ABSTRACT

Title of Document: REVIEW OF THE MOLECULAR BIOLOGY AND EPIDEMIOLOGY OF INFECTIOUS LARYNGOTRACHEITIS (GALLID HERPESVIRUS-1)

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A review of the molecular biology and epidemiology of avian infectious laryngotracheitis (ILT) is conducted due to the outdated state of current ILT review material. The objective of this review is to include updated information on the molecular biology of Gallid herpesvirus 1 (GaHV-1), the causative agent of ILT, and to present the latest information on the molecular epidemiology of ILT. Recent developments in molecular biology specific to GaHV-1 have been made and are highlighted in this review, and the role of current and historical use of live-attenuated vaccines is associated with the global and molecular epidemiology of ILT. Also, target genes for detection and strain differentiation are compiled by region of the world, and the global distribution of ILT is illustrated. Additionally, the field of epigenetics related to virus-host interactions is reviewed, and the molecular, epidemiologic, and epigenetic factors investigated are related to prospects for future eradication of ILT.

REVIEW OF THE MOLECULAR BIOLOGY AND EPIDEMIOLOGY OF
INFECTIOUS LARYNGOTRACHEITIS (GALLID HERPESVIRUS-1)

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Dedication

For my parents, who always let me keep every animal that I brought home and who have

been a source of unending encouragement.

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Chapter 1: Introduction

Rationale and Objectives

Infectious laryngotracheitis (ILT) is an upper respiratory tract disease of chickens, pheasants, and peafowl caused by the alphaherpesvirus Gallid herpesvirus 1 (GaHV-1) (Guy & Garcia, 2008). The virus is shed in respiratory secretions, easily transmitted from bird-to-bird by inhalation of secreted droplets, and commonly carried and additionally transmitted by poultry facility workers and fomites. ILT is characterized by acute respiratory disease and mild to severe clinical signs involving the upper respiratory tract including conjunctivitis, nasal discharge, coughing, sneezing, and expectoration of bloody mucous. ILT occurs worldwide and severity of clinical signs and mortality rate, which can range from 0-70%, depending on virulence of the infective strain (Oldoni *et al.*, 2009). The virus is responsible for frequent outbreaks in high-density poultry producing areas, which are often associated with large economic losses (Bagust *et al.*, 2000).

Since the initial description of ILT in the early 20th century, the disease has remained a problem for the global poultry industry, causing morbidity and mortality related loses each year. Both the layer and broiler industries are affected, although the broiler industry is affected to a larger extent. In the vertically integrated broiler industry, large companies contract growers to raise their birds using company specifications, during which time a grower incurs any loss during the grow-out process. As a result, ILT related losses specifically affect the individual grower. Current control measures, including chicken embryo origin (CEO) and tissue culture origin (TCO) live-attenuated

vaccines, reduce the impact of losses. However, they often perpetuate release of live

virus into the field leading to virulence reversion of vaccine virus, which can cause

outbreaks of vaccinal laryngotracheitis (VLT) (Dufour-Zavala, 2008). However, based

on the low cost and time efficiency of live-attenuated vaccination on a per-bird basis,

contract growers continue to select these vaccines to reduce morbidity and mortality

related losses.

Progress in the areas of ILT prevention and control would significantly decrease

health related production losses in these sectors of animal agriculture, and development

of improved or novel vaccines will ultimately guide these improvements. However

characteristics relating to the molecular biology and epidemiology of GaHV-1 remain

undefined and limit ILT technological advances. While much is known about the

structure of the virus and its genome, the mechanisms responsible for virulence reversion

are yet to be fully understood. Recently, investigation into attenuation and virulence

reversion has begun, and further investigation characterizing genes involved in viral

pathogenesis is a primary focus of current ILT genetics.

In addition to the fields of ILT molecular biology and epidemiology, recent

epigenetic study on host-pathogen interactions of the ILT and chicken genomes has been

launched. Aimed at the transcriptional level of the genome and invested in elucidating

the effects of infection on host gene expression, ILT epigenetics seeks to identify those

factors involved in viral pathogenesis and host resistance to infection. Additionally,

GaHV-1 contains many genetic differences from other related herpesviruses and has host

tropism with high specificity for chickens, indicating potential for ILT-specific

mechanisms to be discovered.

With novel technologies opening new investigative pathways, much of the review literature available for ILT is out of date. The aim of this literature review is to bring together new information, and to touch upon six major issues related to ILT molecular biology, epidemiology, and epigenetics.

1. Review GaHV-1 molecular biology.

2. Outline the historical implications of live-attenuated vaccine development in shaping current molecular epidemiology of GaHV-1.

3. Outline the current implications of live-attenuated vaccine use in shaping the current global and molecular epidemiology of GaHV-1.

4. Review the global and molecular epidemiology of the GaHV-1.

5. Review the current epigenetic findings involving host-virus interactions.

6. Outline the significance of molecular biology, epidemiology, and epigenetics in future eradication of ILT.

Chapter 2: Molecular Biology of Gallid Herpesvirus-1

2.1 Viral Morphogenesis and Chemical Composition

In 1931, experiments first defined the causative agent of ILT as a filterable virus (Beach, 1931), and the nucleic acid content was confirmed to be DNA and similar to that of the herpesvirus group (Tannock, 1965). The molecular weight of the virus was estimated by restriction endonuclease fragment summation and approximated to be between 102.1 x 10^6 to 97.35 x 10^6 Daltons (Kotiw *et al.*, 1982). Electron microscopy confirmed the typical herpesviral morphology of GaHV-1 (Figure 1), and the virus consists of an icosahedral DNA containing capsid at its core and is surrounded by a tegument layer and an outer envelope with embedded surface glycoproteins (Fuchs *et al.*, 2007).

2.2 Viral Genome

The genome of GaHV-1 is composed of a linear, double-stranded DNA molecule. Among the seventeen complete genome sequences currently available on GenBank, the size of the GaHV-1 genome ranges from 148-kb to 155-kb, with size variations attributed to single nucleotide polymorphisms (SNPs) as well as insertions and deletions (INDELs) between strains (Lee *et al.*, 2011a.b; Chakma *et al.*, 2012; Lee *et al.*, 2012; Spatz *et al.*, 2012). The genome is comparable to that of other alphaherpesviruses and contains the prototypic unique long (U_L) and unique short (U_S) regions. However, it does not contain characteristic repeat regions flanking the U_L region of the genome (Waidner *et al.*, 2011), and instead inverted repeats flank only the U_S region (Guy & Garcia, 2008) (Figure 2).
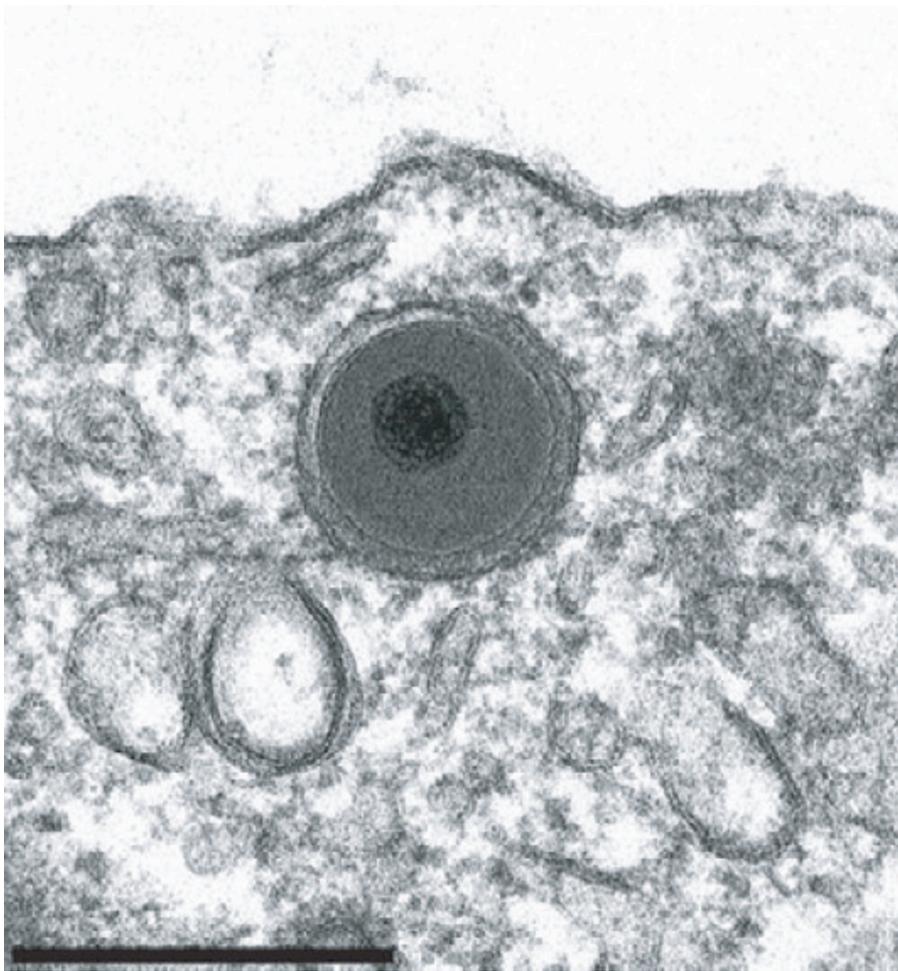
Figure 1: Electron microscopy from Fuchs *et al.* (2007) of GaHV-1 virion exiting the host cell via exocytosis from chicken Leghorn male hepatoma (LMH) cells 18 hours post infection. Bar represents 300 nm.
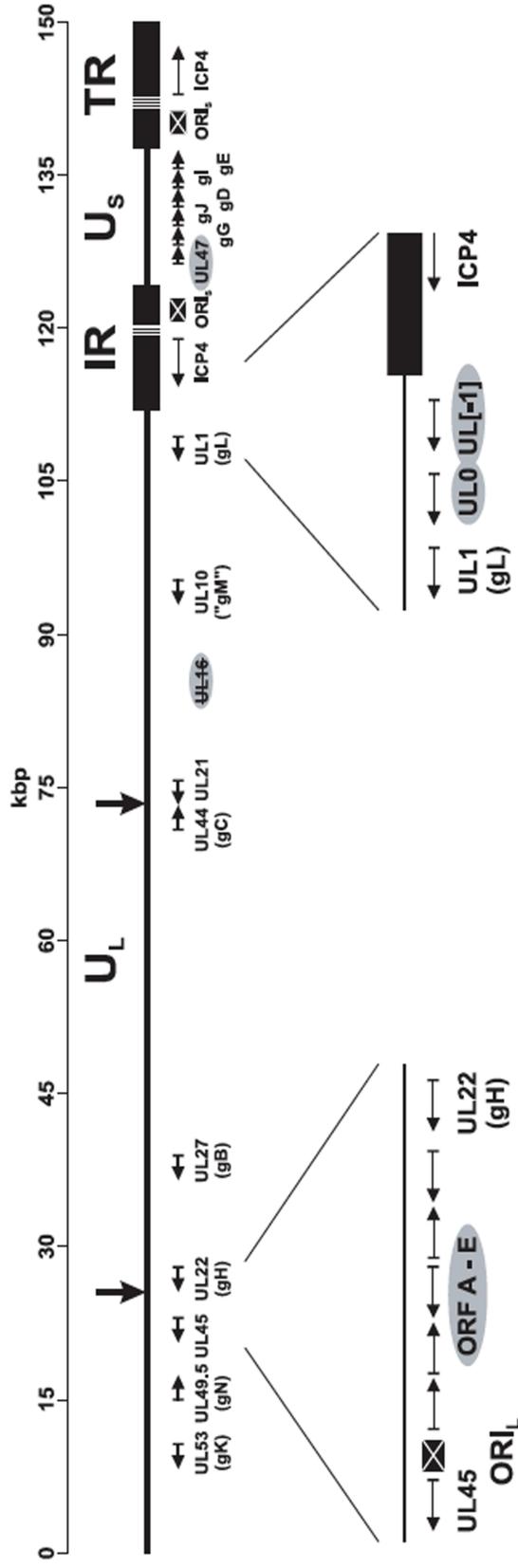
Figure 2: Map of the dsDNA genome of GaHV-1 created by Fuchs et al. (2007) illustrating the unique long and unique short genome regions (U_L, U_S), and the inverted repeats (IR, TR) containing repeat elements (vertical lines). Unique to GaHV-1 and PsHV-1 include the inverted repeat region within the US region between the UL22 and UL44 gene region (vertical arrows), as well as ORF A-E and the paralogous UL0 and UL[-1] genes (highlighted). Specifically unique to the GaHV-1 genome is absence of the UL16 gene and translocation of the UL47 gene (highlighted). Horizontal arrows represent other genes, and the origins of replication are also identified (ORI_L, ORI_S).

Seventy-seven open reading frames (ORFs) encode for either predicted or demonstrated proteins (Guy & Garcia, 2008), sixty-three of which share homology with genes of herpes simplex virus 1 (HSV-1) (Fuchs *et al.*, 2007). The UL3.5 ORF of the GaHV-1 genome is not present within the HSV-1 genome, but is common to other alphaherpesviruses such as porcine pseudorabies virus (PrV) and varicella zoster virus (VZV) (Fuchs *et al.*, 2007). GaHV-1 contains many other unique genomic characteristics, indicating its phylogenetic divergence from other alphaherpesviruses, starting with the absence of a typically highly conserved UL16 gene homolog (Fuchs & Mettenleiter, 1999). The viral genome also contains a large internal inversion similar to one found within the genome of PrV but absent in alphaherpesviruses such as HSV-1, VZV, and equine herpesvirus 1 (EHV-1), and this internal inversion is comprised of a gene cluster spanning from the UL22 to the UL44 ORFs of the U_L region (Ziemann *et al.*, 1998a). A UL47 homolog, typical of the U_L region of many alphaherpesviruses, is absent in the equivalent region of the GaHV-1 genome and is instead translocated between the US3 and US4 ORFs of the U_S region (Helferich *et al.*, 2007c). Five ORFs in the U_L region, ORF A to ORF E, are unique to both GaHV-1 and psittacid herpesvirus (PsHV-1), an alphaherpesvirus of psittacine birds (Thureen & Keeler, 2006). In addition to these 5 ORFs, GaHV-1 and PsHV-1 share similarities in the region between the UL22 and UL44 ORFs, as well as the translocation of the UL47 ORF, defining these viruses as the only two members of the family *Iltoviridae* (Thureen & Keeler, 2006). Lastly, a paralogous pair of genes, UL0 and UL[-1], represent a unique duplication in the GaHV-1 genome and are evidence of an evolutionary duplication of a spliced GaHV-1 distinct

gene (Ziemann *et al.*, 1998b). These characteristics of the GaHV-1 genome are also illustrated in Figure 2.

2.3 Viral Replication

While GaHV-1 entry has yet to be fully explained, structural glycoprotein C (gC) has been confirmed as an accessory entry protein (Pavlova et al., 2010). Based on HSV-1, other proteins involved in attachment and entry include structural glycoproteins gB, gD, gH, and gL, the process beginning with gB or gC interaction with heparin sulfate proteoglycans, followed by interaction of gB, gD, and a gH-gL complex to trigger merger of viral and cellular membranes and release of the tegument and nucleocapsid into the host cell cytoplasm (Akhtar & Shukla, 2009; Thureen & Keeler, 2006). However, an apparent disparity in the entry process exists, as GaHV-1 entry is most likely heparin-independent, pointing to a pathway alternative to that of HSV-1 (Pavlova e*t al.*, 2010).

Following release into the cytoplasm, the nucelocapsid is transported to the nuclear membrane where viral DNA is released, allowing for migration of viral DNA into the nucleus via nuclear pores where transcription and replication of viral DNA occur (Guy & Garcia, 2008). Gene expression has been displayed to occur in a cascade pattern similar to other alphaherpesviruses (Figure 3) (Prideaux *et al.*, 1992), and much information about GaHV-1 DNA replication has also been adapted from HSV-1. Immediately-early (IE or alpha) genes are the first to be expressed in the nucleus of infected cells, the protein products of which stimulate expression of early (E or beta) genes required for DNA replication, subsequently stimulating expression of late (L or gamma) genes encoding for viral structural proteins (Knipe & Cliffe, 2008).
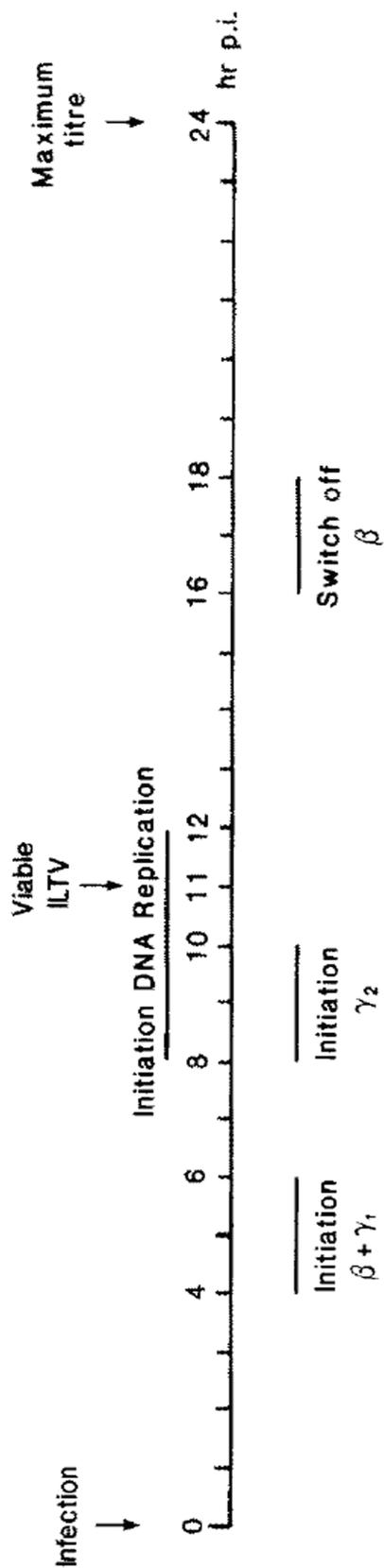
Figure 3: Cascade pattern of GaHV-1 gene expression from Prideaux et al. (1992) illustrating growth kinetics and viral replication following chicken kidney (CK) cell culture infection. Following infection, β and γ1 genes are expressed beginning at 4 hpi, while γ2 gene expression begins 8hpi. α gene expression is required for β and γ gene expression and begins prior to their expression (not pictured).

GaHV-1 nuclear egress begins with translocation of the capsid through the nuclear membrane after pro-capsid packaging of monomeric DNA, followed by addition of an envelop from the inner membrane of the host cell nucleus and movement to the lumen of the endoplasmic reticulum into vacuoles within the cytoplasm (Guo *et al.*, 1993). Mature capsid-less particles are formed in the *trans*-Golgi region of the cytoplasm, where assembly of tegument and secondary envelopment occur, and infectious virions are subsequently released by exocytosis (Fuchs *et al.*, 2007). *In vitro* replication kinetics have shown DNA replication beginning between 8 and 12 hours post infection (hpi), with exponential increases in virus titer between 11 and 24 hpi, indicating the peak of viral replication within this period (Prideaux *et al.*, 1992).

2.4 Viral Proteins

The cascade pattern of GaHV-1 protein expression begins with a short period of alpha (α) polypeptide expression, followed by beta (β) polypeptide expression between 4 to 16 hpi, and gamma (γ) polypeptide expression divided into γ1 and γ2 expression maintained from 4 and 8 hpi respectively (Prideaux *et al.*, 1992). α products are non-structural polypeptides responsible for regulation of β and γ gene products, and are additionally self-dependent on their own production for downregulation of transcription. β products include enzymes critical for DNA synthesis such as DNA polymerase and thymidine kinase (TK), and γ products include structural proteins such as surface glycoproteins (Post *et al.*, 1981; Prideaux *et al.*, 1992).

2.4.1 Alpha and Beta Proteins

Non-structural proteins, majority of which are expressed as α and β proteins, are

critical to regulatory functions of viral infection. While description of these proteins specific to GaHV-1 is far from complete, functional characterizations exist, some of which have been adapted from other herpesviruses.

α genes are able to express in the absence of protein synthesis and consist of regulatory genes such as the infected cell protein (ICP) family. The GaHV-1 gene coding for ICP4 is the only ICP described in detail for GaHV-1 and shares sequence and functional homology to that of HSV-1 (Johnson *et al.*, 1995c). Of the five regions of the GaHV-1 ICP4 protein, two exist with considerable homology to other alphaherpesviruses, with region 4 the most conserved and critical to γ gene expression. Least conserved, region 5 exists as a comparatively larger protein region in GaHV-1 and contains a second serine run, indicating additional sites for phosphorylation and increased overall activation potential when compared to other herpesviruses. Further functional investigation of GaHV-1 ICP4 has yet to be done, however ICP4 resides in the nucleus of HSV-1 infected cells, mediates the switch from α to β and γ expression, mediates down-regulation of its own expression following the onset of viral protein expression, and requires minimal promoters containing simple TATA homologies for transactivation (Dixon & Schaffer, 1980; Knipe *et al.*, 1987; Smith *et al.*, 1993; Helferich *et al.*, 2007a). In addition to ICP4 description, a region homologous to HSV-1 ICP27 has been described for GaHV-1, while sequence homologies of the UL3 and UL4 products suggest colocalization of these proteins with ICP22 in GaHV-1 nuclear inclusion bodies as seen with other herpesviruses (Johnson *et al.*, 1995b; Fuchs & Mettenleiter, 1996; Xing et al., 2011).

Additional amino acid sequence comparisons have revealed GaHV-1 proteins with homology to those of other herpesviruses and with specific roles in DNA replication. The UL2 product encodes two conserved amino acid stretches considered signature uracil-DNA glycosylase (UDG) sequences among alpha-, beta-, and gammaherpesviruses, and indicates that GaHV-1 UL2 retains equivalent UDG functional activity (Fuchs & Mettenleiter, 1996). Also, the GaHV-1 UL5 product contains six functionally conserved helicase motifs, predicting conserved function in DNA replication in the form of a helicase-primase subunit complexed with the UL8 and UL52 proteins as seen in other alphaherpesviruses (Fuchs & Mettenleiter, 1996; Chen et al., 2011). The GaHV-1 UL50 product shows conservation of typical deoxyuridine triphosphatase (dUTPase) function, essential to nucleotide metabolism during DNA replication, despite loss of the first five domains seen among mammalian alphaherpesviruses (Fuchs *et al.*, 2000).

Dependent on α gene expression, β gene expression results in enzymes critical for DNA synthesis. One such enzyme described for GaHV-1 is the TK gene product. With 27.9% amino acid sequence identity to HSV-1 TK, portions of the protein corresponding to the nucleotide binding domain are well conserved, while non-conserved regions suggest GaHV-1 TK to have unique substrate-binding specificities when compared to mammalian herpesviruses (Keeler, 1991).

2.4.2 Gamma Proteins

γ gene expression is responsible for structural protein products including tegument and glycoproteins. Traditionally, herpesviral structural glycoproteins have

been described as mediators of virus entry, cell fusion, and viral egress, as well as important immunogens and targets of cell-mediated immunity (Poulsen & Keeler, 1997). GaHV-1 glycoproteins share homology to those of other alphaherpesviruses, however direct characterization of these proteins specific to GaHV-1 remains incomplete. DNA sequencing has confirmed that GaHV-1 encodes structural proteins homologous to HSV-1 including gK, gN, gH, gB, gC, gM, gL, gG, gJ, gD, gI, and gE, from the UL53, UL49.5, UL22, UL27, UL44, UL10, UL1, US4, US5, US6, US7, and US8 genes respectively (Devlin *et al.*, 2006a). The first full composite genome sequence of GaHV-1 was compiled from fourteen published partial sequences (GenBank accession number NC_006623.1), with identification of genes and their functions based on that of other herpesviruses and indicative of GaHV-1 glycoproteins in functions such as virion morphogenesis, membrane fusion, cell entry, cell-to-cell spread, cell attachment, binding of complement factors, and binding of cell surface receptors (Thureen & Keeler, 2006).

Limited investigation into specific GaHV-1 structural proteins has revealed that gK is encoded from a late transcript that shares significant homology to that of HSV-1 and contains characteristics of a membrane-bound glycoprotein (Johnson *et al.*, 1995b). Conserved herpesviral gene products gM and gN have been shown to form a complex, with correct processing of O-glycosylated gN depending on complex formation with non-glycosylated gM (Fuchs & Mettenleiter, 2005). gB is initially synthesized as a 110 kDa monomeric precursor protein, which is first processed into 100 kDa subunits, and further proteolytically cleaved into two disulphide-linked species of 58 kDa each (Griffin, 1991; Poulsen & Keeler, 1997). gC and gJ have been shown to localize in the outer viral envelope (Veits *et al.*, 2003a). Additionally, gC contains low amino acid sequence

homology to other herpesviruses, as well as an extracellular charged region typical of herpesviral interaction with cell surface proteoglycans, and is a glycosylated protein product of late gene expression (Kingsley *et al.*, 1994).

The gG protein is secreted from GaHV-1 infected cells and plays a role in inflammatory response at the site of infection as a likely viral chemokine binding protein (vCKBP) (Devlin *et al.*, 2006b; Helferich *et al.*, 2007a). gI and gE of other herpesviruses have been displayed to form a heterodimer with Fc receptor binding activity, and are pivotal to cell-to-cell spread of GaHV-1 (Davis-Poynter & Farrell, 1996; Devlin *et al.*, 2006a). The role of gD as a GaHV-1 glycoprotein is based upon regions of significant homology to other herpesviruses, specifically the positioning of six cysteine residues that are conserved among all gD amino acid sequences (Johnson *et al.*, 1995a). gL functional homologues co-processed and complexed with gH can be found in many herpesviruses including GaHV-1, and presence of a N-terminal signal sequence, N-glycosylation site, and two cysteine residues suggest similar function for the GaHV-1 protein (Fuchs & Mettenleiter, 1996).

Herpesviral tegument proteins, which form the protein layer between the nucleocapsid and viral envelope, are primarily structural in function but additionally regulate functions such as capsid transport during viral entry and egress, targeting of the capsid to the nucleus, regulation of transcription, translation and apoptosis, DNA replication, immune modulation, cytoskeletal assembly, and viral assembly and egress (Kelly *et al.*, 2009). While each of these roles has not been individually described for GaHV-1, a few have been identified. The membrane associated UL11 tegument protein, located in both mature virons and cytoplasm of infected chicken cells, has been shown to

be essential for secondary envelopment of GaHV-1 (Fuchs *et al.*, 2012). UL37 as well as UL46, UL47, UL48, and UL49 are also identified GaHV-1 tegument proteins, each with unique characteristics (Helferich *et al.*, 2007a). UL37, UL46, and UL49 accumulate in the cytoplasm of syncytia but are absent from infected nuclei, confirming cytoplasmic location of tegument addition relevant to secondary envelopment of the virion. However, UL47 and UL48 accumulate in the cytoplasm and in larger portions within the nuclei of GaHV-1 infected cells, consistent with a role in viral gene expression as well as secondary envelopment. Additionally, UL48 is important in the onset of viral gene expression and enhances α gene promoters such as those for ICP4.

In addition to tegument and glycoproteins, other GaHV-1 γ genes capable of nuclear accumulation include UL0, UL[-1], and UL31. As previously described, UL0 and UL[-1] result from a duplication unique to the GaHV-1 genome, and their nuclear targeting is hypothesized to be a product of polypeptide sequences rich in arginine, with functions possibly involved in host gene expression, encapsidation of viral DNA, or as structural components of nucleocapsid assembled within the host cell nucleus (Ziemann *et al.*, 1998b). Nuclear accumulation of UL31 has been alternatively correlated with possible function in nuclear egress due to peripheral localization along the nuclear membrane of host cells (Helferich *et al.*, 2007a).

While structure-function analysis remains incomplete, protein function in relation to *in vivo* viral virulence has been investigated for a few of the GaHV-1 proteins. In the absence of gG, clinical sings, mortality, and effects on weight gain are reduced, while an increase in tracheal thickness is representative of an increase in inflammatory cell infiltration and supportive of the role of gG as a vCKBP (Devlin *et al.*, 2006b). A

decrease in clinical symptoms, in addition to a decrease in microscopic tracheal lesions, is also seen in the absence of TK (Han *et al.*, 2002), while little to no clinical signs and viral shedding occurring in the absence of UL0 (Veits *et al.*, 2003b). Similar effects occur with UL47, with a decrease in clinical signs, viral shedding, and tracheal lesions in the absence of the gene (Helferich *et al.*, 2007b). Additionally, good protection efficiency for TK, UL0, and UL47 against virulent virus challenge suggests vaccine candidacy for mutants of these genes, while *in ovo* vaccine delivery of gG deficient virus to embryos at 18 days of incubation is able to protect birds from challenge 20 days post hatch (Legione *et al.*, 2012). While these vaccines suggest safe and efficacious new vaccine candidates, the issue of vaccine virus tracheal replication and shedding is still apparent, as exemplified by gG deficient virus retention of tracheal replication, and the capacity of these viruses to revert to virulence when passed from bird-to-bird has yet to be sufficiently investigated.

2.5 Host Immunity and Viral Immune Evasion

Following GaHV-1 infection, the humoral immune response produces detectable levels of antibody. Following infection, virus-neutralizing antibodies are detectable within 5 to 7 days and peak at 21 days, after which they begin to decline but remain detectable for up to 1 year (Guy & Garcia, 2008), possibly generated by long lasting plasma cells. Secreted antibodies within the trachea are detectable beginning at 7 days, and IgA- and IgG-plasma cells begin to increase between days 3 and 7 after infection. Although a role of humoral immunity in GaHV-1 infection is apparent, the importance of cell mediated immunity over humoral is exemplified by the ability of bursectomized

birds, unable to produce specific antibodies, to resolve primary infections as efficient as birds retaining functional bursae of Fabricius (Fahey & York, 1990). Additionally, poor correlation is typically seen between antibody titers and immune status of flocks (Guy & Garcia, 2008). However, little research directly investigating the cell-mediated immune response to GaHV-1 has been done. In terms of passive immunity, maternal antibodies are transferred to offspring, however protection is not conferred and transferred antibodies do not interfere with vaccination (Hayles *et al.*, 1976).

Evaluation of enzyme-linked immunosorbent assay (ELISA) titers in birds has been a useful method of diagnosis in the past, and titers are typically detectable within 2 weeks of exposure and for 4 to 7 weeks following (Sander & Thayer, 1997). However, currently more precise methods of diagnosis such as histopathology and quantitative PCR (qPCR) using GaHV-1 gene specific primers are utilized for definitive diagnosis.

The virus itself codes for immune evasion mechanisms characteristic of herpesviruses, and alpha-, beta-, and gammaherpesviruses each encode for proteins with functions including inhibition of complement, antibody function, cellular immunity, and the cytokine network, as wells as coding for functional homologues of cytokines, chemokines, and their receptors (Davis-Poynter & Farrell, 1996). Specific to alphaherpesviruses are the functions of gC, gE, gG, and gI. A complex protein consisting of gE and gI has been functionally described as having Fc receptor binding activity of immunoglobulins such as IgG, and gC has been associated in the blocking of complement activation through binding of component C3, and derivatives such as C3b, for alphaherpesviruses such as HSV-1 (Davis-Poynter & Farrell, 1996). The functions of gC, gE, and gI have not been specifically described for GaHV-1, although their sequence

homologies suggest that they do in fact retain functional homology, however host immune mediation by GaHV-1 gG has been displayed and function of the protein as a vCKBP is currently accepted (Devlin *et al.*, 2006b).

2.6 Viral Pathobiology

GaHV-1 typically gains entry via the mucous membranes of the upper respiratory tract or ocular tissue of the host. Following GaHV-1 infection, an average incubation period of 4 days occurs, with onset of clinical signs occurring between 2 and 12 days post-infection (dpi), a shorter time period typical of experimental infection, and with severity of clinical signs dependent on host age as well as infective dose and strain (Hughes *et al.*, 1987; Guy & Garcia, 2008; Tablante & Menendez, 2010). Peak viral shedding occurs between 2 and 4 dpi, directly correlated to the viral replication cycle. Induction of mild to severe histopathological lesions within the tracheal epithelium occurs concurrently to clinical sign development, and level of severity is also associated with infective strain. Figure 4 illustrates a histopathological section of a chorioallantoic membrane from a virulent GaHV-1 United States Department of Agriculture (USDA) reference strain infected embryo showing mild diffuse heterophilic cellulitis and typical herpesviral syncytia including intranuclear eosinophilic inclusion bodies.

ILT clinical signs include conjunctivitis, nasal discharge, and decreased production efficiency, and in more severe forms, gasping, coughing, and expectoration of bloody mucus may develop (Figure 5). While genes specifically responsible for development of clinical signs have not been fully elucidated, the loss of or decrease in clinical signs in the absence of gG, TK, UL0, and UL47 directly correlates these genes to
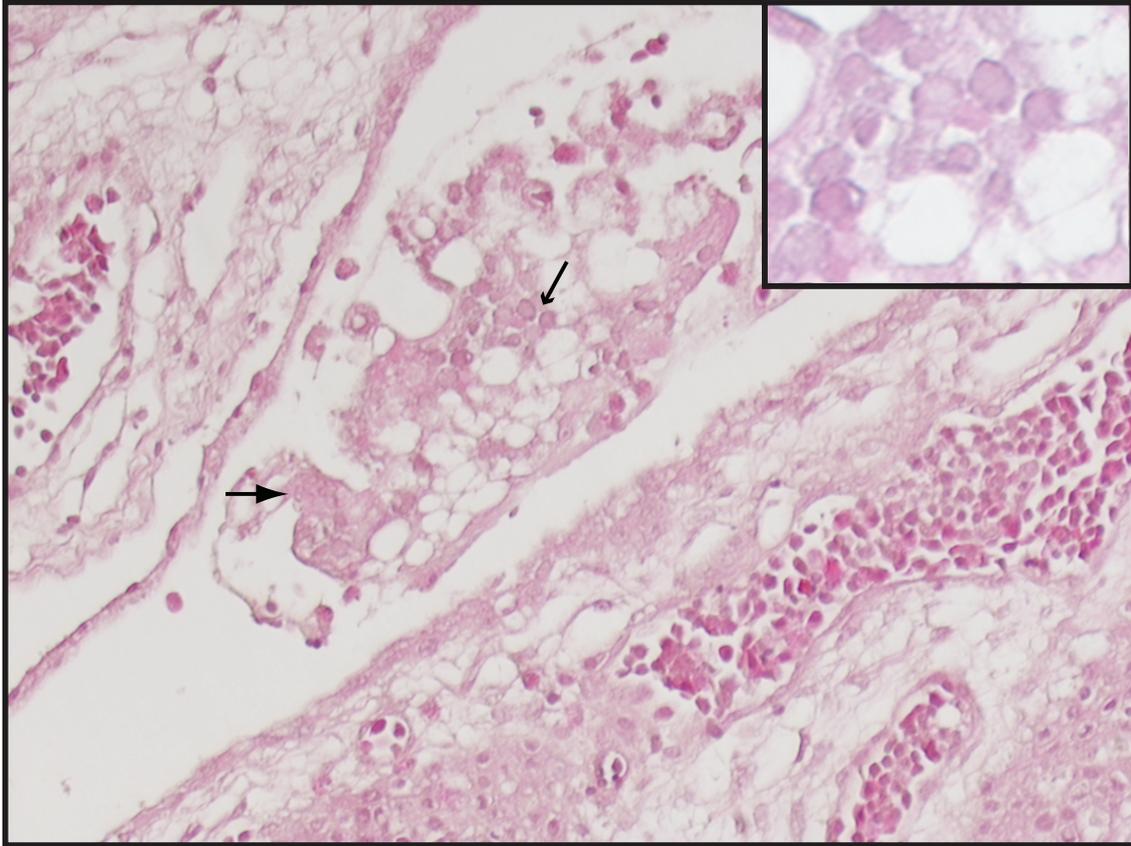
Figure 4: (20x magnification) Chorioallantoic membrane (CAM) from a chicken embryo infected at 10 days of development with USDA reference strain GaHV-1. At 5 dpi, CAM tissue was harvested and fixed in buffered formalin. Specimens were further processed, embedded in paraffin, sectioned to 4-µm thickness, and stained with Hematoxylin & Eosin (H&E). Tissues were microscopically evaluated by an ACVP-certified veterinary anatomic pathologist in a blinded fashion for evidence of herpesviral infection. Sloughed epithelial cells exhibited prominent eosinophilic intranuclear inclusions (small arrow) and formation of syncytia (large arrow) typical of herpesviral infection. Inset: Herpesviral inclusion bodies at higher (40x) magnification demonstrate peripheralization of chromatin.
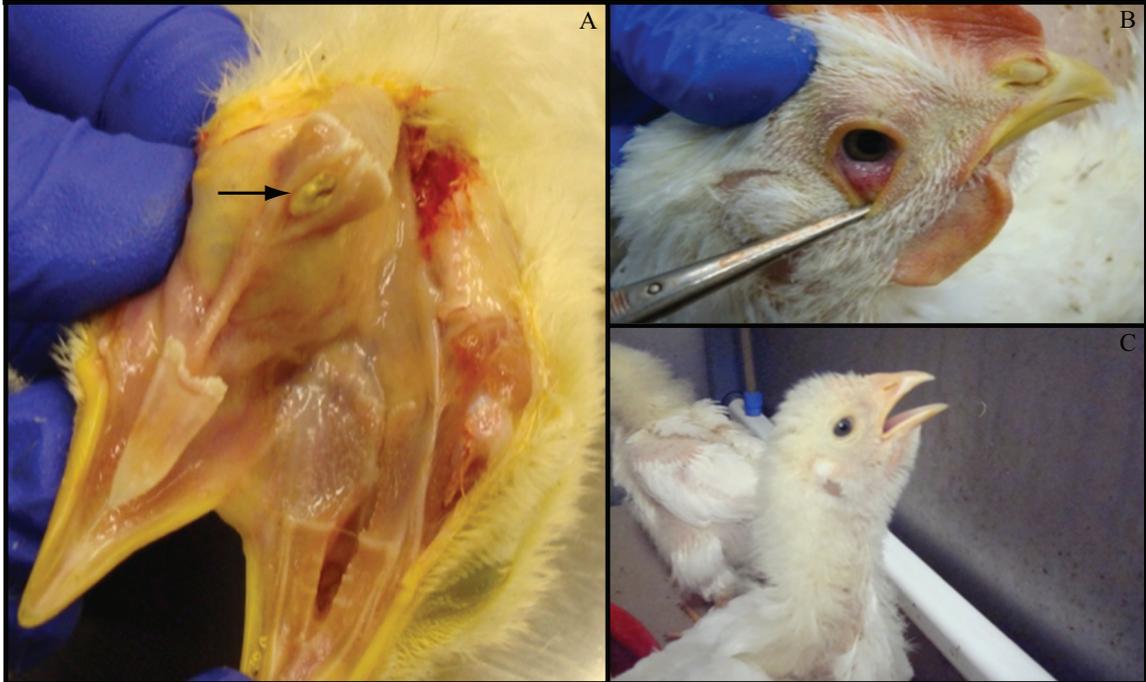
Figure 5: In a study to investigate incubation period, shedding, and immune response of commercial broiler chickens to GaHV-1 infection, 15 specific pathogen free birds were divided into 5 groups and inoculated with differential doses of the virulent United States Department of Agriculture (USDA) reference strain of GaHV-1 at 14 days of age. Group 1 were inoculated with a dose level of 1.7×10^3 $TCID_{50}$/ml, group 2 a dose level of 3.4×10^3 $TCID_{50}$/ml, group 3 a dose level of 5.1×10^3 $TCID_{50}$/ml, group 4 a dose level of 6.8×10^3 $TCID_{50}$/ml, and group 5 sterile phosphate buffered saline at a volume of 0.1 ml to serve as the control group. All infected birds displayed varying clinical sings beginning at 4 days post infection, including coughing, sneezing, caseous plug (arrow) formation due to increased exudate within the tracheal lumen (A), inflamed conjunctival tissue (B), and/or ILT characteristic extension of the neck associated with caseous plug formation and respiratory distress (C) (Tablante & Menendez, 2010).

host response to infection. Morbidity and mortality of ILT related to clinical signs and severe respiratory disease range from 5% to 70%, however a range of 10% to 20% is more typical of outbreaks due to mild strains of the virus (Guy & Garcia, 2008).

Morbidity and mortality related losses negatively affect the global poultry industry each year and are still present despite vaccine related control efforts. Additionally, development of a carrier state in birds and establishment of latency, following infection or vaccination, coupled with viral reactivation further complicate the issue (Hughes *et al*., 1987; Tablante & Menendez, 2010). Development of vaccine alternatives has yet to provide a suitable alternative to the industry, with further developments reliant on molecular advances, leaving biosecurity as the most critical factor to preventing current outbreaks that are typically capable of spreading rapidly.

The mechanism of spread has not been fully explained, however wind-borne spread of the virus has been associated with transmission, as well as vehicle related farm-to-farm traffic, farm employee hygiene and personal protective equipment use, and farm equipment such as tunnel ventilators and shared litter removal equipment (Johnson *et al*., 2005; Volkova *et al*., 2012). Strict adherence to simple hygiene measures and biosecurity are capable of abrogating spread of the virus, and inactivation of the virus outside of the host is easily attained using low levels of heat or disinfectants (Bagust *et al*., 2000). For the California broiler industry, a strategy involving extended downtime of flocks, in addition to implementation of an extensive biosecurity audit in response to ILT outbreaks beginning in 2005, has substantially decreased the occurrence of ILT in the entire state to a rate of 1.25% between May 2010 and April 2012 (Chin *et al*., 2009; Shivaprasad, 2012).

Chapter 3: Historical and Current Vaccine Strategies Related to ILT

Molecular Epidemiology

3.1 Discovery of ILT and Early Vaccine Development

To introduce the molecular epidemiology of ILT, an understanding of the origin and usage of ILT vaccines is necessary. The disease ILT was first confirmed in 1925 in Canada, followed by the United States in 1926, Australia & Great Britain in 1935, and Europe in 1940 (Cover, 1996). By 1962, ILT was described in at least 40 countries in the FAO-WHO-OIE Animal Health Yearbook (Pulsford, 1963). In 1934 C.S. Gibbs described the first vaccination method for ILT (Gibbs, 1933, 1934). Brush vent application with live virulent virus from tracheal scraping preparations was recommended and shown to provide year-long protection to birds showing takes, or inflammation of the cloacal mucosa typically seen 3 to 8 days after brush vent application. However, vent vaccination was also described to release live virulent virus into the field, allowing for continued spread of the virus.

3.2 Live-attenuated Vaccine History

Development of the chorioallantoic (CAM) virus propagation method in 1935 by C.A. Brandly gave way to efforts in the 1950s and 1960s to attenuate field strains for the development of strains of weaker virulence for vaccine use, higher environmental safety, and improved efficacy (Brandly, 1935). Worldwide adoption of this method, coupled with successive *in ovo* passage of field viruses, gave rise to various strains of attenuated virus. The Cover, Hudson, Samberg, SA-2, A20, and Serva vaccine strains (Table 1), all

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Vaccine Name, Company	Country of Origin	Strain
Poulvac Laryngo A20, Fort Dodge	Australia	A20
Avipro ILT vac, LAHi	USA	Hudson
Avivac ILT, Avivac	Russia	VNIIBP
BIO Laringo PV, Merial	Italy	PV 09
Himmvac, KBNP	South Korea	IVR-12
ILT, Abic	Israel	Samberg
ILT Vac, Merial	France	T20
Infectious Laryngotracheitis Vaccine Living, Qilu Animal Health	China	K317
Izovac ILT, IZO	Italy	PV/64
Laringovac, Pasteur Institute	Romania	LT-79-2
Larivac, Romvac	Romania	ILT 90
Laryngo-Vac, Pfizer	USA	Cover
Living Vaccine of Fowl Laryngotracheitis, Qingdao Yebio	China	K317
LT-Blen, Merial	USA	Hudson
LT-IVAX, Merck	USA	ASL L-6*
Medivac ILT, Medion	Indonesia	A 94
Nobilis ILT Vaccine, Intervet	Netherlands	Serva
Poulvac, Pfizer	UK	Salisbury 146
Poulvac Laryngo A20, Pfizer	Australia	A20
Poulvac Laryngo SA2, Pfizer	Australia	SA2
Rinbio ILT, Ringpu	China	K317
Poulvac Laryngo SA2, Fort Dodge	Australia	SA-2
Trachivax, Merck	USA	Hudson
Vaksi ILT, Vaksindo Satwa Nusantara	Indonesia	Hudson
Vir 101, Biovac	Israel	Samberg
Volvac LT MLV, Boehringer Ingelheim	Mexico	N-71851

Table 1: Examples of live-attenuated ILT vaccines from across the globe. Many of the strains utilized to make the TCO vaccine (LT-IVAX) and the many CEO vaccines available today originated in the 1950s and 1960s. Since that time, the ability of these vaccines to spread from bird-to-bird and cause vaccine-related outbreaks of ILT has been described and remains a large problem for the poultry industry. *While the current LT-IVAX product label does not cite the strain name, the original strain utilized to create the TCO vaccine was the ASL L-6 strain. (Gelenczei & Marty, 1964; Elkin, 2012)

still in current use, were derived using variations of the CAM attenuation method, and are currently referred to as chick embryo origin (CEO) vaccines.

Cover and Benton first described United States field strains in 1958 with lower levels of virulence when administered to birds, capable of protecting birds against challenge 19 days post intratracheal inoculation (Benton *et al.*, 1958), later giving rise to the Cover strain CEO vaccine. In a United States patent submitted by C.B. Hudson in 1969, the Hudson strain CEO vaccine was created after 191 *in ovo* passages, producing an attenuated virus intended for ocular, intranasal, or intratracheal vaccination. Methods included inoculation of the CAM of 9 to 12 day old embryonated chicken eggs, followed by collection of infected CAMs 5 days post inoculation, and preparation of masticated membranes for vaccination (Hudson, 1969).

In Israel, virus from acute field cases of ILT were utilized to create a vaccine from 18th passage CAM material, produced in a similar fashion as the Hudson methods using chicken, duck, and turkey eggs. The resulting Samberg strain CEO vaccine was intended for intra-ocular or vent-brush application (Samberg & Aronovici, 1969a). In Australia, the SA2 vaccine strain was also developed as a chicken embryo attenuated strain in 1966 from Australian field isolates, and was later further attenuated in chicken embryonic cell culture to generate the A20 vaccine in 1983 (Kirkpatrick *et al.*, 2006a). The Serva vaccine, also of chick embryo origin, was developed using European based GaHV-1 strains. Each of these vaccines not only addressed outbreaks according to regional strains, but were time saving in their application routes and, for the first time, presented vaccine options that decreased production losses associated with clinical sign manifestation following live virus vaccination.

3.3 Effect of Live-attenuated Vaccines on Viral Epidemiology

While positive factors associated with the newly labeled CEO vaccines accomplished many of the goals of the time, Samberg described two negative factors still controversial with CEO use today. In addition to noting a failure in the drinking water vaccination method (Samberg & Aronovici, 1971), the tendency of the attenuated virus to spread to unvaccinated in-contact birds was also described. This phenomenon, also described by others developing CEO vaccines at that time, was attributed to an increase in virulence beginning with the 7th back passage of the Samberg strain in unvaccinated birds (Samberg & Aronovici, 1969b).

Despite these indications, a large portion of the poultry industry adopted both CEO vaccination and the drinking water application method, which when combined, perpetuated the spread of virulent virus. This spread was, and still is, largely attributed to back passage through birds inadequately vaccinated, perpetuating release of live virus into the field and the creation of carriers just as live virus vaccination had been negatively attributed to beginning in the 1930s.

The first tissue culture-modified vaccine originated in 1964 (Gelenczei & Marty, 1964), representing a hopeful new vaccine option, however it too was associated with similar drawbacks as the CEO vaccines. Attenuation of the virulent ASL L-6 virus strain was successful in providing birds immunity after 50 serial passages in primary avian cell monolayers. Birds were protected against direct challenge for up to 22 weeks after ocular or intranasal application, and the TCO vaccine did so with a decrease in clinical signs. However, as with the CEO vaccine, the ability of this virus to spread to unvaccinated

birds was described, and the goal of eliminating vaccine associated spread and subsequent outbreaks remained unfulfilled.

3.4 Current Chicken Embryo Origin Vaccines

In today's industry, live-attenuated vaccine technology has remained vastly the same. Despite the negative implications, birds are still vaccinated with either the TCO vaccine or one of the many CEO vaccines currently available in the United States and across the globe (Table 1). This is either performed in a preventive manner, typical of the layer industry or with breeding stock, or in the face of an outbreak, as in the case of the broiler industry. Due to the lack of preventive measures in the broiler sector, a large majority of outbreaks occur in broiler operations and are directly correlated to their CEO-centered vaccine strategies. In the face of an outbreak, CEO vaccine is commonly delivered via drinking water to broilers, a method of mass application that relies on contact of the vaccine with the nasal cavity during the act of drinking (Robertson & Egerton, 1981; Loudovaris *et al.*, 1991a; Devlin *et al.*, 2008). However this method does not provide uniform flock vaccination and often results in uneven protection of birds allowing for the spread of vaccine virus from vaccinated to non-vaccinated birds. As a result, vaccine responses are prolonged, leading to outbreaks of VLT and potential spread to surrounding broiler operations.

3.5 Vaccinal Laryngotracheitis

Clinical signs associated with VLT outbreaks can range from mild to severe, however most broiler operations are willing to bear the comparatively low production

losses associated with vaccination as opposed to risking potential losses that would occur

if the disease were left untreated (Zavala, 2011). These practices, coupled with a short

two-week acute infection phase, followed by a classic herpesviral latent infection, result

in a cyclical pattern of outbreaks triggered by stress-induced reactivation of the virus.

Subsequently, the potential for spread to surrounding operations is common, allowing the

virus to increase in virulence with each successive passage, and resulting in damaging

strains of the virus.

3.6 Tissue Culture Origin Vaccines

The TCO vaccine produces a robust immune response equivalent to that of the

CEO vaccine, but is milder in its reverted virulent form and is subsequently isolated from

the field to a significantly lower extent (Rodríguez-Avila *et al.*, 2007). However, because

mass application of the TCO vaccine is not an option, with direct delivery methods

necessary (Gelenczei & Marty, 1964), use in large operations is typically unfavorable for

producers. Additionally, the TCO vaccine retains the ability to replicate in the trachea,

conjunctiva, cecal tonsils, trigeminal ganglia, and cloaca to an equal extent as the CEO

vaccine. Localized replication is sustained in the conjunctiva and trachea after eye-drop

vaccination, and thus the ability to transmit from bird-to-bird, albeit to a lower extent

than the CEO vaccine, perpetuates VLT outbreaks even with use of the TCO vaccine

(Rodríguez-Avila *et al.*, 2007).

3.7 Viral Vector ILT Vaccines

Viral vector vaccines, such as herpesvirus of turkey (HVT) and fowlpox (FP)

vectored vaccines, present an alternative option to live-attenuated vaccines altogether.

They are increasingly safe due to their inability to revert to virulence, can be

administered *in ovo*, and lack an impact on production performance. However, they are

comparatively high in cost and mass application in adult birds is not possible, rendering

these vaccines inadequate for broiler industry associated outbreaks. Recent studies have

found that these vaccines provide partial protection and reduce clinical signs, but do not

decrease challenge viral load in the trachea most likely due to an inherent inability to

induce a sufficient local immune response within the trachea (Johnson *et al.*, 2010). As a

result, many poultry industry veterinarians are reluctant to use vaccines that do not elicit

a robust immune response, leaving birds susceptible to infection, even if these vaccines

do not revert to virulence and cause VLT outbreaks that are typical of CEO vaccines.

However, further improvement of viral vector vaccines is warranted, and the desirable

inability of these vaccines to revert to virulence deserves further development.

3.8 Recombinant ILT Vaccines

Recently, GaHV-1 recombinant viruses involving deletion or alterations of genes

such as gG, TK, UL0, or UL47 have been investigated and implicated as suitable targets

for recombinant vaccine development due to their phenotypic properties (Han *et al.*,

2002; Veits *et al.*, 2003b; Devlin *et al.*, 2006b; Devlin *et al*, 2007; Helferich *et al.*, 2007b;

Legione, *et al.*, 2012). Of these genes, deletion of gG has been most thoroughly

described and demonstrated to be a favorable target for vaccine candidacy (Devlin *et al.*,

2007). gG deficient mutant virus strains of GaHV-1 have been developed, described *in*

vivo, and vaccination via eye-drop and drinking water have been validated (Devlin *et al.*,

2007; Devlin *et al.*, 2008; Devlin *et al*., 2011).   Additionally, while the gG deficient candidate strain after eye-drop application can still pass from bird-to-bird, it is able to prevent spread of infection with challenge virus, remains attenuated after one passage to unvaccinated birds, and is comparable in efficacy to the A20, SA2, and Serva live-attenuated vaccines (Coppo *et al.*, 2011; Devlin *et al.*, 2011). However, further investigation using larger bird numbers, increased *in vivo* passage numbers, and in different production settings is critical to the progression of this and other recombinant vaccine strains.

3.9 CEO and Recombinant ILT Vaccination

In recent years, a combination of recombinant and CEO vaccination has been investigated in the United States broiler industry. While intuitively greater in expense, CEO vaccination of birds within the zone and live haul routes of outbreak areas, in addition to simultaneous *in ovo* recombinant vaccination at hatchery facilities, sets up for the first time a combination treatment and preventive strategy against ILT (Burleson, 2012).

3.10 Future ILT Vaccines

Potential for future vaccine development and utilization performed in a manner more strategically executed compared to past vaccines is apparent, and synchronization of poultry health and production in a way that controls outbreaks of GaHV-1 is in the horizon (Devlin *et al*., 2011). However, ease of use, cost, effectivity, and availability ultimately motivate the majority of producers and poultry health personnel across the

globe to use live-attenuated vaccines, thus perpetuating VLT outbreaks and continually shaping the epidemiology of the virus. Furthermore, continued host-to-host passage and spread of the virus due to past and current vaccine strategies continues to mold the epigenome of the virus. Understanding these changes, based on virulence reversion, holds potential for better discernment of the genes responsible for continued spread of the virus and point to targets for further genomic investigation.

Chapter 4: Global and Molecular Epidemiology of ILT

4.1 Global Epidemiology

Based on outbreak data from the World Organization for Animal Health (OIE) and other sources, the current distribution of ILT is described in Figure 6 (Pal *et al.*, 2009; Cabezas, 2012; Community, 2012; Health, 2012; Heinonen, 2012). Red regions indicate countries positive for ILT between 2000 and 2012, and those illustrated with a grid pattern have been ILT positive for 10 years or greater. Most apparent from this distribution is the proximity of these regions (marked in red and those with grids) to major poultry producing areas and to those areas that currently use or have used live-attenuated vaccines (Table 2). While licensed uses of live-attenuated vaccines are determined at the country, state, or province level, depending on the region of the world, presence of vaccine strains has been confirmed in the field even in some countries that disallow use of live attenuated-vaccines (Table 2) (Chacon & Ferreira, 2009; Neff *et al.*, 2008).

4.2 Molecular Epidemiology

Continual use of live-attenuated vaccines has shaped the molecular epidemiology of the virus, more so than outbreaks caused by wild-type strains. Currently, the majority of outbreak related strains from commercial poultry are either indistinguishable from or closely related to vaccine strains (Oldoni *et al.*, 2008), while outbreaks caused by wild-type strains occur to a much lesser extent than those attributed to live-attenuated vaccine strains in commercial poultry.
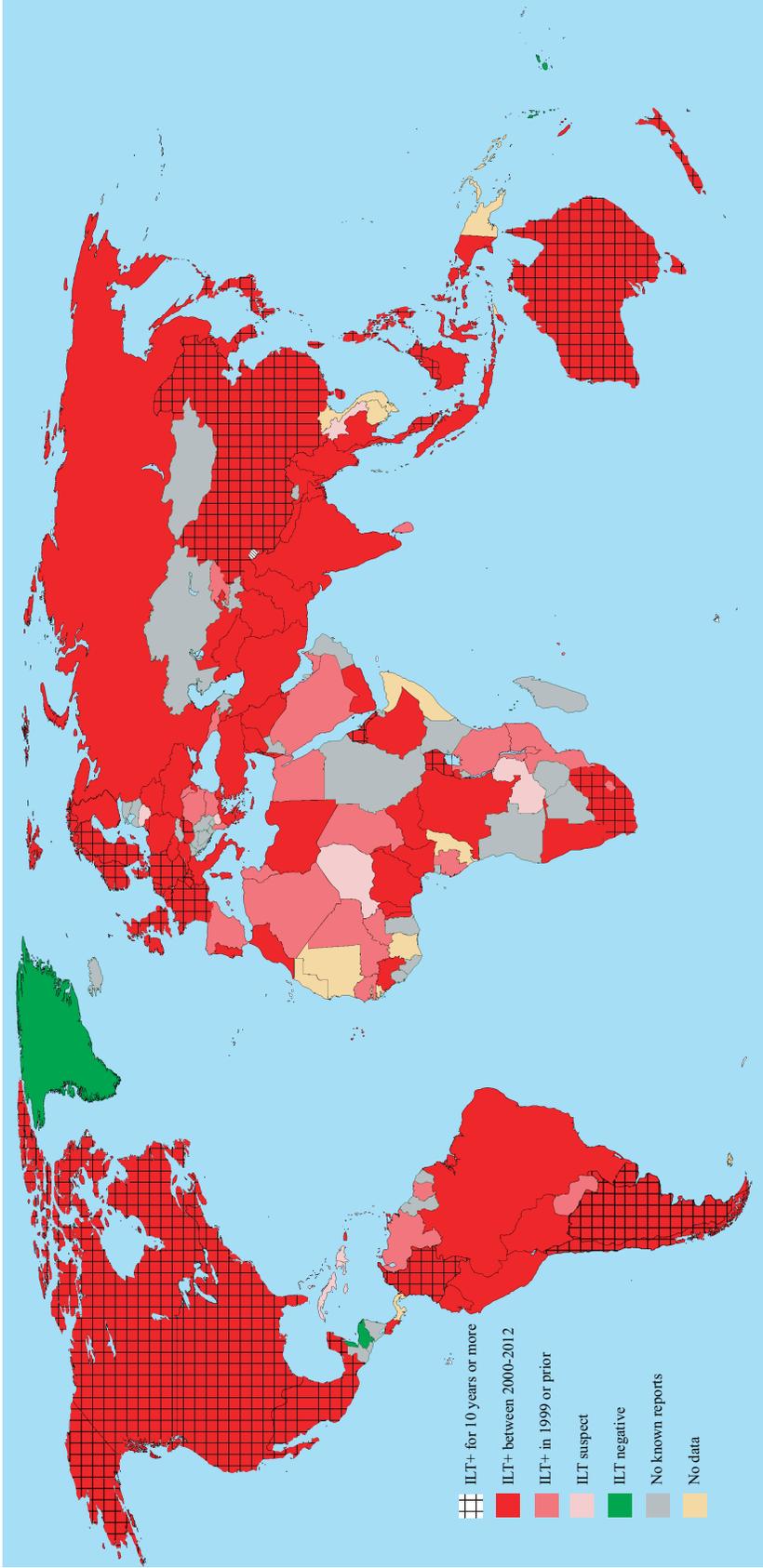
Figure 6. An extensive online search utilizing the World Organization for Animal Health (OIE) World Animal Health Information Database (WAHID) interface, in addition to PubMed and other reports of ILT outbreaks, was performed in order to illustrate the global distribution of ILT as of 2012. Those countries positive for ILT between the year 2000 to 2012 are marked in red, and those positive in the year 1999 or prior are marked in dark pink. In order to display the contribution of countries with endemic ILT, those countries positive for ILT for 10 years or greater are marked additionally with a grid pattern. Marked in pink, countries that are suspected positive by the OIE are marked as such most likely due to their proximity to ILT positive countries or high poultry producing areas, however no official reports exist for these countries to date. Only three countries are currently ILT negative, including Belize, Greenland, and Honduras, which are marked in green. Countries with no known reports or no data are marked in grey and tan respectively; a corresponding list of ILT statuses by country is available in the appendix as supplementary list 1 on page 58. (Pal et al., 2009; Community, 2012; Health, 2012)

Geographical Region	Examples of Currently Used Live-Attenuated Vaccines	Licensed Uses	Identification of Vaccine Strains in the Field
North America	Trachivac, LT-Blen, Laryngo-vac, Laryngotracheitis Vaccine, Broilertrake-M, Laryngo-vac, LT-Ivax	Commercial & non-commercial; regulated by state or province	Yes
Central America	VolvacLT MLV	CEO and TCO use dependent on country regulations	No data
South America	LT-Ivax, Trachivax, LT-Blen, Laryngovac, Avipro	Commercial & non-commercial; regulated by country	Yes
United Kingdom	Poulvac ILT	Commercial & non-commercial	Yes
Europe	LaryngoVac, Nobilis ILT, LI-Ivax, Hipraviar-ILT	Commercial & non-commercial; regulated by country	Yes
Africa	No data	Libya: not used as of 2011; no other data	No data
Middle East	ILT-Abic	Israel: commercial & non-commercial; Turkey: not used as of 2007; no other data	Yes
East Asia	LT-Blen	Commercial & non-commercial; regulated by country	Yes
South Asia	Noblilis ILT, Gallivac LT	Commercial use regulated by country	Yes
South East Asia	BAL-ILT™, Belstar	Philippines as of 2002, banned; no other data	Yes
Australia	SA-2, A20, Serva	Commercial & non-commercial	Yes
New Zealand	Laryngo-vac	Commercial and non-commercial	No data

Table 2: Examples of current live-attenuated vaccines and licensed uses. While live-attenuated vaccine use is not licensed in some regions of the world, vaccine strains have still be isolated from the field in countries without licensed use of these vaccines. This illustrates the possibility of these strains lingering in host reservoirs from past points in time when live-attenuated vaccines may have been permitted in these countries, and the ability of live-attenuated vaccines to move across borders regardless of licensing and biosecurity. (Chang *et al.*, 1997; Han & Kim, 2001; Saepulloh & Rovira, 2003; Kirkpatrick *et al.*, 2006b; Noormohammadi & Kirkpatrick, 2006; Creelan *et al.,* 2007; Oldoni & Garcia, 2007a; Neff *et al.*, 2008; Chacon *et al.*, 2010; Diallo *et al.*, 2010; Islam *et al.*, 2010; ; Sadeghi *et al.*, 2011).

Recently, investigation into molecular markers responsible for reversion of GaHV-1 to virulence has unveiled or confirmed genomic regions suspected to be viral virulence factors. Initially, methods to differentiate vaccine and wild-type viruses established different groupings of GaHV-1 strains based on polymerase chain reaction restriction fragment length polymorphisms (PCR-RFLP) and sequencing data in different regions of the world. By means of these distinctions, sequence comparison of low and high virulence strains revealed SNPs and INDELs among strains, with specific attention given to non-synonymous amino acid changes found within gene products associated with classic herpesviral immune evasion strategies and viral virulence, as well as those unique to GaHV-1 (Lee et al., 2011c; Spatz *et al*., 2012). Discovery of the molecular markers responsible for GaHV-1 reversion to virulence will potentially identify targets for genetic manipulation and point to a promising future for the development of novel control strategies. If advances are to be made in the control of the disease, it will be pivotal to incorporate associations related to virulence and attenuation through epidemiological investigation of GaHV-1 at the molecular level.

At the foundation of ILT molecular epidemiology lie techniques aimed at differentiation of virus genotypes. However, because strains of ILT have no serospecificity, molecular methods such as PCR-RFLP and DNA sequencing have been used to draw epidemiological conclusions. While identification of strain type does not necessarily stop or change current control measures, defining strains responsible for disease allow for poultry companies involved in or near an outbreak to take specific actions regarding biosecurity and vaccination programs. Modification of vaccination strategies, biosecurity, and clean-out techniques are pivotal in controlling continuous

outbreaks. However, efforts made to improve outbreak response remain challenging. In Australia, the introduction of the European-origin Serva CEO vaccine to a population of birds previously vaccinated with the native vaccines, SA-2 and A20, resulted in emergence of virulent strains responsible for outbreaks of the disease. Full genome sequencing clearly indicated that a recombination event between the native SA-2 and A20 with the CEO Serva strain may have influenced the emergence of the new virulent genotypes identified as classes 8 and 9 (Lee *et al*., 2012b). In the United States, outbreak-related strains are mostly derived from CEO vaccines that circulate in the field due to sub-optimal vaccine administration in combination with poor biosecurity measures. Both DNA sequencing and PCR-RFLP have been critical in strain differentiation and in understanding the emergence of virulent virus.

4.2.1 Strain Genotyping by PCR-RFLP

Target genes for detection and strain differentiation are regionally dependent, with each region of the world requiring its own optimal set of genes for differentiation. Table 3 outlines target genes for GaHV-1 detection, as well as PCR-RFLP and sequencing differentiation by region of the world. However, these gene specifications are not concrete and, as made apparent by the recent recombination of vaccine viruses in Australia, changes in these targets may occur with time and as vaccine strategies evolve.

Initially, PCR-RFLP was the method of choice for strain differentiation and involves differentiation of virus strains by restriction enzyme cleavage patterns of targeted genes. Within the United States, 9 groups with unique PCR-RFLP patterns have been identified using genes ORFB-TK, gM, ICP4, and gG (Oldoni & Garcia,

| Geographical Region | Strain Differentiation | | Diagnostic Testing |
	PCR-RFLP Target Genes & Restriction Enzymes	Sequencing & PCR Target Genes	Target Genes for Detection
North America	gM, gG, UL47, ICP4, ORF B-TK* (*Hae*III, *Mwo*I, *Hin*PII, *Bst*F5I)	ICP4, UL47, gG, gM, gB	gC, gE, ICP4
South America	TK, UL47/gG, ICP4 (*Hae*III, *Msp*I, *Hin*P1I)	ICP4	gE
United Kingdom	TK, ICP4 (*Hae*I, *Sau*96, *Nci*I, *Msp*I)	TK, ICP4	ICP4
Europe	gE, gG, ICP18.5, TK, ORFB-TK (EaeI, *Msp*I, *Hae*III, *Fok*I)	TK, ORFB-TK, ICP18.5, gE, gG, ICP4	gC
Africa	No Data	ICP4	gE
Middle East	gG, TK (*Bam*HI, *Hae*III)	ICP4	TK, ICP4
East Asia	gG, TK, ICP4 (*Msp*I, *Hae*III, *Hinp*1I)	gC, gG gE, gJ, TK, ICP4	TK
South Asia	No data	TK	TK
Australia	gG, TK, ICP4, ICP18.5, ORFB-TK* (*Msp*I, *Hae*III, *Fok*I)	TK	UL15

Table 3: Target genes for PCR-RFLP strain differentiation, sequencing and PCR strain differentiation, and detection of GaHV-1. Due to single nucleotide polymorphisms (SNPs) and insertions and deletions (INDELs) among strains of different geographical origin, target genes for strain differentiation and detection differ by region of the world. However, due to the constant evolution of field strains and vaccination programs, as exemplified by the recombination of three vaccine strains in Australia (Lee *et al.*, 2012b), target genes should be evaluated prior to investigation. *Contains ORFB, ORFC, ORFD, ORFE, gH, and TK genes. (Chang *et al.*, 1997; Vogtlin *et al.*, 1999; Han & Kim., 2001; Humberd *et al.*, 2002; Pang *et al.*, 2002; Han & Kim, 2003; Kirkpatrick *et al.*, 2006b; Noormohammadi & Kirkpatrick, 2006; Ojkic *et al.*, 2006; Callison *et al.*,, 2007; Creelan *et al.*, 2007; Gulacti *et al.,* 2007; Oldoni & Garcia, 2007a; Oldoni & Garcia, 2007b; Chacon & Ferreira, 2008; Neff *et al.*, 2008; Callison *et al.* 2009; Chacon & Ferreira, 2009; Rashid *et al.*, 2009; Chacon *et al.*, 2010; Diallo *et al.*, 2010; Moreno *et al.*, 2010; Xie *et al.*, 2010; Mahmoudian *et al.*, 2011; Sadeghi *et al.*, 2011; Cabezas, 2012; Chen *et al.*, 2012; Halami *et al.*, 2012; Sridevi *et al.*, 2012).

2007a). The resulting groupings consisted of the USDA reference strain in group I, the

TCO vaccine strain in group II, field isolates closely related to the TCO vaccine in group

III, CEO vaccine strains and CEO identical commercial poultry isolates in group IV,

commercial poultry isolates closely related to the CEO vaccine in group V, vaccine-

unlike commercial poultry isolates in group VI, and unique backyard flock isolates in

groups VII, VIII, and IX.

Alternatively, in Australia five classes of strains were originally compiled based

on PCR-RFLP differentiation using a combination of gG, TK, ICP4, and ICP18.5 target

genes. Class 1 consisted of the SA-2 and A20 CEO vaccines as well as related strains,

classes 2 and 3 of vaccine-unlike field strains, class 4 of the Australian CSW virulent

field strain, and class 5 of vaccine-like and –unlike field strains (Kirkpatrick et al.,

2006b). In 2011, four new classes were identified, including class 6 strains isolated from

the region of Victoria, the Nobilis (Serva) ILT vaccine in class 7, and the SA-2, A20,

Serva recombinants in classes 8 and 9 (Blacker, 2011; Lee et al., 2012b).

In South America, based on PCR-RFLP of the TK and gG genes, five patterns

were identified among Brazilian and Peruvian field isolates (Chacon & Ferreira, 2009).

Pattern A consisted of isolates from the Sao Paulo state of Brazil, pattern B isolates

originated in southern Brazil, pattern C isolates originated in Peru, pattern D

corresponded to the TCO vaccine, and pattern E to the CEO vaccine.

In Taiwan, based on PCR-RFLP of gG, TK, and ICP4, three groups of strains

were identified (Chang *et al.*, 1997). Group 1 consisted of the TCO vaccine and TCO-

like field strains, Group 2 of the CEO vaccine and CEO-like field strains, and Group 3 of

vaccine-unlike field strains. In Korea, three groups of field strains were differentiated

using the TK gene alone (Han & Kim, 2001). Group 1 consisted of virulent strains, group 2 of low-virulence strains, and group 3 of vaccine strains. In the latter example, the level of differentiation did not match that of other similar studies because only one target gene was utilized, outlining the importance of using multiple genes in PCR-RFLP differentiation. However, despite the level of differentiation, the PCR-RFLP method initially revealed the presence of circulating vaccine-like strains as a source of disease outbreaks across the globe. A summary of the target genes and corresponding restriction enzymes utilized for PCR-RFLP analysis are displayed in Table 3 according to region of the world.

4.2.2 Strain Genotyping by DNA Sequencing

In recent years, PCR-RFLP has been steadily replaced with DNA sequencing for strain differentiation, although this technique remains a less costly option for certain regions of the world. One main advantage of sequencing over PCR-RFLP is that the data produced is easier to document, analyze, and maintain, whereas PCR-RFLP can be highly subjective. Also, sequencing is also more precise, especially when multiple target genes are utilized for differentiation.

Like PCR-RFLP, target genes amplified and sequenced for strain differentiation are regionally dependent and are summarized in Table 3. In North America, target genes, sequenced either in their entirety or partially, include ICP4, UL47, gB, gG and gM (Ojkic *et al.*, 2006; Oldoni & Garcia, 2007b; Callison *et al.*, 2009). In South America, the ICP4 gene has been sequenced (Chacon & Ferreira, 2009; Chacon *et al.*, 2010), and in the United Kingdom the TK and ICP4 genes (Creelan *et al.*, 2007). In Europe, larger scale

investigation has been performed including the target genes TK, ICP4, gG, gE, ORFB-TK (containing the gene regions ORFB, ORFC, ORFD, ORFE, gH, to TK), and ICP18.5 (Neff *et al.*, 2008; Moreno *et al*., 2010). The target gene ICP4 has been successfully utilized in both Africa and the Middle East (Sadeghi *et al*., 2011; Halami *et al*., 2012), and the TK gene in South Asia (Sridevi *et al*., 2012). Strains in East Asia have been successfully sequenced and differentiated using the target genes TK, ICP4, gC, gG, gE, gJ (Chen *et al*., 2012; Han & Kim., 2001), and in Australia the genes TK and gG have been used (Diallo *et al*., 2010). No sequencing data has been published for Russia or Southeast Asia.

4.2.3 Optimal Methods for Strain Genotyping

Between PCR-RFLP and sequencing, two of the genes that have been most widely used for differentiation and molecular epidemiologic analysis of GaHV-1 are the TK and ICP4 genes. In addition to being costly and time consuming, sequencing of multiple genes requires large amounts of viral DNA, which may require further virus isolation. Alternatively, sequencing of TK and ICP4, either in their entirety or as partial gene sequences, is common and has been successful in differentiating field and vaccine strains of GaHV-1. Although this method is not optimal, lacking some of the discriminatory power necessary to differentiate among GaHV-1 isolates, sequencing these genes in their entirety or partially has been a useful, cost effective, and rapid method to differentiate strains from several regions of the world. Table 4 outlines by region of the world those laboratories that have differentiated strains by the amplification and sequencing of the TK and ICP4 gene segments.

Geographical Region	Genes	Amplified Region	GenBank Accession No.
North America	ICP4	1807-3052	L32139
South America	TK	183-831	JN580313
	ICP4	205-822	JN580313
	ICP4	3796-4381	JN580313
United Kingdom	ICP4	714-935	JN580313
Europe	TK	3379-5546	DD00565
Middle East	ICP4	181-856	JN580313
	ICP4	3773-4395	JN580313
Australia	TK	691-1085	JN580313

Table 4: Amplified regions of TK and ICP4 for DNA sequencing. In recent years, PCR-RFLP has been steadily replaced with DNA sequencing for strain differentiation, although PCR-RFLP remains a less costly option for certain regions of the world. While there is a range of target genes used for sequencing differentiation, as displayed in Table 3, TK and ICP4 have been successfully used across regions. Sequencing of these two genes alone can cut cost and time, although with a reduction in the level of discriminatory power. Included are the regions of each gene amplified in previous publications and the corresponding GenBank accession number. (Creelan *et al.*, 2007; Neff *et al.*, 2008; Callison *et al.*, 2009; Chacon, *et al.*, 2010; Chakma *et al.*, 2010; Diallo *et al.*, 2010; Sadeghi *et al.*, 2011).

Despite the advantages of partial sequencing of the TK and ICP4 genes, use of

PCR-RFLP plus sequencing is a more precise method for differentiating vaccines from

vaccine derived field isolates, and multilocus analysis of at least two genes is optimal

(Table 3). Scientists from across the world have employed a combination of these

methods in order to obtain maximal information to analyze differences between vaccine

and non-vaccine strains. In the United States, a combination of reverse restriction

fragment length polymorphism (RRFLP), a method using both PCR-RFLP and real-time

PCR, and DNA sequencing of the ICP4 gene have be exploited to genotype strains

(Callison *et al*., 2009). In Canada, PCR-RFLP of target genes ICP4, UL47, gE, and gG

has been combined with sequencing of UL47 and gG (Ojkic *et al*., 2006), while in Brazil

PCR-RFLP of the TK, ICP4, gG, and gE genes has been combined with sequencing of

the TK and ICP4 genes (Chacon & Ferreira, 2009; Chacon, *et al*., 2010). In the United

Kingdom, PCR-RFLP of TK and ICP4 has been combined with sequencing of ICP4

(Creelan *et al.*, 2007), and in Europe PCR-RFLP of gE, gG, ICP18.5, ORFB-TK, and TK

has been combined with sequencing of ICP4, TK, gE, gG, ORFB-TK, and the gene

region spanning from ICP18.5 to UL43 (Neff *et al*., 2008; Moreno *et al.*, 2010). In

Korea, PCR-RFLP and sequencing of the TK and gG genes has been combined (Han &

Kim., 2001), and in Australia PCR-RFLP and sequencing have been done using only the

TK gene (Diallo *et al*., 2010).

4.2.4 Full Genome Sequencing

So far, none of the genotyping methods outlined above has been successful in

relating strain genotype to pathotype. Some evidence indicates that changes in TK may

be related to virulence of Korean isolates (Han & Kim., 2001). However, it is essential

that this be further investigated and verified by introduction of changes related to

attenuation in the TK gene into a virulent strain to determine if this confers some degree

of attenuation. In 2011, the first non-composite genome of GaHV-1 was sequenced for

the Serva vaccine strain (Lee *et al*., 2011c), and Table 5 shows the 17 strains to date for

which full genome sequences have been reported in GenBank and their corresponding

pathotype.

In an attempt to identify genetic determinants of attenuation in vaccine strains and

virulence in field isolates, comparison of full genome sequences among vaccine and field

isolates has been recently documented. Comparison of the Australian Serva vaccine

strain to four virulent GaHV-1 strains from the United States genotype groups I-VI

revealed non-synonymous amino acid changes exclusive to the vaccine. While some

changes occurred among structural glycoproteins, suspected to account for geographical

differences between strains, those found in the non-structural proteins UL28, UL5, and

ICP4 are suspected to relate to virulence or attenuation due to their roles in genetic

function of the virus (Spatz *et al*., 2012). Additionally, the effect of further attenuation of

the SA-2 vaccine was investigated by comparison of full genome sequences of the related

SA-2 and A20 vaccines from Australia. Only two non-synonymous amino acids changes

were identified in the ORF B and UL15 non-structural proteins, representing two genes

specifically affected by attenuation (Lee *et al.*, 2011b). Further comparison of complete

genome sequences from differing genotypic classes, in addition to specific investigation

of ICP4, UL28, UL5, ORF B, UL15 and other identified genes of interest, will ultimately

Isolate	Virulence	Origin	GenBank Accession No.
A20 Vaccine	High Attenuation	Australia	JN596963
Australia Class 8	Virulent	Australia	JN804826
Australia Class 9	Virulent	Australia	JN804827
CEO High Passage	Virulent	United States	JN80316
CEO Low Passage	Moderate Attenuation	United States	JN580317
CEO TRVX	Moderate Attenuation	United States	JN580313
Laryngo-vac CEO Vaccine	Moderate Attenuation	United States	JQ083494
LT-Blen CEO Vaccine	Moderate Attenuation	United States	JQ083493
SA2 Vaccine	Moderate Attenuation	Australia	JN596962
Serva Vaccine	High Attenuation	European-Origin	HQ_630064
TCO High Passage	Mild Virulence	United States	JN580314
TCO IVAX	High Attenuation	United States	JN580312
TCO Low Passage	High Attenuation	United States	JN580315
USDA Reference Strain	Virulent	United States	JN542534
1874C5 Broiler Isolate	Virulent	United States	JN542533
63140 Broiler Isolate	Virulent	United States	JN542536
81658 Broiler Breeder Isolate	Mild Virulence	United States	JN542535

Table 5: GaHV-1 full genome sequences. The first full genome sequence of GaHV-1 became available in 2011 (Lee *et al.*, 2011c), and to date there are 17 full genome sequences of varying genotypes, each provided in this table with their corresponding GenBank accession numbers. The availability of the full sequences of these various genotypes has allowed for high and low virulence strain comparisons, identifying potential genes involved in virulence reversion (Lee *et al.*, 2011b; Spatz *et al.*, 2012). Further genomic investigation is important to advancing understanding of GaHV-1 reversion.

reveal genes associated with attenuation and virulence of GaHV-1 and increase

knowledge on the molecular epidemiology of the virus.

Chapter 5: ILT Host-Pathogen Interactions

In recent years, in addition to genomic characterization of GaHV-1 genotypes, and investigation into virus-specific attenuation mechanisms, investigation into host specific pathways following infection has begun. Little research has been done regarding host-pathogen interactions of GaHV-1 and the chicken genome, however recent research into GaHV-1 epigenetics has investigated changes in host gene expression based on infection. Identification of genes that result in host resistance to infection, and selecting for these genes in lines of commercial poultry, represents an additional strategy that could be developed in order to prevent infection. The basis for these epigenetic investigations began with past field observations of breeds with more or less resistance to infection.

5.1 Past Field Observations

According to past field observations, differences in susceptibility to GaHV-1 infection have been described. Single comb white leghorns have been noted as a more resistant breed, while broiler breeds are typically less resistant, and reproduction of infection in a laboratory setting easier in broilers than in layers or specific pathogen free (SPF) chickens (Zavala, 2011). One possible source of these differences in susceptibility is the increased level of Cornish type genes which broilers carry today.

5.2 MHC Allele-based Resistance

In further genetic investigation of these differences in susceptibility, birds with defined major histocompatibility complex (MHC) phenotypes have shown differential response to infection (Loudovaris *et al.*, 1991a). Specifically, birds expressing the B^{113} MHC allele are relatively resistant to GaHV-1 infection and birds expressing the B^{114} MHC allele are relatively susceptible to infection. In comparison to birds expressing the B^{114} allele, those expressing the B^{15} MHC allele are increasingly susceptible to infection. Additionally, macrophages from birds expressing the $B^{113/113}$ MHC allele or the $B^{114/114}$ MHC allele express a greater proportion of GaHV-1 antigen after *in vitro* infection compared to those expressing the $B^{15/15}$ MHC allele, suggesting macrophages from the two relatively resistant genetic B^{113} and B^{114} lines of birds may be able to better recognize, process, and present viral antigen to the immune system (Loudovaris *et al.*, 1991b). Furthermore, birds expressing the B^2B^2 MHC allele can mount a more efficient protective immune response to low infective doses of virulent infection, while birds expressing the B^2B^{15} MHC allele require a 10-fold higher dose to mount a protective immune response, and birds expressing the $B^{15}B^{21}$ MHC allele are altogether unable to mount a protective immune response (Poulsen *et al.*, 1998).

5.3 Epigenetics

Changes in host gene expression, based on GaHV-1 infection, have also recently been investigated and functional pathways responsive to infection uncovered. Based on *in vitro* infection with virulent virus, Lee *et al.* (2010)

identified 789 host genes which are differentially expressed during GaHV-1 infection, with 275 of these genes connected to 21 possible gene networks classified in functional groups including cancer, genetic disorders, cellular growth, cellular proliferation, and cell death. Of the 21 gene networks, 6 gene networks are identical at all time points. Further investigation of these networks by Lee *et al.* (2010) revealed the Network 1 pathway to be closely associated with the IL6 signaling pathway, suggesting that GaHV-1 increases IL6 expression and subsequently inhibits cellular proliferation through downregulation of the proliferation enhancer Janus kinase 1 (JAK). Network 2 contains several downregulated heat shock proteins (HSP) thought to be responsible for erroneous viron structures and a source of low GaHV-1 titers typical to tissue culture infection. The genes of network 3 include growth factors and matrix metalloproteinases (MMPs), with expression profiles consistent with those seen during other herpesvirus infections and involved in extracellular remodeling, tissue invasion, and angiogenesis. Networks 4, 5, and 6 contain genes encoding for IFNβ, IL1β, CCL20, CCL4, NF-κβ, NFIB, IL1, and ID1 specific to the host immune response to pathogenic infection.

In comparison to virulent infection, *in vitro* infection with vaccine virus by Lee *et al.* (2012a) revealed 213 differentially expressed host genes, divided into 10 possible gene networks, and grouped into functional categories including tissue development, cellular growth, cellular proliferation, cellular movement, and inflammatory response. Additionally, of the 213 differentially expressed host genes, bone morphogenetic protein 2 (BMP2), chromosome 8 open reading frame 79 (C8orf79), coagulation factor X (F10), and neuropeptide Y (NPY) are expressed

distinctly during vaccine infection when compared to virulent infection.

While genetic markers responsible for host response and resistance to infection have been identified, broad integration of these findings into production strategies has yet to be undertaken. Research into these scientific sectors remains in the early stages related to GaHV-1, limiting current developments. However, possibilities such as incorporation of breed resistance genes in production lines of birds represents an additional protective strategy which could ultimately be combined with vaccine strategies to enhance overall defense against ILT outbreaks.

Chapter 6: Potential for Future Eradication of ILT

6.1 Eradication Potential of ILT

In 1995, Bagust & Johnson examined the virus-host interactions of ILT as related to the prospect for eradication of the pathogen by the year 2000 (Bagust & Johnson, 1995). As it stands, we are 12 years past this postulated date for eradication and GaHV-1 remains a worldwide pathogen that causes significant economic damage to the poultry industry on an annual basis. Establishment of latency remains a critical issue regarding spread of both wild type and vaccine strains of the virus. The current vaccine options, coupled with current management practices, are not sufficient in combating the ability of the virus to establish latency and thus result in a constant pool of infection upon highly predictable reactivation.

6.2 Factors Associated with Eradication Potential

Bagust and Johnson (1995) cited eight factors associated with the eradication potential of ILT including, (1) the virus is not egg transmitted, (2) infection is essentially confined to chickens, (3) levels of infectivity are usually low, (4) spread of infection can be strongly confined by industry precautions, (5) virus infectivity is easily inactivated outside of the host, (6) immunity will absolutely protect against challenge, (7) immunity to GaHV-1 is cell-mediated, (8) and GaHV-1 strains are antigenically homogeneous, which will be discussed in the following sections.

6.2.1 Host Restriction

While ILT has been reported in turkeys, pheasants, and peafowl (Hilbink, 1985; Kaleta & Redmann, 1997; Portz *et al.*, 2008), these bird populations are comparatively small versus broiler, layer, and breeder populations in major poultry producing countries. Additionally, there have yet to be major outbreaks of ILT reported in a non-chicken species. While chickens remain the primary host reservoir of the virus, control of disease outbreaks in commercial and backyard flocks could help towards eradication of the disease, a goal that could ultimately be attained if vaccines which do not permit establishment of latency, development of carrier birds, and reversion to virulence were created. Additional consideration for wild populations of birds, however, would also have to be taken into account.

6.2.2 Egg Transmission

As discussed in section 2.5, while maternal antibodies are passed to offspring, they do not confer protection, nor do they interfere with vaccination. Additionally, ILT is not vertically transmitted from parent to offspring *in ovo*. The lack of egg transmission is hugely advantageous to prospective eradication efforts and the consideration of parental disease state does not interfere with current disease control programs.

6.2.3 Viral Infectivity

On average, high levels of viral shedding are sustained for approximately 7 dpi. However, during this period, the virus is highly transmissible and easily able to

spread from bird-to-bird and via personnel, equipment, and other fomites commonly present in a production setting. While the period of viral shedding is relatively short, control of the spread of disease is critical and successes in places like California have been described through strict biosecurity measures (Shivaprasad, 2012). Since the discovery of ILT in the early 20th century, biosecurity has been an essential factor in disease control and will remain so in eradication efforts.

6.2.4 Industry Precautions

While the above-mentioned industry precautions are successful in decreasing disease, strict adherence to biosecurity regulations can be both difficult to regulate and costly. New reservoirs of virus have also recently been uncovered, including retention of CEO vaccine in water drinker lines due to biofilm formation, leading to transmission of disease to birds up to 21 days following drinking water vaccination (Ou *et al.*, 2011). Additionally, wind-borne transmission of the virus and other intangible forms of spread complicate control efforts (Johnson et al., 2005). However, industry precautions remain a top priority, and if strictly followed, are successful in eliminating disease on-site and in a local radius. Precautions include but are not limited to use of personal protective equipment (PPE), proper disinfection of housing and production equipment, limited on-farm traffic, and all-in-all-out production policies in which flocks of birds enter and exit a farm as a whole and birds of different immune status are not intermingled. Additionally, effective disinfection programs are critical, including sufficient contact time and

biofilm-reducing sanitizers, and employee education is pivotal to assuring an understanding of disease transmission and ways to improve flock health and production efficiency.

6.2.5 Viral Inactivation

Following a disease outbreak and/or vaccination, complete disinfection and cleanup becomes increasingly critical compared to the end of a typical production cycle. As discussed in section 2.6, GaHV-1 is easily inactivated outside of the host using common disinfectants or low levels of heat. However, the importance of following manufacturers' guidelines in terms of disinfectant dilution and contact times are important, and the presence of organic matter during disinfection poses an additional complication that increases required contact times beyond typical manufacturers' recommendations (Ruano *et al.*, 2001). However, the fact that GaHV-1 inactivation is attained with relative ease using disinfectants and low-level heat decreases overall carryover and spread of the disease (Bagust *et al.*, 2000).

6.2.6 Host Immune Protection

Following live-attenuated vaccination, protection against challenge virus exposure is complete 6 to 8 days after vaccination and sustained for 15 to 20 weeks post vaccination, although waning of immunity has been described as early as 8 weeks post vaccination (Guy & Garcia, 2008). Established immunity will absolutely protect birds against challenge, thus preventing establishment of latency and of carrier state birds. However, the value of revaccination following the 15 to 20 week

marker is not definite, and prevention of vaccine virus replication due to neutralization is suspected to inhibit protection (Izuchi *et al.*, 1984; Fahey & York, 1990). Additionally, vaccination is not a standard practice, typically only performed in endemic ILT regions, leaving many populations of birds susceptible as the virus spreads.

Overall, while immunity will absolutely protect birds against infection as Bagust and Johnson suggest, length of that immunity elicited by current vaccine options is insufficient. The prospect of alternative vaccine options that elicit sustained protection, or permit effective revaccination, is dependent on further developments in ILT vaccinology. Other vaccine options such as viral vector vaccines exist, however as discussed in section 4.4, issues with establishment of an immune response that is robust enough to fully protect birds still remain.

6.2.7 Host Cell-mediated Immune Response

As discussed in sections 2.6 and 7.2.6, the main response to GaHV-1 infection is cell mediated. While the fact that maternal antibodies do not interfere with vaccination is advantageous as Bagust and Johnson suggest, the inability to revaccinate due to virus neutralizing antibodies is hugely disadvantageous to ILT protection programs. Account for this fact must be taken in development of future vaccines, in addition to issues such as reversion to virulence and establishment of latency.

6.2.8 Antigenetic Homogeneity

At the core of the GaHV-1 virion is a dsDNA genome, which is relatively stable and provides an advantageous backbone for vaccine development. GaHV-1 innately lacks the quasi species conundrum of many RNA viruses because the virus does not display antigenic variation, and cross-neutralization and cross-protection of known GaHV-1 strains has been shown to occur (Russell & Turner, 1983). This factor conveys a large advantage to scientists and has allowed ILT vaccine research to flourish, past and present, and ultimately defines the prospect of ILT eradication as a possible feat.

In addition to these eight factors, Bagust and Johnson suggest consideration of the benefit-cost ratio and time scale for eradication of ILT. Their projection for eradication of ILT from production sites by the year 2000 was based on quarantine, and hygiene measures, in addition to genetically engineered vaccines. Quarantine and hygiene, in the form of strict biosecurity, have proven to successfully prevent the spread of the virus. However, vaccines have yet to be successfully developed in a manner that would move poultry producers to discontinue live-attenuated vaccine use. As most field strains are indistinguishable from vaccine strains, discontinuing live-attenuated vaccine use would remove the largest source contributing to annual outbreaks of disease, and would ultimately foster eradication of the virus.

Chapter 7: Summary & Conclusions

GaHV-1 is a virus that remains analyzed to a much lesser extent than other alphaherpesviruses, with much of biology of the virus based on HSV-1. Further development in the molecular characterization of the virus is pivotal to novel vaccine development, an area currently concentrating on genetic engineering and necessary to the end of live-attenuated vaccine use. Vaccine related outbreaks have been described since the development of ILT vaccines and remain the largest source of virus in the field. If spread of the virus is to be stopped to a significant extent, stopping the use of live-attenuated vaccines represents a direct way to remove a major source of virus from the environment. However, because the cost, ease of use, and availability of the CEO and TCO live-attenuated vaccines meets the needs of the poultry industry, their use is still prevalent and continues to shape the molecular epidemiology of the virus.

For each region of the world, there is an optimal set of target genes for detection and differentiation of GaHV-1 related outbreak strains. However, with recombination events like that which occurred with the SA-2, A20 and Serva vaccine strains in Australia, modification of these target genes may be necessary with time and change in local epidemiology of virus strains. New potential exists in epigenetic research of host-virus interactions, and those interactions involved in resistance and susceptibility to the virus could be integrated into production lines of birds to strengthen ILT protection programs. However, if continued thought of ILT eradication is to be considered, ultimately, development of vaccines that provide

sufficient immune protection while retaining the inability to revert to virulence, establish latency, and create a carrier state in birds, will allow successful eradication and control of the disease.

Appendix

Supplemental List 1: Global Distribution of ILT as of 2012

Year of last report of disease in parenthesis; 2012 status yet to be reported
in completion for all countries; sited from the OIE-WAHID ILT disease
timeline unless otherwise noted (Health, 2012).

2000 to 2012 ILT+
1. Afghanistan (2012)
2. Argentina (2012)⌘
3. Armenia (2005)
4. Australia (2011)⌘
5. Austria (2007)*
6. Bahrain (2011)❖
7. Bangladesh (2010)*
8. Belarus (2005)
9. Belgium (2011)⌘
10. Benin (2010)*
11. Bolivia (2011)
12. Brazil (2010)
13. Cameroon (2010)❖
14. Canada (2012)⌘
15. Cape Verde (2009)*❖
16. Central African
    Republic (2011)
17. Chile (2012)⌘
18. China (2012)⌘
19. Columbia (2012)⌘
20. Congo (Dem. Rep. of)
    (2011)
21. Costa Rica (2011)
22. Cyprus (2011)
23. Czech Republic (2011)
24. Denmark (2012)⌘
25. Ecuador (2012)
26. Eritrea (2009)*⌘
27. Ethiopia (2000)
28. Finland (2012)
29. France (2006)*⌘
30. French Polynesia(2011)
31. Germany (2011)⌘
32. Greece (2000)
33. Guam (2001)*
34. Guinea (2004)*
35. Hong Kong (2010)
36. Hungary (2011)⌘
37. India (2009)[1]

38. Indonesia (2007)*
39. Iran (2000)
40. Iraq (2012)
41. Ireland (2012)
42. Israel (2012)
43. Italy (2011)❖
44. Japan (2012)⌘
45. Kiribati (2012)☉
46. Korea, North (2010)
47. Korea, South (2010)*⌘
48. Kuwait (2005)
49. Lebanon (2011)⌘
50. Libya (2010)
51. Malaysia (2007)⌘
52. Malta (2008)
53. Mexico (2011)⌘
54. Morocco (2004)*
55. Myanmar (2010)*
56. Namibia (2007)
57. Netherlands (2011)⌘
58. Nepal (2009)
59. New Caledonia (2008)
60. New Zealand (2012)⌘
61. Nigeria (2004)*
62. Norway (2012)⌘
63. Pakistan (2011)
64. Palestine (2010)
65. Peru (2012)
66. Philippines (2011)⌘
67. Poland (2007)
68. Portugal (2008)
69. Puerto Rico (2003)
70. Reunion (2003)
71. Russia (2006)
72. Rwanda (2009)
73. Samoa (2005)❖
74. South Africa (2008)⌘
75. Swaziland (2001)
76. Sweden (2012)⌘
77. Switzerland (2012)⌘

78. Syria (2008)*
79. Taiwan (2010)⌘
80. Thailand (2004)
81. Togo (2010)
82. Trinidad & Tobago
    (2004)
83. Turkey (2003)*
84. Turkmenistan (2010)*
85. Uganda (2011)⌘
86. United Kingdom
    (2011)⌘
87. United States of
    America (2012)⌘
88. Ukraine (2004)
89. Uruguay (2012)⌘
90. Uzbekistan (2004)
91. Wallis & Futuna
    (2002)☉
92. Yemen (2006)*

1999 and prior ILT+
1. Albania (1996)*
2. Algeria (1989)*
3. Brunei (1996)
4. Bulgaria (1994)
5. Burkina Faso (1999)*
6. Chad (1972)*
7. Cook Islands (1996)*☉
8. Egypt (1991)
9. Gabon (1997)
10. Georgia (1990)
11. Kyrgyzstan (1992)
12. Lesotho (1996)
13. Luxembourg (1999)
14. Malawi (1996)
15. Mali (1996)*
16. Mauritius (1994)
17. Moldavia (1992)
18. Mozambique (1998)
19. Paraguay (1996)

20. Romania (1999)
21. Saudi Arabia (1998)*
22. Senegal (2000)
23. Singapore (1989)
24. Spain (1999)❖
25. Sri Lanka (1994)
26. Suriname (1997)
27. Tanzania (1996)*
28. Tunisia (1998)
29. United Arab
    Emirates (1997)
30. Venezuela (1977)

Suspected
1. Andorra
2. Anguilla
3. Antigua & Barbuda
4. Aruba
5. Bahamas
6. Barbados
7. British Virgin
    Islands
8. Cayman Islands
9. Cuba
10. Curacao
11. Dominica
12. Dominican Republic
13. F.Y.R. of Macedonia
14. Grenada
15. Guadeloupe
16. Haiti
17. Jamaica
18. Laos
19. Lithuania
20. Martinique
21. Montserrat
22. Niger
23. Sao Tome &
    Principe

24. St. Barthélemy
25. St. Kitts & Nevis
26. St. Lucia
27. St. Maarten
28. Turks & Caicos
29. U.S. Virgin Islands
30. Zambia

Disease Negative
1. Belize
2. Greenland
3. Honduras

Disease Never Reported
1. Angola
2. Azerbaijan
3. Bhutan
4. Bosnia &
    Herzegovina
5. Botswana
6. Burundi
7. Comoros
8. Croatia
9. Djibouti
10. El Salvador
11. Equatorial Guinea
12. Estonia
13. Fiji
14. French Guiana
15. Ghana
16. Guatemala
17. Guyana
18. Iceland
19. Jordan
20. Kazakhstan
21. Kenya
22. Kosovo
23. Latvia

24. Liberia
25. Liechtenstein
26. Madagascar
27. Maldives
28. Micronesia
29. Mongolia
30. Montenegro
31. Nicaragua
32. Oman
33. Qatar
34. San Marino
35. Serbia
36. Seychelles
37. Sierra Leone
38. Slovakia
39. Slovenia
40. Sudan
41. Tajikistan
42. Vanuatu
43. Zimbabwe

No Data
1. Cambodia
2. Congo (Rep. of)
3. Cote d'Ivoire
4. East Timor
5. Falkland Islands
6. Gambia
7. Guinea Bissau
8. Mauritania
9. Monaco
10. Panama
11. Papua New Guinea
12. Solomon Islands
13. Somalia
14. Tuvalu
15. Vietnam
16. Western Sahara

Legend
* Data unavailable after last GaHV-1+ reported year; current status unknown, however no literary evidence or reports of becoming disease free.

❖Last reported year reported as a suspected year, however previous years confirmed GaHV-1 positive (Health 2012).

⌘ GaHV-1 positive for 10 years or greater (Health, 2012).

⊙The Secretariat of the Pacific Community currently sites "serologic evidence [of GaHV-1] in Cook Islands, Kiribati, Tonga, and Wallis and Futuna" (Community, 2012)

[1] In a search of all available GaHV-1 DNA sequences on GenBank, the GaHV-1 partial p32 gene sequence for isolate HBL/viral/AP/02/10 (GenBank accession number FN811131) was referenced as an isolate from a poultry farm in Tamilnadu, Namakkal, India from 19-Dec-2009 (Pal *et al.*, 2009).

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