaccines. We do not believe this is accurate since a different person was used to determine the scoring number corresponding to clinical signs presented in infected birds. Although statistical differences were only found on two days (7 and 8 days PI), the mean daily score observes that the IVAX vaccine is less pathogenic as compared to 88-627 and the TVAX vaccine. Isolate 88-627 and the TVAX cause a similar degree of sickness as also confirmed by % susceptibility.

Blood was collected from the major wing vein on day 14 PI. Serum antibody titers were determined by ELISA. Geometric mean titers for the 88-627, TVAX, IVAX, and control groups were: 3417, 1243, 1766, and 2, respectively. As expected all groups seroconverted except for the control.

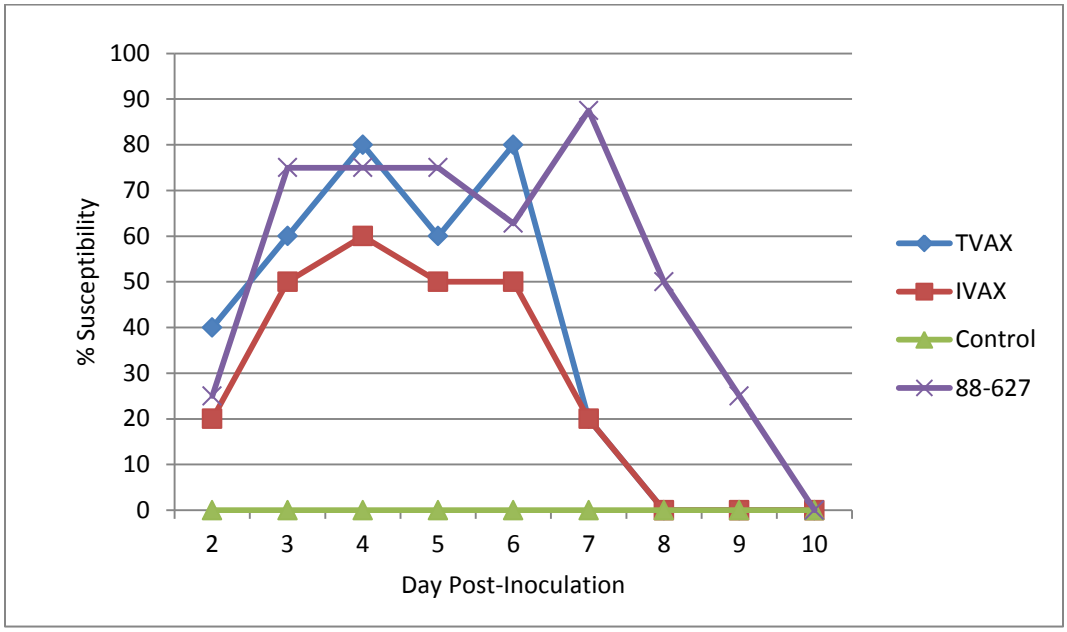


Figure 2. Susceptibility of broiler chickens infected with ILTV. Broiler chickens were inoculated with either field isolate 88-627, a CEO vaccine (TVAX), a TCO vaccine (IVAX) or were mock infected (control). Chickens were scored for clinical signs 2-10 days PI. Birds that presented a score of 1 or higher were considered sick on that day.

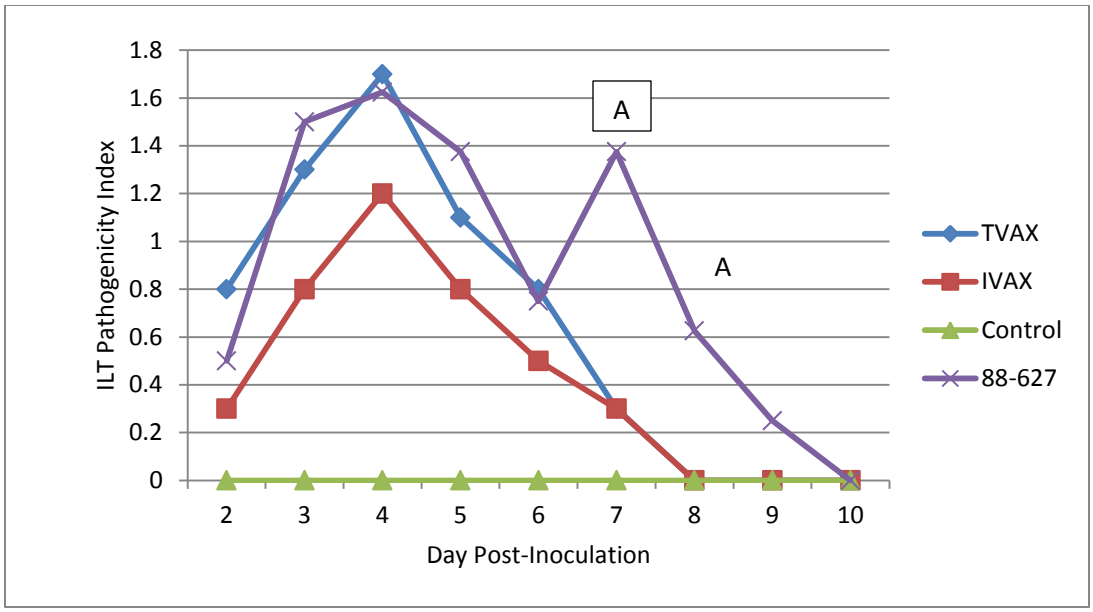


Figure 3. Severity of ILT clinical signs for chickens infected with 88-627. Broiler chickens were inoculated with either field isolate 88-627, a CEO vaccine (TVAX), a TCO vaccine (IVAX) or were mock infected (control). Chickens were scored for clinical signs 2-10 days PI. The pathogenicity index represents the mean daily score. A one way analysis of variance was performed to determine statistical significance at the 5% level ($P \leq 0.05$). (A), statistically different from all other inoculation groups.

3.3 Pathogenicity Testing of UDCEOD1/CEL20

An ILTV CEO vaccine derivative (UDCEOD1) was passaged 29 times in 15-day old chicken embryos liver cells (CEL) as described in Materials and Methods to create UDCEOD1/CEL20.

UDCEOD1/CEL20 DNA was amplified through ILTV and adenovirus PCR as also described in Materials and Methods. ILTV and adenovirus PCR products ran on a 0.8% agarose gel and was electrophoresed for 1.5 hrs at 60 volts. As shown in Figure 4, Lane M contains an E-Gel 1 kb DNA Plus ladder to size DNA fragments on the gel. In lane 1, a UDCEOD1/CEL20 ILTV PCR product corresponding to 1847 bp was observed indicating the presence of ILTV. In lane 3, a UDCEOD1/CEL20 adenovirus PCR product was not observed at 897 bp indicating the absence of adenovirus. 792 P1 served as a positive control in lane 2 (ILTV) and lane 4 (adenovirus). A band was observed at 1847 bp and 897 bp, respectively.

Sixteen chickens were inoculated separately with the USDA challenge virus and UDCEOD1/CEL20. Nine chickens were inoculated separately with the UDCEOD1 and TVAX vaccine. Thirteen chickens were mock infected (control). Clinical signs were scored 2-11 days PI. Clinical signs ranging in severity were observed in all groups and included nasal discharge, conjunctivitis, and open mouth breathing. No mortality was recorded during the course of the trial. Total susceptibility (number of sick birds/total number of birds per group) was observed to be 70% (UDCEOD1/CEL20) and 100% (UDCEOD1, USDA, and TVAX vaccine). No morbidity was observed in the control

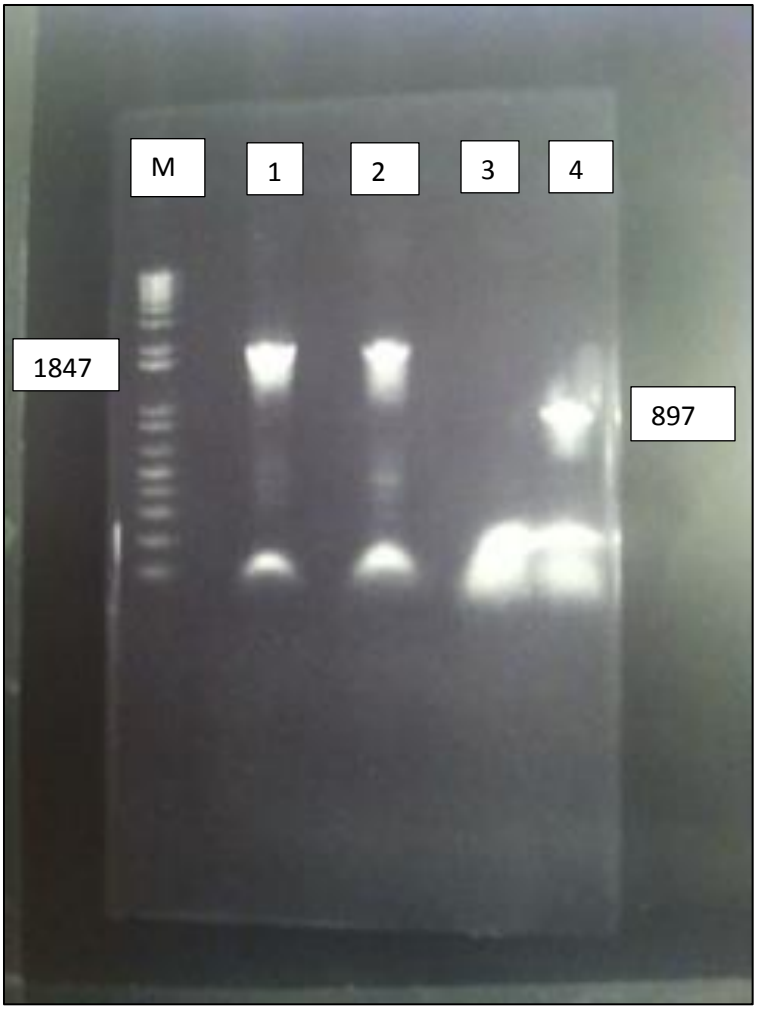


Figure 4. ILTV and adenovirus PCR. M: E-Gel 1 Kb DNA Ladder; lane 1: UDCEOD1/CEL20 ILTV PCR; lane 2: field isolate 792 ILTV PCR; lane 3: UDCEOD1/CEL20 adenovirus PCR; lane 4: field isolate 792 adenovirus PCR.

Group. As shown in Table 5 birds were 40% susceptible to the UDCEO1/CEL20 strain by day 4 PI and remained that way until day 7 PI. As expected birds were 100% susceptible to the TVAX vaccine and USDA virus by day 3 PI and day 5 PI, respectively. 100% of the birds were also susceptible to UDCEOD1 by day 5 PI. Although statistical differences ($P \leq 0.05$) were not observed, UDCEOD1/CEL20 is considerably less pathogenic from all other inoculation groups (not including the control) on days 2-8 PI. Figure 6 depicts the mean daily scores of clinical signs. Following statistical analyses of mean daily score, UDCEOD1/CEL20 was statistically lower ($P \leq 0.05$) in pathogenicity from the USDA virus strain on 2, 3, 4, 5, 7, and 11 days PI. UDCEOD1/CEL20 was statistically lower ($P \leq 0.05$) in pathogenicity from the UDCEOD1 on 3, 4, 5, and 6 days PI. UDCEOD1/CEL20 was statistically lower ($P \leq 0.05$) in pathogenicity from the TVAX vaccine on 3 and 4 days PI. All birds were challenged with the USDA strain 14 days PI. As expected the mock infected birds (control) developed clinical signs and reached 87.5% morbidity. The TVAX group observed 14.28% morbidity. Normally CEO vaccines provide full protection. The UDCEOD1 and UDCEOD1/CEL20 strains were 100% protected from the USDA challenge virus.

Birds were sacrificed from the USDA and UDCEOD1/CEL20 inoculation group on days 2, 3, 4, 5, 7, and 10 PI as well as from the control group on days 2, 4, 7, and 10 days PI. Trachea and eyelids were collected and examined for INIB and syncytia. As shown in Table 3 only one eyelid (USDA, day 5 PI)(Figure 7) and one trachea (USDA, day 4 PI)(Figure 8) exhibited INIB and syncytia.

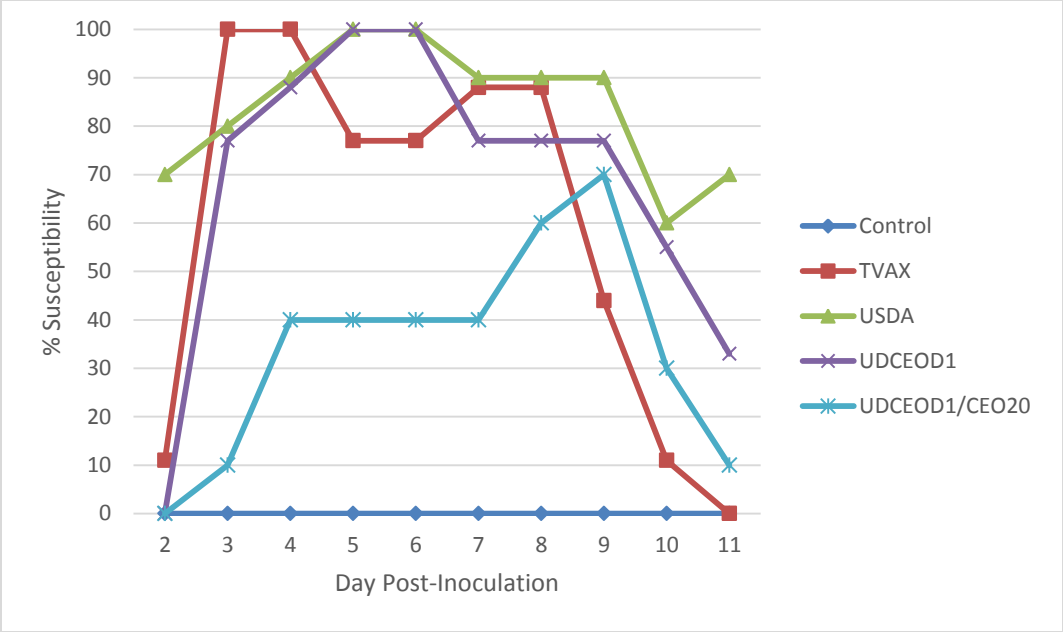


Figure 5. Susceptibility of broiler chickens infected with ILTV. Broiler chickens were inoculated with either UDCEOD1, UDCEOD1/CEL20, USDA, CEO vaccine (TVAX), or were mock infected (control). Chickens were scored for clinical signs 2-10 days PI. Birds that presented a score of 1 or higher were considered sick on that day.

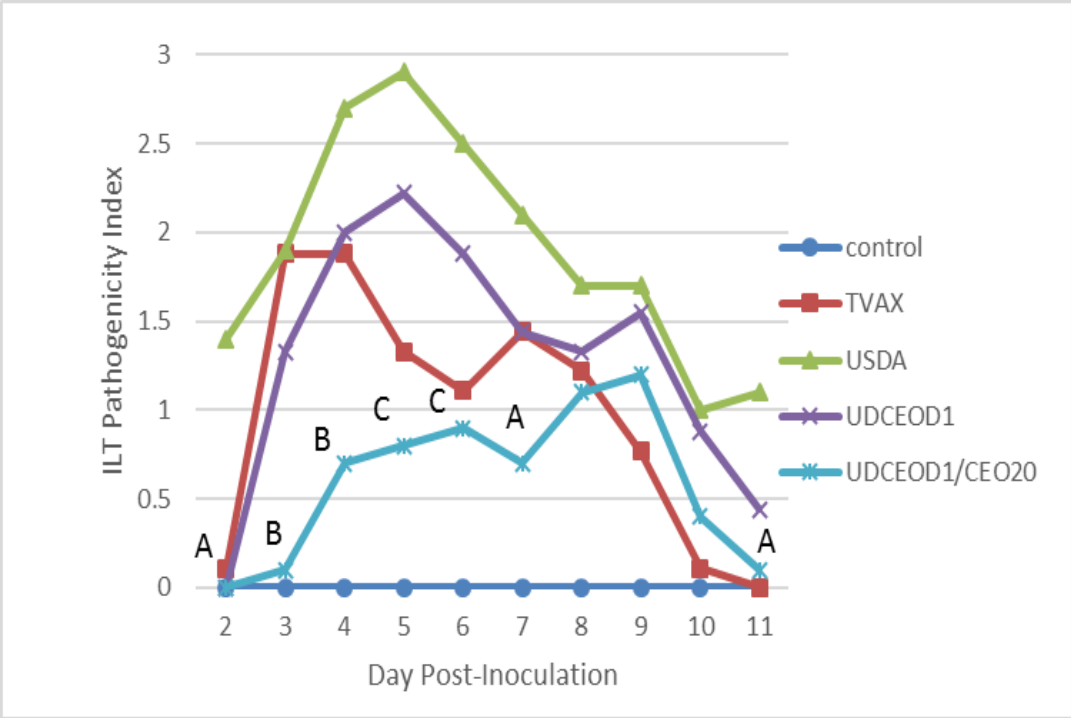


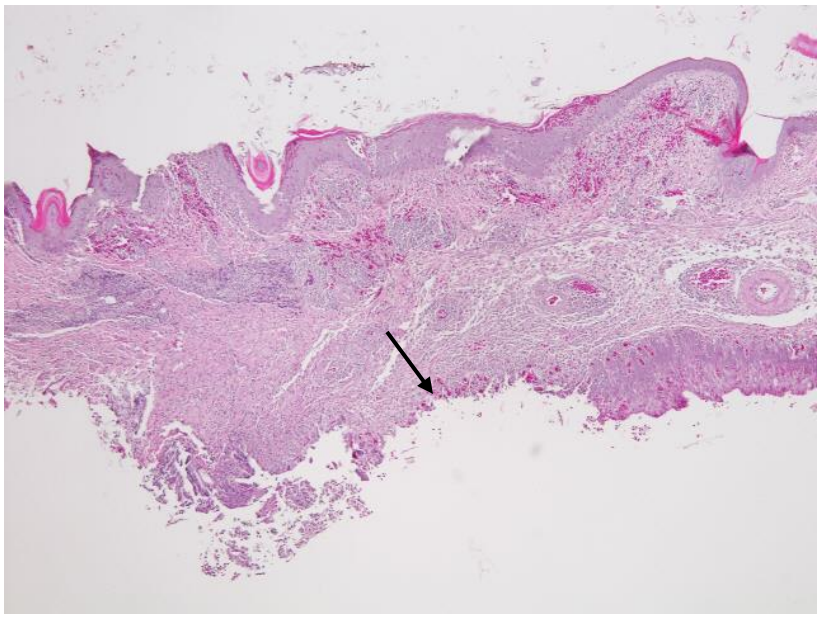
Figure 6. Severity of ILT clinical signs in broiler chickens infected with UDCEOD1/CEL20. Broiler chickens were inoculated with either UDCEOD1, UDCEOD1/CEL20, USDA, CEO vaccine (TVAX), or control (untreated). Chickens were scored for clinical signs 2-10 days PI. The pathogenicity index represents the mean daily score. A one way analysis of variance was performed to determine statistical significance at the 5% level ($P \leq 0.05$). (A), statistically different from the USDA virus. (B), statistically different from the USDA virus, the TVAX vaccine, and UDCEOD1. (C), statistically different from the USDA virus and UDCEOD1.

Table 3. Histologic examination of tracheas (tr) and eyelids (el) from broiler chickens infected with UDCEOD1/CEL20. Tracheas and eyelids were collected at the days indicated post-inoculation as described in Materials and Methods. Specimens were observed for characteristic herpesviral intranuclear inclusion bodies and syncytia.

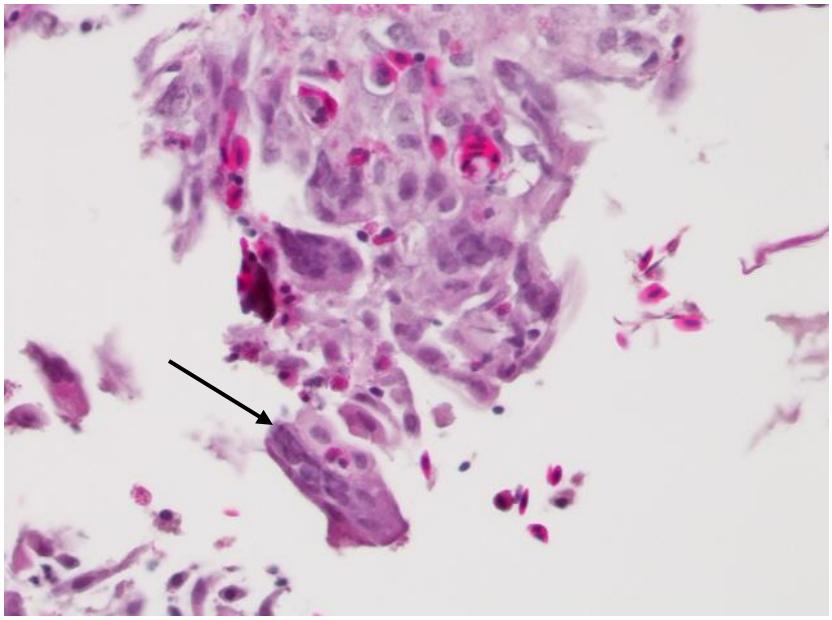
Inoculation group	2 days PI		3 days PI		4 days PI		5 days PI		7 days PI		10 day PI	
	tr	el	tr	el	tr	el	tr	el	tr	el	tr	el
USDA	0/1[a]	0/1	0/1	0/1	1/1	0/2	0/1	1/1	0/1	0/2	0/1	0/2
UDCEOD1/CEL20	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1	0/2	0/1	0/2
Control	0/1	0/1	ND	ND	0/1	0/1	ND	ND	0/1	0/2	0/1	0/1

[a] Positive INIB samples/total sample

ND- Not determined

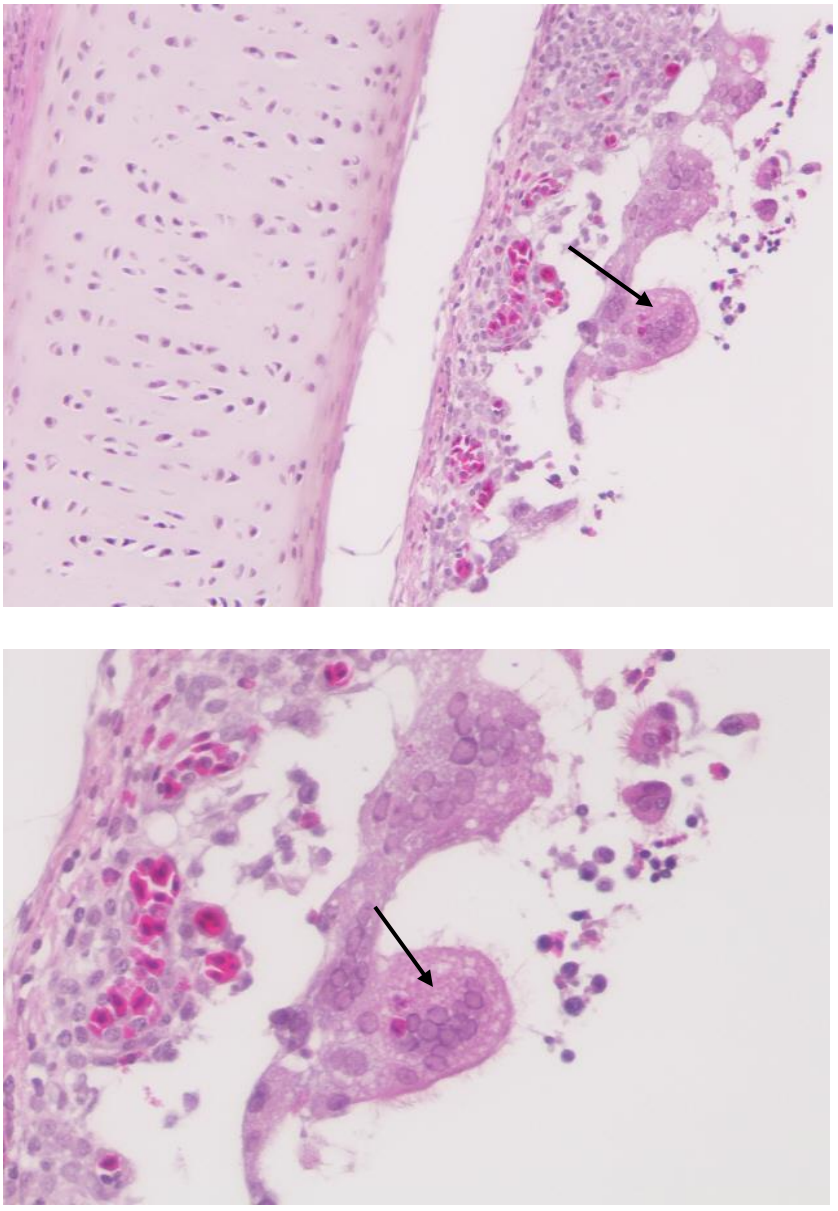


A



B

Figure 7. Eyelid from a broiler chicken infected with USDA virus 5 days PI (H&E stain): Affected conjunctival epithelium displays ulcerative-erosive changes which is infiltrated by a mixture of lymphocytes, plasma cells, and heterophils (A) 40x magnification. Epidermis is present on the top and conjunctival epithelium is on the bottom (B) 400x magnification. Formation of pathognomonic herpesviral epithelial syncytia and intranuclear eosinophilic inclusion bodies.



A

B

Figure 8. Trachea collected from a broiler chicken infected with USDA virus 4 days PI (H&E stain): Formation of pathognomonic herpesviral epithelial syncytia and intranuclear eosinophilic inclusion bodies at (A) 200x and (B) 400x magnification. Affected epithelium is ulcerated from underlying mucosa which is infiltrated by a mixture of lymphocytes, plasma cells, and heterophils.

3.4 DNA Sequence of ILTV UDCEOD1/CEL20

Sequencing of the UDCEOD1/CEL20 viral genome was accomplished using the Illumina sequence-by-synthesis method (see Materials and Methods) which generated a total of 42,457,948 reads. The UDCEOD1/CEL20 sequence was aligned against the chicken genome and 15,307,664 reads were removed. The remaining 27,150,284 reads were aligned against a ILTV CEO TVAX vaccine sequence using DNA (Genbank accession #JN580313). 6,536,386 reads were assembled into a UDCEOD1/CEL20 genome. The UDCEOD1/CEL20 ILTV genome was determined to be 153,694 nucleotides in length with an average depth of coverage of 2,090. Three single-nucleotide polymorphisms (SNPs) were identified in the ILTV UDCEOD1/CEL20 genome when compared against ILTV CEO TVAX vaccine sequence (Table 4). The nucleotides in reference positions 118,177 (T), 135,011 (C), and 152,543 (A), were called as C, T, and G, respectively in UDCEOD1/CEL20. Reference position 118,177 and 152,543 both corresponded to the ICP4 gene, located within the inverted repeat of the ILTV genome. This SNP results in an amino change from a threonine (ACT) to an alanine (GCT) (amino acid 1,132). Reference position 135,011 is within the US5 gene (glycoprotein J) and results in no amino acid change (isoleucine at amino acid 258).

Table 4. Single nucleotide polymorphisms (SNPs) of the UDCEOD1/CEL20 sequence. The UDCEOD1/CEL20 sequence was compared to the ILTV CEO TVAX vaccine sequence (Genbank accession # JN580313). SNPs were analyzed for gene correspondences and amino acid changes within the gene as described in Materials and Methods.

Reference Position	Reference Base	Called Base	Gene	Amino Acid Change
118177	T	C	ICP4	Threonine-Alanine
135011	C	T	US5-Glycoprotein J	Isoleucine-Isoleucine (no-change)
152543	A	G	ICP4	Threonine-Alanine

Chapter 4

DISCUSSION

The chicken industry relies on the use of tissue culture origin (TCO) vaccines, chicken embryo origin (CEO) vaccines, and recombinant vaccines to control active outbreaks of ILT . Although proven successful in the reduction of the disease, TCO vaccines do not provide adequate protection. CEO vaccines are suggested to increase in virulence upon bird passage and are difficult to distinguish from field isolates [38]. Both TCO and CEO vaccines can result in latent viral infections [51]. Recombinant vaccines, although safer, take several weeks to induce a protective immune response. However, there is a need and an eagerness to develop a new live attenuated vaccine strains of ILTV due to the several reasons listed in the introduction. For the present study, our goal was to identify a strain of ILTV that demonstrates both reduced pathogenicity and virulence. Two approaches were used to identify a strain of reduced pathogenicity. Field isolates exhibiting low pathogenicity were identified from the LIMS database and attempts were made to attenuate a current CEO vaccine by passage in CEL tissue culture.

4.1 Identification of Field Isolates of ILTV as Potential Vaccine Candidates

Two field isolates (88-627 and 11-11349) were identified for evaluation for *in vivo* bird studies. Field isolate 88-627 was evaluated in this study. From the pathogenicity study strain 88-627 has been proven a poor candidate for the use as a potential modified live vaccine for commercial use to control outbreaks of ILT. Field isolate 88-627 demonstrated high morbidity during the course of the trial (75%).

Although statistical significance was not determined between the commercially-available vaccines and field isolate 88-627, in regards to pathogenicity, the data suggests that isolate 88-627 is similar in severity of clinical signs to currently used CEO vaccines.

Our approach to identifying potential vaccine strains of ILT through the LIMS database was problematic. Recorded mortality of the flock may be misleading. Clinical samples submitted from flocks only represent a single time point in the stage of disease. It is possible that field isolates such as 88-627 were submitted in the initial stages of the disease and an increase in morbidity would have been reported later during the course of infection. Secondly, there were contamination issues of adenovirus with many of the field isolates. All samples needed to be tested for adenovirus for reliable confirmation.

4.2 Attenuation of ILTV by Passage in Tissue Culture

An attempt was made to attenuate an ILTV CEO vaccine derivative (UDCEOD1) by passaging 20 times in 15-day old chicken embryos liver cells to create UDCEOD1/CEL20. UDCEOD1/CEL20 was found to be a potential vaccine candidate due to its low pathogenicity when evaluated in birds. On days 2, 3, and 4 PI, UDCEOD1/CEL20 is statistically ($P \leq 0.05$) less pathogenic than the virulent USDA challenge virus and the moderately pathogenic TVAX vaccine. UDCEOD1/CEL20 also protected 100% of the birds when challenged with the USDA strain. However, this study should be repeated and evaluated in a larger group of birds. A larger sample size would improve the statistical analysis of the results.

Trachea and eyelid samples from infected birds were analyzed for herpesviral intranuclear inclusion bodies and syncytia. Two samples (USDA, day 5, eyelid) (USDA,

day 4, trachea) presented INIB and syncytia. It is not uncommon in experimental studies for specimens not to observe INIB when analyzed. The USDA likely exhibited INIB due to its high level of virulence. The TVAX and the UDCEOD1/CEL20 strain are observed to be less virulent in studies and therefore INIB are likely to be absent. The absence of INIB could also be due to day of sampling, the sampling site, or the route of inoculation.

The UDCEOD1/CEL20 genome was sequenced and was found to be 153,694 nucleotides in length. Three SNPs (118,177, 135,011, and 152,543) were identified in the ILTV UDCEOD1/CEL20 genome when compared to ILTV CEO TVAX vaccine sequence (Genbank accession # JN580313). Nucleotide 135,011 of the ILTV UDCEOD1/CEL20 genome corresponds to glycoprotein J. Glycoprotein J is encoded by the US5 gene located within the unique short region. Within glycoprotein J gene the change from a "C" to a "T" resulted in a silent mutation (isoleucine to isoleucine). Mundt et al., [60] demonstrated that glycoprotein J was involved in the egress of the virions through kinetic studies while Fuch et al., [61] showed that glycoprotein J is directly involved in the spread of ILTV. A mutant ILTV lacking the glycoprotein J gene exhibits reduced virus titer when measured by plaque size. However, this SNP does not alter the sequence of glycoprotein J.

Nucleotides 118,177 and 152,543 are located within the ICP4 gene. This gene is present in two copies, as it is in inverted repeats of the ILTV gene [62]. ICP4 is suggested to play a role in the regulation of immediate early protein synthesis and the shift from immediate early to early protein synthesis [63]. Within the ICP4 gene, this SNP resulted in an amino acid change from a threonine (polar, hydrophilic) to an alanine (nonpolar,

hydrophobic). Both copies of ICP4 in UDCEOD1/CEL20 contain the same SNP as expected. Due to the critical role of ICP4 in herpesvirus growth and replication this mutation may have functional significance. It is possible that this SNP results in a mutations that effects ILTV immediate early gene expression making it less virulent. This strain should be further characterized with regards to its growth characteristics and the functionality of its ICP4 protein.

Chapter 5

CONCLUSION

Future research should focus on evaluating field isolate 11-11349 which has been identified as a potential ILTV vaccine candidate as outlined in table 2. Using the criteria outlined in Material and Methods, 44 other field isolates have been identified for further consideration. These isolates should be further characterized by ILTV PCR, adenovirus PCR, or growth in eggs. Field isolates positive for ILTV and negative for adenovirus should be evaluated in birds. Field isolates demonstrating similar susceptibility to the disease, severity of clinical signs, and duration of recovery to the IVAX vaccine would then be evaluated for their ability to elicit a protective immune response when challenged by a virulent USDA strain.

CEL passages should also be continued in order to further attenuate the ILTV CEO vaccine derivative (UDCEOD1), however it may take hundreds of more passages before a potential vaccine candidate is identified. Garcia et al. has recently reported (personal communication) an attempt to attenuate virulent field isolate of ILTV by passing 100 times in CEL.

Ultimately, screening ILTV field isolates and serial passage of an CEO vaccine derivative could potentially overcome current shortcomings of commercially available vaccines. This research could aid in the development of a novel ILT vaccine that is low in pathogenicity and provides good coverage.

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Appendix

UNIVERSITY OF DELAWARE

COLLEGE OF AGRICULTURE AND NATURAL RESOURCES

AGRICULTURAL ANIMAL CARE AND USE COMMITTEE

Application for Use of Agricultural Animals

In Teaching or Research

AACUC Protocol Number: (14)04-27ORa

TITLE OF PROJECT: Integrative approaches for ILTV vaccines

INSTRUCTOR/PRINCIPAL INVESTIGATOR

Calvin L. Keeler, Jr.	[signature]	4/21/10
Printed Name	Signature	Date

(This section for Committee use only)

Application Approved (date) 5-12-2010

Application Rejected (date) _____

Reason for Rejection _____

[signature]	5-12-2010
Signature, Animal Care and Use Committee	Date