Chapter 1

Review of Literature

1.1 Scope of the Problem

Turkey Hemorrhagic Enteritis Virus (THEV) is an officially recognized member of the viral family *Adenoviridae,* genus *Siadenovirus.* Strains of THEV responsible for three distinct clinical diseases have been isolated from chickens, turkeys, and pheasants. Most experimental evidence indicates that all three diseases are caused by a single species of virus.

Hemorrhagic Enteritis (HE) is a disease of turkeys caused by THEV resulting in depression, splenomegaly, bloody diarrhea, immunosuppression, and mortality. Intestinal lesions and mortality vary by host and by virus strain, and are immune-mediated. HE is the most economically significant disease caused by any strain of THEV. It was originally described in the 1930's and 1950's in turkeys located in Ohio and Minnesota, respectively (Pomeroy and Fenstermacher, 1937; Gale and Wyne, 1957). By the 1960's, HE had become a serious problem for commercial turkey growers in Virginia and seven other states, with flock mortality ranging from 1-60% (Gross and Moore, 1967; Domermuth and Gross, 1971). By the mid-1970's, severe cases of clinical HE in Virginia became less common. Naturally occurring avirulent strains of THEV were isolated in turkey flocks refractory to experimental infection with virulent THEV. These avirulent strains and pheasant origin THEV strains were utilized in live-virus vaccines (Domermuth *et al*., 1977). These vaccines proved to be extremely effective at preventing HE in young turkey poults. Development of an *in vitro* propagation system for THEV in the early 1980's permitted expansion of HE vaccine production into the commercial sector (Nazerian *et al*., 1982; Nazerian and Fadly, 1982; Fadly *et al*., 1985).

Unfortunately, the avirulent strains of THEV cause a transient period of immunosuppression 8-14 days post-vaccination (Larsen *et al*., 1985). Colibacillosis in turkey poults greater than six weeks-of-age is often exacerbated by THEV infection. In

1984, losses due to colibacillosis were estimated at $40 million each year (Sponenberg *et al.*, 1985; Larsen *et al.*, 1985; Sharma, 1991). THEV-induced immunosuppression also has the potential to interfere with vaccination protocols for other infectious agents. Alternatives to live-virus vaccines have been explored, but none have proven to be as effective.

1.2 Adenoviruses

1.2.1 Taxonomy Overview

Adenoviruses are non-enveloped icosahedral viruses with linear, double-stranded DNA genomes. They are known to be involved in infectious diseases of virtually every class of vertebrates. Adenoviruses are divided into four genera based on phylogenetic differences: *Mastadenovirus*, *Aviadenovirus*, *Atadenovirus*, and *Siadenovirus*. Mastadenoviruses infect a wide range of mammalian species including humans, monkeys, cows, dogs, horses, mice, sheep, and pigs. Aviadenoviruses infect a wide range of avian species including chickens, quail, ostriches, pigeons, psittacines, geese, turkeys, and ducks. The most recently accepted genera, *Atadenovirus* and *Siadenovirus*, are composed of "orphan" adenoviruses whose genetic structure and evolutionary history are not consistent with the host-defined hierarchy of mast- and aviadenoviruses. Atadenoviruses are known to infect reptilian and avian species in addition to sheep, cows, and goats. Siadenoviruses are known to infect frogs and avian species. Designation of a fifth genus composed of fish adenoviruses is currently under consideration by the ICTV (Benko and Harrach, 1998; Davison *et al.*, 2003; Wadell, 2002; Harrach, 2002; Both, 2002; Benko *et al.*, 2002; Davison and Harrach, 2002). A thorough review of adenovirus taxonomy has been published (Davison *et al.,* 2003).

1.2.2 General Adenovirus Mechanisms

The majority of research involving the mechanisms of the adenovirus infectious cycle has been done on members of the genus *Mastadenovirus*. Despite the differences in

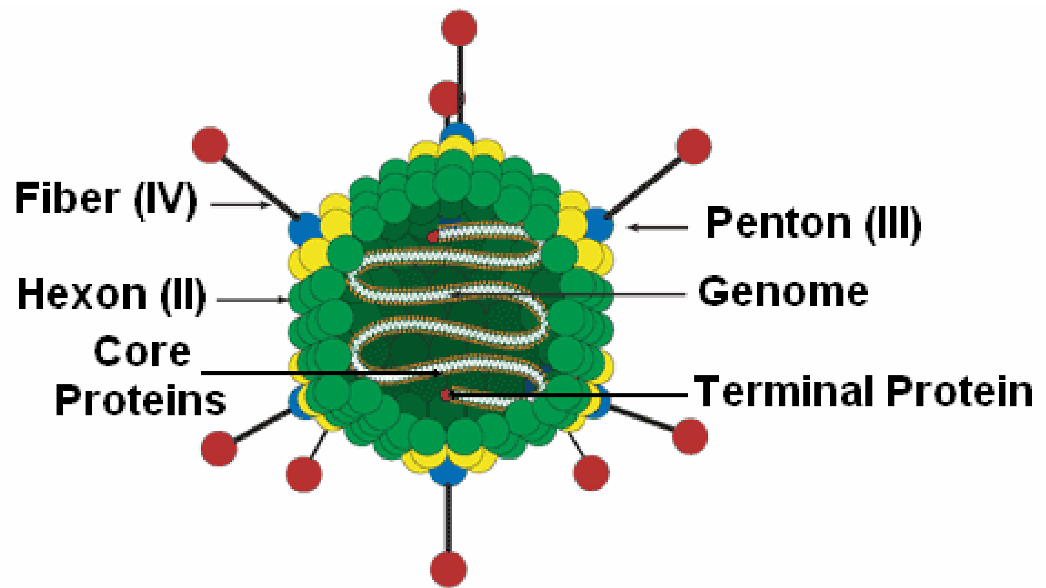

Figure 1-1: Major Structural Features of the *Adenoviridae*
The major capsid protein in each virion is the hexon. Penton proteins are located at each vertex, with one or two non-covalently bound trimeric fibers extending from each. The core is composed of viral proteins pVII, pX (mu), and pTP.

Image source: superman.blogs.sapo.pt/arquivo/114634

mammalian, avian, and reptilian cells, the basic mechanisms are assumed to be the same for all adenovirus infections. A detailed review of the adenovirus infectious cycle is available (Shenk, 1996).

The first step during infection is the attachment of the virion to a host target cell. Adenoviruses recognize target cells within the host organism via specific cellular receptors. The viral fiber is primarily responsible for interaction with the receptor, and attaches non-covalently. The virion must then penetrate the plasma membrane via receptor-mediated endocytosis. Amino acid motifs located on the external portions of the penton proteins located at the base of each fiber interact with integrins located on the external surface of the target cell, triggering endocytosis. Changes in the chemical environment within the endosome begin the uncoating process. Penton-integrin interactions trigger release of the virion into the cytosol. The viral protease becomes activated, and begins to degrade internal protein VI, which destabilizes the capsid further. The nucleocapsid, made up of the genome coated with protein VII and two covalently bound terminal proteins, is transported into the nucleus of the cell via nuclear pore complexes (Greber *et al.,* 1997; Shenk, 1996).

The next step is the takeover of the host cell transcription machinery and expression of genes necessary for viral genome replication. Transcription of viral genes is divided into early and late phases, in which mRNA populations accumulate at different times after infection (Binger and Flint, 1984). Early phase viral proteins are known to interact with cellular transcription factors and activate viral transcription via enhancer sequences in the viral promoters (Hearing and Shenk, 1983, 1986; Fax *et al*., 2000; Parker *et al*., 1997). The viral DNA polymerase (AdPOL), preterminal protein (pTP), and DNA binding protein (DBP) are produced, and begin replication of the genome (Liu *et al*., 2003). IVa2 transcription is repressed by a cellular factor, and is not produced immediately during the early phase. Instead, accumulation of viral genomes resulting in titration of the repressor is required before IVa2 is produced (Huang *et al*., 2003; Lin and Flint, 2000). As the amount of IVa2 increases, transcription from the major late promoter (MLP) is activated. In this way, IVa2 mediates the time course of infection, triggering the

transition from the early phase to the late phase (Jansen-Durr *et al*., 1988, 1989, 1990; Lutz and Kedinger, 1996; Pardo-Mateos and Young, 2004 a, b; Tribouley *et al*., 1994).

All of the late genes are transcribed from the MLP, via alternative splicing from a single polycistronic mRNA. In addition to activation by IVa2, the MLP is further activated by cellular transcription factors that bind to specific enhancer sequences within and upstream of the promoter (Sheppard *et al*., 1998; Song *et al*., 1996; Song and Young, 1998; Lu *et al*., 1997; Young, 2003; Zain *et al*., 1979). Early genes are down-regulated after the onset of transcription from the MLP, though the mechanism in unknown (Fessler and Young, 1998). Accumulation of late gene products, including structural proteins and packaging machinery, triggers the assembly of progeny virions. IVa2 is known to bind specific sequences at the left end of the genome, and interacts with proteins VII, VIII, and 52K to facilitate packaging (Hammarskjold and Winberg, 1980; Grable and Hearing, 1990, 1992; Ostapchuck and Hearing, 2003; Schmid and Hearing, 1997; Singh *et al*., 2005; Zhang and Arcos, 2005; Gustin *et al*., 1996; Gustin and Imperiale, 1998). Virions accumulate within the nucleus of the infected cells, sometimes forming semi-crystalline arrays that are visible by light microscopy as intranuclear inclusion bodies. After cleavage of protein precursors with the viral endoprotease, mature virions lyse the infected cell and are released.

1.2.3 Common Genes

Sixteen genes are conserved in all members of the family *Adenoviridae* based on comparative analysis of a variety of DNA sequences (Reddy *et al*., 1998; Morrison *et al*., 1997; Nagy *et al*., 2001; Roy *et al*., 2004; Farkas *et al*., 2002; Benko *et al.,* 2002; Wellehan *et al*., 2004; Davison *et al*., 2000; Pitcovski *et al*., 1998). Eight of these have been confirmed to be structural components of infectious virions. The others perform functions that are indispensable to virus replication. All of the conserved genes that are transcribed early after infection are non-structural, and are involved in genome replication and transcriptional activation of the late genes. The conserved late genes are

either structural components or are involved in the packaging of genome and assembly of infectious virions.

Early

Three of the early genes are involved primarily in replication of the double-stranded DNA genome. AdPOL, pTP, and DBP are involved in formation of the pre-initiation complex. First, DBP coats the DNA and enhances binding of nuclear transcription factors to the origin of replication. A heterodimer made up of pTP and AdPOL then binds specific sequences within the origin. The origin is located within the inverted terminal repeats, and the AdPOL-pTP binding site is located at nucleotide (nt) 9-18. Replication is then initiated by covalent binding of the terminal dCMP and a serine residue within pTP. This cytosine serves as a primer for DNA synthesis, and initiates separation of the pTP from AdPOL. pTP remains covalently bound to the terminal cytosine throughout replication and packaging (Botting and Hay, 2001; Webster *et al*., 1997; Freimuth and Ginsberg, 1986). DBP is known to coat double stranded DNA as it is formed, protecting it from degradation. An additional function of the DBP may be transcriptional activation of viral promoters (Chang and Shenk, 1990).

The IVa2 protein is involved in several aspects of the viral infection cycle. IVa2 is required for assembly of progeny virions. IVa2 binds to the terminal packaging sequences, and there is evidence that capsid proteins may assemble around the genome (Zhang and Imperiale, 2000, 2003; Zhang *et al*., 2001). IVa2 is known to interact with several other viral proteins, including VII, VIII, and 52K (Singh *et al*., 2005; Zhang and Arcos, 2005; Gustin *et al*., 1996; Gustin and Imperiale, 1998). In addition to packaging, IVa2 plays a critical role in the regulation of transcription of late genes from the MLP. IVa2 binds sequences that are downstream of the MLP and thereby mediates the transition from early to the late phase of the infectious cycle (Lutz and Kedinger, 1996; Pardo-Mateos and Young, 2004a, b; Tribouley *et al*., 1994).

Late

Four of the twelve late genes are non-structural. The viral 52K protein is required for assembly of progeny virions. It is thought to ensure stable association between DNA and capsid proteins, and interacts with IVa2. It is possible that 52K also interacts with IVa2 at an earlier stage in infection, perhaps involved in MLP trans-activation (Gustin *et al*., 1996; Gustin and Imperiale, 1998). The viral endoprotease, also called adenain, plays an important role during uncoating and maturation of progeny virions. It is responsible for cleavage of viral protein precursors, especially capsid proteins, into mature forms (Ruzindana *et al*., 2002). The viral 100K and 33K proteins are involved in some aspect of the virion assembly process, although they are not well-characterized. The 100K protein in particular is thought to assist in hexon trimerization.

The remaining eight late genes are structural components of the virion. Adenovirus virions are made up of two distinct parts: the core and the capsid. The core is composed of viral proteins pVII, pX (mu), and pTP. The pTP protein is covalently bound to each terminus of the linear genome. pVII and pX bind the DNA genome similar to histone proteins, creating a chromatin-like nucleocapsid. Furthermore, pVII interacts with IVa2 and 52K to facilitate packaging (Zhang and Arcos, 2005). pVII may also interact with nuclear pore complexes to facilitate the entry of viral genome into the nucleus of infected cells (Greber *et al*., 1997).

The major capsid protein in each virion is the hexon. Each facet of the icosahedron is made up of nine trimeric hexons, called a "group of nine" (GON). During assembly of the capsid, GONs are combined with penton, pVIII, and pIIIa proteins. pIIIa proteins link adjacent facets of the icosahedron. Pentameric penton base proteins are located at each vertex, with one or two non-covalently bound trimeric fibers extending from each. It is unclear whether the genome is packaged into empty viral capsids, or if the capsid forms around the nucleocapsid core. The core is attached to the inside of the icosahedral capsid by viral protein VI, which is a DNA binding protein that interacts with the hexon (Shenk, 1996; van den Hurk, 1992).

The adenovirus fiber is responsible for initial attachment to host cells via a primary receptor protein. The fiber has three domains: the tail, shaft, and knob. The tail is responsible for non-covalent attachment of the fiber to the penton base at each vertex of the virion. The shaft is made up of repeats of a triple beta-spiral motif approximately 15 amino acids in length (Stouten and Sander, 1992; van Raaij *et al*., 1999). The knob is the receptor binding domain and is responsible for binding specificity. Chimeric fibers have been shown to change cell tropism, and recombination in the knob domain has been proposed as a possible mechanism of sudden shifts in host species tropism (Glasgow *et al*., 2004; Nagy *et al*., 2002, Magnusson *et al*., 2001). The structure of the knob has been determined, as well as domains required for trimer formation (Henry *et al*., 1994; Hong and Engler, 1996). The structure of the knob domain is susceptible to change by only a few amino acid changes. Several distinct receptors have been described for mastadenoviruses including the Coxsackie and Adenovirus Receptor (CAR), MHC class I, and sialic acid residues (Arnberg *et al*., 1997, 2000a, b; Stevenson *et al*., 1995; Howitt *et al*., 2003; Roelvink *et al*., 1999; Tan *et al*., 2001; Hong *et al*., 1997).

1.2.4 Genus *Siadenovirus*

The name of the genus *Siadenovirus* is derived from a genus-specific open reading frame (ORF) in the E1 region whose putative gene product has high sequence similarity with bacterial sialidases. The two recognized members of this genus are THEV and Frog adenovirus 1 (FrAdV-1)(Davison *et al*., 2000; Davison and Harrach, 2002). It has been speculated that siadenoviruses originated in amphibians and then adapted to avian species (Davison *et al*., 2003). There is no experimental evidence to confirm this speculation.

Members of the genus *Siadenovirus* have several putative genus-specific genes that share no sequence similarity with any proteins studied in other organisms. It is not known what functional importance these genes may have (Davison *et al*., 2000). Very little is known about the replication of FrAdV-1 *in vivo* and, although THEV has been studied extensively for decades, detailed information about the molecular mechanisms of

replication and pathogenesis is still lacking. An understanding of the role of genus-specific genes in viral replication and pathogenesis is essential in order to determine mechanisms by which siadenoviruses cause disease in their respective hosts.

1.3 Turkey Hemorrhagic Enteritis Virus

1.3.1 Morphology

THEV is an officially recognized member of the viral family *Adenoviridae,* genus *Siadenovirus.* As such, it has morphologic characteristics in common with other adenoviruses. The capsid of THEV is primarily made up of three major proteins: the hexon, penton, and fiber. There is one fiber structure (made up of a trimer of fiber protein monomers) non-covalently attached to the penton protein at each vertex (van den Hurk, 1992). The buoyant density of THEV is 1.32-1.34 g/cm^3 (Ossa *et al*., 1983a; Iltis *et al*., 1977; van den Hurk, 1992). Electron microscopy of intranuclear inclusion bodies and cesium chloride-purified virions from several strains of the virus revealed non-enveloped, icosahedral virions with diameters ranging in size from 60-90 nanometers (nm)(Itakura and Carlson, 1975a; Carlson and Al-Sheikhly, 1974; Tolin and Domermuth, 1975; Ossa *et al*., 1983a; Gomez-Villamandos *et al*., 1994; Trampel *et al*., 1992; van den Hurk, 1992; Iltis *et al*., 1975a; Wyand *et al*., 1972; Iltis *et al*., 1977; Iltis and Daniels, 1977; Carlson *et al*., 1973). Three distinct populations of virions were observed, differentiated by electron-density of their cores: dense osmiophilic, loose osmiophilic, and empty (Trampel *et al*., 1992; Wyand *et al*., 1972; Itakura and Carlson, 1975a; Tolin and Domermuth, 1975). Differences in core density were thought to be evidence of stages of virion assembly. Particles with “loose” and empty cores were not formed completely, and lacked nucleic acid and other core components. Particles with a dense osmiophilic core were thought to represent complete, infectious virions and were seen at a much greater frequency than the other two types. The electron micrographs showed a virion consistent in shape and structure with other adenoviruses.

As a non-enveloped virus, THEV is capable of withstanding a wide range of temperatures and environments. THEV is able to remain infectious for long periods of time in fecal material and contaminated litter. It is susceptible to chlorine and drying, but only after proper removal of all organic material (Domermuth and Gross, 1971, 1972).

The structural proteins of THEV have been characterized in the literature three times, with some conflicting results. In all of the studies, viral proteins were separated using SDS-PAGE and visualized by Western blot, but there was variation between the results of the studies. Two studies looked at the structural proteins present in cesium chloride-purified virions. In the first study, eleven polypeptides were determined to make up each virion, ranging in size from 9.5-96 kilodaltons (kDa)(van den Hurk, 1992). In the other, eight polypeptides were determined to make up each virion, ranging in size from 18-97 kDa (Zhang *et al.,* 1991). However, preparations from different isolates did not match, and the bands on the gels were very unclear. A third study examined the viral proteins present in infected cell lysates, probed with anti-THEV serum. There were eleven polypeptides that reacted with the polyclonal serum, ranging in size from 14.6-88 kDa (Nazerian *et al*., 1991).

1.3.2 Characteristics of HE in Turkeys

Susceptibility

Several spontaneous outbreaks of HE have been documented in commercial turkey flocks dating back to the mid-1930's. The ages of the birds affected range from 6-12 weeks old. HE was first described in 1936 in 35 turkey poults, 7-12 weeks-of-age, from several different flocks with mortality approaching 10% (Pomeroy and Fenstermacher, 1937). The causative agent was unknown at the time, as the existence of viruses had not yet been discovered. HE was later described in 1957 in two experimental turkey flocks with 1100 birds each; one was 7 weeks old, the other was 11 weeks old. Mortality for the two flocks was 1.6% and 3.5%, respectively (Gale and Wyne, 1957). By the early 1970's, the disease had become a widespread problem in the commercial turkey flocks of the United States and Canada. There were extensive problems with HE in

turkey flocks in Ontario, in which 8-12 week-old birds exhibited widespread watery diarrhea with some blood, and mortality three days later (Itakura *et al*., 1974). A natural outbreak of HE was described in 41, 6 week-old turkey poults, with 17% mortality in the first 48 hours (Domermuth *et al*., 1973). Five separate outbreaks were described in 8-11 week-old turkeys (Wilcock and Thacker, 1975). Production of vaccines to prevent HE in turkeys in the late 1970's and 1980's led to fewer losses related directly to the virulent strains of THEV (THEV-V). There were some reported cases of HE as it spread from North America to Europe. In 1994, a 7-8 week-old flock in Spain broke with a 3-4% spike in mortality associated with depression, splenomegaly, and bloody diarrhea (Gomez-Villamandos *et al*., 1994).

The time at which turkey poults are most likely to develop clinical disease in the field is 6-12 weeks-of-age, though it is not known whether this is due to age-dependent susceptibility or coincidence. Several experiments have been conducted to determine the characteristics of susceptibility in SPF turkeys. THEV has been shown to spread horizontally to susceptible poults following exposure to infected birds (Itakura *et al*., 1974; Itakura and Carlson, 1975a; Sharma, 1994). One study concluded that viral distribution and magnitude of infection varies from bird to bird, and is not necessarily influenced by route of inoculation or incubation period (Iltis *et al*., 1975a).

Egg inoculation and inoculation of poults as young as 1 day-old with THEV has resulted in a productive infection. In one experiment, 24 day-old turkey embryos and 1 day-old turkey poults inoculated with a vaccine strain had a good protective antibody response by 4 weeks post-inoculation (pi)(Ahmad and Sharma, 1993). In other experiments, newly hatched poults were shown to be susceptible to viral infection, but refractory to intestinal lesions and mortality associated with clinical disease. In 3 day-old poults orally inoculated with THEV-V, the only lesions 5 days post-inoculation (dpi) were splenomegaly with intranuclear inclusion bodies (INIs) and hyperplasia of the white pulp (Nazerian and Fadly, 1982; Fadly and Nazerian, 1982). Turkey poults 16-18 days-of-age were also shown to be susceptible to vaccine strains, indicated by a protective antibody response (Chary *et al*., 2002).

At 2 weeks-of-age, turkey poults apparently mature to the point where they can develop intestinal hemorrhage in response to THEV infection. In 13 day-old poults inoculated intravenously (IV) with THEV-V, 2/5 had clinical HE, and one died 3 dpi. In 24 day-old poults, 5/5 developed clinical HE, and one died 2 dpi. In 17 day-old poults orally inoculated with THEV-V, 5/8 had intestinal hemorrhage at 3-4 dpi, and one died at 3 dpi (Rautenschlein *et al*., 1998). THEV-V was also shown to cause 100% clinical HE with 50% mortality in susceptible 2 and 5 week-old poults (Fadly and Nazerian, 1982). Moreover, the incidence of hemorrhage in maternal antibody-free poults was higher after 2 weeks-of-age than at an earlier age (Fadly and Nazerian, 1989). Early age resistance of turkey poults to intestinal hemorrhage was shown to be independent of maternal antibody, and is likely cell maturation dependent (Fadly and Nazerian, 1989).

There is some evidence that is contradictory to the studies above. In one study, maternal antibody-free 2, 7, and 14 day-old turkey poults injected intramuscularly with a field isolate of THEV-V had no gross or histopathologic lesions, and none were protected against virulent challenge at 8 weeks-of-age. Poults that were 3, 4, or 8 weeks old at the time of injection had INIs at 3 dpi, and were protected from virulent challenge (Beasley and Wisdom, 1978). This suggests that age related resistance to viral infection is not maternal antibody-dependent.

Age of susceptibility is an important factor to consider when developing vaccination protocols. In one of several field trials of vaccine strains of THEV, 4 and 5.5 week-old turkeys were successfully vaccinated (Fadly *et al*., 1985). Maternal antibody present in young turkey poults is known to interfere with viral infection in an age-dependent manner. In one study, maternal antibodies protected poults from clinical HE up to 6 weeks-of-age, and interfered with successful vaccination until at least 5 weeks-of-age (Fadly and Nazerian, 1989). It is important to vaccinate poults against HE after the decline of maternal antibody titers, but prior to exposure to virulent field strains.

Cell Tropism

The complete range of cells susceptible to THEV and the identity of the cellular receptor is currently unknown. Based on tissue tropism and histopathology, the general characteristics of susceptible cell populations have been determined. Splenectomy prevented mortality and clinical lesions, but did not completely prevent an antibody response to THEV (Ossa *et al*., 1983b). Cells containing INIs in the spleen of infected poults were identified in one report as "splenic bound macrophages" (Fitzgerald *et al*., 1992). Cells with INIs in turkeys from an outbreak of HE in Spain were described as mononuclear phagocytes and lymphocytes. Macrophages were characterized by "abundant cytoplasm containing moderate numbers of lysosomes, phagosomes, and cell organoids." Lymphocytes were described as having "little cytoplasm, containing no phagosomes or lysosomes, few mitochondria, and numerous ribosomes, together with rough E.R. at varying stages of development" (Gomez-Villamandos *et al*., 1994). INIs were seen in non-proliferating, mature lymphocytes throughout the system 4-6 dpi (Saunders *et al*., 1993). Adherent and non-adherent leukocytes from peripheral blood were found to be susceptible to infection by THEV, though the virus could only be serially passaged a limited number of times. The percentage of infected cells was highest at 3 dpi, which corresponds to the incubation period of IV inoculated turkeys *in vivo* (van den Hurk, 1990).

Although these studies revealed general cell morphology, further studies investigating cell markers associated with infected cells were required to confirm the identity of the cells. B-lymphocytes and macrophages were found to be the primary target cells for viral replication *in vivo*. THEV can be propagated in a Marek's Disease Virus (MDV)-induced lymphoblastoid cell line (MDTC-RP19), as well as in turkey peripheral blood leukocyte cultures (Nazerian *et al*., 1982; Nazerian and Fadly, 1982; van den Hurk, 1990). Splenectomy and chemical bursectomy each prevent intestinal hemorrhage without complete prohibition of viral replication (Beasley and Wisdom, 1978; Fadly and Nazerian, 1982; Ossa *et al*., 1983b; Suresh and Sharma, 1995). The ability of the virus to replicate in chemically bursectomized turkeys is most likely due to infection of other IgM

bearing cells, including macrophages. Replication of THEV was confirmed in IgM bearing B-lymphocytes and macrophage-like cells by polymerase chain reaction (PCR) and *in situ* hybridization (ISH) (Suresh and Sharma, 1996). IgM bearing cells are diminished during peak THEV infection in the spleen. It is not known whether this decrease is a direct result of viral cytolysis.

T-lymphocytes are not a major target cell type for THEV replication. Studies on fractionated cells from THEV infected spleens revealed no viral DNA in $CD4^+$ or $CD8^+$ T-lymphocytes (Suresh and Sharma, 1996). There are minimal lesions associated with THEV infection in the thymus. Small numbers of INIs in mature and immature lymphocytes have been documented, but the overall structure of the tissues was not affected (Iltis *et al*., 1975a; Itakura *et al*., 1974; Itakura and Carlson, 1975a; Fadly and Nazerian, 1982; Saunders *et al*., 1993; Silim and Thorsen, 1981). Furthermore, THEV is incapable of infecting T-lymphoblastoid cell lines in the same way as it infects MDTC RP-19 cells (Nazerian and Fadly, 1982).

<u>Dose-dependence of Lesions</u>

One factor that affects the incidence of lesions and mortality in infected turkeys is the quantity of virus in the inoculum. There is a linear relationship between dosage and formation of gross lesions in infected turkeys. In one study, 1 mL of inoculum produced HE lesions in 90% of infected birds. An inoculum of 0.1 mL produced HE lesions in 60% of infected birds, and 0.01 mL of inoculum produced HE lesions in 25% of infected birds (Gross and Moore, 1967). In another study, dose-dependence of intestinal lesions and mortality was determined. Inoculation with 10^6 Tissue culture infectious dose 50% ($TCID_{50}$) caused HE lesions in 100% of infected birds, and 80% mortality. Inoculation with 10^5 $TCID_{50}$ caused HE lesions in 90% of infected birds, and 50% mortality. Inoculation with 10^4-10^2 $TCID_{50}$ caused HE lesions in 50-80% of infected birds and no mortality. Inoculation with 10^1 $TCID_{50}$ did not cause any lesions (Nazerian and Fadly, 1986). The relationship between dose and splenomegaly has also been investigated. Turkey poults receiving 10^{-3}-10^{-4} dilutions of the viral inoculum had more than twice the

spleen:body weight ratio of un-inoculated controls. Poults receiving a 10^{-5} dilution had an 80% increase in spleen:body weight ratio, and those receiving a 10^{-6} dilution had a 45% increase. Poults receiving 10^{-7} and 10^{-8} dilutions did not have an increase in spleen:body weight ratios (Ossa *et al*., 1983a). Although incidence of lesions is dose-dependent, the dose does not affect the incubation period of the virus. Moreover, a high dose of an avirulent strain has not been shown to result in clinical disease.

Pathology

Splenic Lesions

The first accounts of the splenic lesions caused by THEV infection in turkeys were given in 1937 and 1957 from birds that died in outbreaks in Minnesota and Ohio, respectively. Interestingly, the findings were not significant, as spleens were only dark and sometimes congested but not enlarged (Pomeroy and Fenstermacher, 1937; Gale and Wyne, 1957). By the 1970s, new histopathologic techniques and electron microscopy permitted the characterization of splenic lesions that would soon become pathognomonic for THEV infection. One of the early electron microscopy studies on spleens from field cases of HE revealed massive focal necrosis in the germinal follicle areas, with lymphoid necrosis and degeneration of reticular cells. Enlarged cells were found with INIs and eccentric margination of the chromatin. These INIs contained semi-crystalline arrays of THEV virions (Carlson and Al-Sheikhly, 1974). Several other reports of splenic lesions in experimentally infected turkey poults were published in the 1970s. Oral inoculation of turkey poults with a field isolate of THEV-V resulted in marked splenomegaly at 3-6 dpi. INIs were found 3-7 dpi, with none found after day 7, through the end of the study at 16 dpi. The white pulp was found to be enlarged with many lymphocytes and swollen cells peaking at 5-6 dpi, with all lesions decreasing 7 dpi and later (Itakura *et al*., 1974). Another study examined poults inoculated intravenously (IV) with a diluted homogenate of THEV infected spleens. Spleen size increased 1-4 dpi, and then decreased dramatically on day 5. Most of the lesions were seen in the white pulp 1-4 dpi, with lymphoid necrosis beginning on 1 dpi. A massive proliferation of immature cells was observed 2-4 dpi,

followed by disintegration of the white pulp associated with the appearance of small lymphocytes beginning on day 4. By 5 dpi, only arteries and reticular cells remained in the spleen, and normal white pulp had been restored by 8 dpi (Gross and Domermuth, 1975). A study comparing the effects of avirulent THEV (THEV-A) and THEV-V in turkeys found no major differences in splenic lesions between the two strains. Oral inoculation with both strains resulted in proliferation of the white pulp beginning 3 dpi, and reaching a maximum level by 5 dpi. Numerous INIs were found at 4-5 dpi in lymphoblasts and lymphocytes. Lymphoid necrosis began 4-5 dpi, with a higher level of necrosis seen with THEV-A than with THEV-V. Plasma cells were seen in the red pulp zones 6-7 dpi, and normal splenic architecture had been restored by 10 dpi (Saunders *et al*., 1993). A report on a spontaneous outbreak of HE in turkeys in Spain included descriptions of splenic lesions. Spleens were grossly enlarged, with a mottled gray/white color. Microscopically, spleens had large areas of necrosis, mainly affecting the germinal centers of the lymphoid follicles. Numerous mononuclear cells had Cowdry Type A INIs (Gomez-Villamandos *et al*., 1994).

A series of studies in the 1990's examined changes in specific cell populations following oral inoculation with THEV-V. In the first study, IgM^+ cells were found to be reduced in numbers in the spleen 2, 3, 4, and 9 dpi compared to uninfected controls. The percentage of $CD4^+$ cells was elevated 4-6 dpi, and the percentage of $CD8^+$ cells was increased 16 dpi (Suresh and Sharma, 1995). In another study, IgM^+ cell reduction and $CD4^+$ cell increase was confirmed at 4 dpi. THEV DNA was detected by ISH in the nucleus of 1% of unsorted mononuclear cells and 2.6-3.3% of adherent splenic cells, likely macrophages. Viral DNA was only detected in IgM^+ B-lymphocytes, and not in $CD4^+$ nor $CD8^+$ T-lymphocytes (Suresh and Sharma, 1996). In 13 and 24 day-old turkey poults inoculated IV with THEV-V, there was a profound decrease in the number of B-lymphocytes in the spleen 3-4 dpi (Rautenschlein *et al*., 1998). Another study examined 2 week-old turkey poults orally inoculated with THEV-V. 5/7 had splenomegaly 3 dpi, and 5/5 had splenomegaly 4 dpi. Apoptotic cells were found in the spleen 3-4 dpi, with a

greater number on day 4. The number of THEV infected cells was greatest 4 dpi (Rautenschlein *et al*., 2000).

Several studies have attempted to determine cytokines that are released in response to THEV infection. In 3 week-old turkeys orally inoculated with THEV-V, an interleukin (IL)-6-like factor is secreted by splenocytes 2-3 dpi, less on day 4 pi. Stimulation of explanted splenocytes with Concanavalin A triggered the release of a tumor necrosis-like factor (TNF) and a nitric oxide inducing factor (NOIF). The NOIF was found to be at least partially blocked by the addition of a chicken anti-interferon-gamma antibody (Rautenschlein *et al*., 2000).

Two studies examined the effect of experimental immunosuppression on the splenic lesions of THEV infection in turkeys. Selective B-lymphocyte deficiency was induced using cyclophosphamide (CY), followed by infection with THEV-V. In two separate studies, CY treatment prevented splenomegaly, splenic lesions, and drastically decreased number of infected cells (Fadly and Nazerian, 1982; Suresh and Sharma, 1995). Selective T-lymphocyte deficiency induced by cyclosporine A (CsA) did not affect the degree of splenomegaly or mean viral antigen titer in the spleen of infected poults (Suresh and Sharma, 1995). These results indicate a strong dependence on B-lymphocyte populations during viral replication and splenic lesion formation, and conversely a strong independence of lesion formation on T-lymphocyte functionality.

Some interesting results were seen when comparing the protective ability of various vaccine strains of THEV. One study compared the response of turkey poults to a tissue-culture attenuated product with the response to two splenic strains. At 28 dpi, the spleens of birds infected with the splenic strains were significantly smaller. Despite the smaller spleens, the birds still produced a protective antibody response against THEV (Sharma, 1994). Another study observed that vaccinated poults surviving challenge with THEV-V had significantly smaller spleens than unvaccinated or unchallenged survivors. No sign of necrosis or atrophy was detected in these spleens (Nazerian and Fadly, 1986).

Gastrointestinal Lesions

Intestinal lesions associated with THEV are only caused by virulent strains of the virus. The initial published description of the gastrointestinal lesions of HE in 1937 indicated two stages of lesion formation. Stage one involved the infiltration of the lamina propria with red blood cells and round cells, with distended villi. Stage two involved the separation of the basement membrane and desquamation of the villus epithelium (Pomeroy and Fenstermacher, 1937). Another description in the 1950's reported the lumen of the small intestine filled with a bright red sanguineous material. This material did not contain parasites or protozoa. The duodenal epithelium was edematous and hemorrhagic (Gale and Wyne, 1957). The earliest description of HE lesions in experimentally infected turkey poults was published in 1967. Enteritis characterized by desquamation of the duodenal epithelium was observed. The highest level of congestive lesions was seen at 5 dpi, and lesions were greatly diminished by 6 dpi. Hemorrhage was found to be sudden, and lasted approximately 24 hours. Hematocrit values dropped sharply in birds with HE lesions, and did not return to normal until 15 dpi. Peripheral lymphocytes decreased in number 6 dpi, and were restored to normal by 8 dpi. Peripheral heterophil counts were decreased 7 dpi, increased to 160% of normal 11 dpi, and were normal again by 15 dpi (Gross and Moore, 1967). At 5 dpi, the tips of the villi were congested with few red blood cells outside the vessels. The mucosa was uniformly dark red with congestion. Edema added to the thickness of the villi, and the epithelium began to separate from the villus. An opening was formed at the tip of the epithelial layer so blood could move directly into the lumen of the intestine. Hemorrhage continued for a 24 hour period, during which the tips of some villi became necrotic. By mid-6 dpi, hemorrhage ceased and epithelium was regenerated on the villi. Macrophages were commonly seen at that time. Hemorrhage seemed to be due to red blood cells passing through the vessel walls, rather than ruptured vessels. The author suggested that the lesions were caused by a toxin rather than an infectious agent (Gross, 1967). Another published report from a field case described the intestinal lesions of HE. Mostly blood filled small intestine and petechial mucosal hemorrhage was observed grossly.

Microscopically, there was intense villus congestion, and edematous separation of epithelium from the lamina propria (Wilcock and Thacker, 1975).

In all cases, mortality caused by THEV infection correlates with intestinal lesions, although several studies have concluded that there is not a great deal of viral proliferation at the site of hemorrhage. This is true for field cases and experimental infections. In one case report, the lamina propria had ballooned cells with INIs identical to those found in the spleen, but in smaller numbers (Carlson and Al-Sheikhly, 1974). In a field outbreak in Spain, the small intestines of affected poults were dilated with bloody contents. The mucosa was thickened, edematous, and congested, and cecal tonsils were also hemorrhagic. Heavy bleeding and necrosis in the small intestine was accompanied by abundant mononuclear infiltration of macrophages, lymphocytes, and plasma cells. Some of these cells contained INIs identical to those commonly found in the spleen (Gomez-Villamandos *et al*., 1994). In turkey poults orally inoculated with THEV-V, small numbers of INIs were seen 3-6 dpi. There was also an observed increase in heterophil and lymphocyte numbers in areas of the lamina propria (Itakura *et al*., 1974). One study compared lesions in turkey poults orally inoculated with THEV-V and THEV-A. The duodenum of poults receiving THEV-V was normal until 5 dpi. 2/3 poults had gross hemorrhage in the villus tip and free red blood cells in the lamina propria. The blood vessels were intact, so it appeared that leakage of the red blood cells had occurred by diapedesis. Bleeding was associated with unidentified necrotic cells in the lamina propria of the villus. The necrotic cells did not have INIs. Poults infected with THEV-A had normal duodena throughout the study (Saunders *et al*., 1993).

Several experiments were conducted to determine the timing of lesion formation and factors influencing lesion severity. In one such study, the influence of route of infection on lesion formation was examined. Intestinal lesions were seen at 4 days post-IV inoculation, and 6 days post-cloacal inoculation. Death occurred 3-5 days post-IV inoculation (Domermuth *et al*., 1973). Lesions appeared 3 days post-IV inoculation following peak antigen level in the spleen. There was no correlation between the number of INIs in the spleen and intestinal lesions (Gross and Domermuth, 1975).

The specific biological mechanism by which THEV-V causes duodenal lesions is unknown despite several studies to determine key pathways involved in lesion formation. Turkey poults infected with THEV-V were found to have significantly higher numbers of duodenal mast cells than uninfected poults. Only infected poults showed signs of increased vascular permeability. This permeability was localized to the lamina propria, also the location of the mucosal mast cell increase. Heat stress increased the incidence of HE lesions: 14/20 stressed poults had clinical HE; 3/20 unstressed poults had clinical HE (Opengart *et al*., 1992). Stress is known to alter immunocyte populations and the way they react to infection. The clinical lesions of HE are related to T-lymphocyte activation and TNF. Selective T-lymphocyte depletion using CsA was found to prevent HE lesions in turkeys infected with THEV-V. Viral replication and splenic lesions were not apparently affected (Suresh and Sharma, 1995). Thalidomide, which down-regulates TNF production in mammals, was found to prevent HE lesions in turkeys infected with THEV-V (Rautenschlein and Sharma, 2000). In some way, THEV-V is able to stimulate T-lymphocytes and TNF release, which leads to mast cell proliferation and degranulation.

Systemic Lesions

Lesions are present in tissues other than the spleen and gastrointestinal tract, although there is variation between studies. One early study examining the tissues from birds killed in field outbreaks saw no significant lesions in the kidney, liver, heart, pancreas, bursa, or breast muscle (Carlson and Al-Sheikhly, 1974). In subsequent studies, INIs characteristic of THEV infection were observed in several tissues. One study determined the incidence of INIs in peripheral tissues was directly related to the number of INIs observed in the spleens of infected poults (Iltis *et al*., 1975a).

The bursa of Fabricius is thought to be one of the first locations of viral replication in birds infected orally. Despite this, very few lesions have been documented during THEV infection in the bursa. Several studies have detected small numbers of cells with THEV-specific INIs concurrent with the peak viral load in the spleen (Itakura *et al*., 1974; Saunders *et al*., 1993; Iltis *et al*., 1975a; Fadly and Nazerian, 1982). These cells are

similar in appearance to those cells in the spleen with INIs. Other descriptions of bursal lesions are less consistent. Mild lymphoid necrosis in the follicles was observed in one study (Fadly and Nazerian, 1982). Mild lymphoid depletion caused by THEV-V at 6 days post-oral inoculation was observed in one study (Saunders *et al*., 1993). An increase in lymphocytes in follicles 5-6 days post-oral inoculation was observed in another study (Itakura *et al*., 1974). The bursa is not greatly affected during THEV infection, regardless of the virulence of the THEV strain.

Very few lesions are associated with THEV infection in the thymus and bone marrow. Small numbers of INIs in mature and immature lymphocytes have been documented in both, but ultra-structure was not affected (Iltis *et al*., 1975a; Itakura *et al*., 1974; Itakura and Carlson, 1975a; Fadly and Nazerian, 1982; Saunders *et al*., 1993; Silim and Thorsen, 1981).

Lesions in the liver are not generally associated with clinical HE. The first accounts of HE in 1937 found that the livers were most often normal in birds who had died (Pomeroy and Fenstermacher, 1937). Some case reports of field outbreaks have observed petechial hemorrhage and occasional necrosis with mononuclear infiltration, but it is not known whether this is directly related to THEV infection (Itakura *et al*., 1974; Wilcock and Thacker, 1975; Saunders *et al*., 1993). The most common observations are small numbers of INIs in immature cells, especially during peak viral load in the spleen (Itakura *et al*., 1974; Itakura and Carlson, 1975a; Saunders *et al*., 1993; Iltis *et al*., 1975a; Silim and Thorsen, 1981).

The kidneys are not generally affected during THEV infection. INIs were found in small numbers of mature lymphocytes in the kidney 4-6 days post-oral infection (Saunders *et al*., 1993; Silim and Thorsen, 1981). INIs in kidney cells have also been found in normal turkeys in commercial turkey operations at 6-15 weeks-of-age. In one study, rare to numerous INIs were found in the epithelial cells lining the distal convoluted renal tubules, and confirmed by immunoperoxidase assays (IPA) to be THEV-specific. INIs were found in the kidneys of 25-67% of poults in each submission (Trampel *et al*., 1992). A retrospective study reviewed histologic records in the California Veterinary

Diagnostics Laboratory System for correlations between age, serology, and INIs in the spleen and kidney of unvaccinated turkey poults greater than 5 weeks-of-age. The results showed that in young poults (6-8 weeks-of-age), 58% had splenic inclusions, 37% were THEV sero-positive, and only 21% had INIs in the kidney. In 8-10 week-old poults, only 15% had splenic INIs, 85% were sero-positive, and 81% had renal INIs. In 10-12 week-old turkeys, none had splenic INIs, all were sero-positive, and all had renal inclusions. In 12-15 week-old turkeys, none had splenic INIs, all were sero-positive, and only 50% had renal INIs. Overall, INIs in the kidney were frequent in birds 8-12 weeks-of-age, without the splenic INIs associated with active THEV infection (Meteyer *et al.,* 1992). It is unknown whether THEV is capable of replicating in the kidney. The primary natural route of infection of THEV is fecal-oral. Intestinal contents have been shown to be infectious only briefly during peak viral load in the spleen. Intestinal permeability is thought to return to normal after this period. Infection of the kidney may allow THEV to be shed via urates at any time post-infection. "Histologic results suggest that the renal tubule cells containing inclusions desquamate into the tubule lumen and may be a source of viral shedding in the excrement beyond the short duration of intestinal involvement" (Meteyer *et al*., 1992). However, this has not been confirmed experimentally.

Lesions of the lungs are not generally associated with clinical HE in turkeys. The lung is generally thought to be more important in the clinical lesions in field cases of Marble Spleen Disease (MSD) in pheasants and, to a lesser degree, clinical lesions of *Aviadenovirus* Type II-Associated Splenomegaly (AAS) in chickens (Domermuth *et al*., 1982; Wyand *et al*., 1972). There have been some observations of lesions in the lungs of turkeys with HE. The earliest reports of clinical HE in the 1930's and 1950's described the lungs of affected turkeys as pale, edematous, and sometimes congested (Pomeroy and Fenstermacher, 1937; Gale and Wyne, 1957). Experimentally infected turkeys sometimes had pulmonary INIs in small numbers of mature lymphocytes, with occasional heterophil migration into alveolar walls (Iltis *et al*., 1975a; Saunders *et al*., 1993; Itakura *et al*., 1974). These lesions are very similar to those seen in field MSD of pheasants, and are likely caused by similar molecular pathways.

Viral Distribution vs. Time

The pattern of viral distribution and corresponding serologic response has been studied in turkeys. Most of those studies focus on the early acute stage of infection, the first 5-6 days post-inoculation. The distribution of the virus in the host is appropriately widespread, considering it is capable of infecting macrophages and lymphocytes that can travel throughout the body.

Early experimental infection of turkey poults with THEV was by the cloacal route. Viral antigen was detected in the spleen of infected birds by agar gel immunodiffusion (AGID). Only 8% of infected birds had viral antigen in the spleen at 4 dpi. By 6 dpi, 66% of birds had detectable antigen in the spleen, which then decreased to 33% by 8 dpi (Domermuth *et al*., 1973). Intestinal contents from poults were able to transmit clinical HE to other poults only at 5 days post-cloacal inoculation (Gross and Moore, 1967).

One study examined turkeys inoculated intraperitoneally (IP), and used an indirect IPA to detect virus in INIs, primarily in the spleen. Virus was detected at a high level in the spleen on days 2-5 pi. One out of three birds had INIs in the liver, intestine, and kidney on days 3, 4, and 5, respectively. The authors concluded that the virus proliferated primarily in the spleen, and was subsequently distributed to peripheral tissues (Silim and Thorsen, 1981).

By far, the most common route of infection described in the literature is oral. Oral inoculation most closely imitates natural infection. In several studies observing incubation period following oral inoculation, the period of peak splenomegaly and viral antigen load in the spleen was at 4-5 dpi (van den Hurk, 1986; Suresh and Sharma, 1996). One study examined the first four days of infection in great detail. At 1 dpi, viral DNA was detected by ISH in the duodenum, cecal tonsil, and the bursa. Viral DNA was detected in the spleen by 2 dpi, with high levels of virus found in the spleen and cecal tonsil by 3 dpi. The highest level of THEV DNA was detected in the spleen at 4 dpi, accompanied by lymphoid depletion and hyperplasia of mononuclear phagocytes in the spleen. The activity in the spleen coincided with the onset of intestinal hemorrhage.

Despite necrosis, hemorrhage, and sloughing of the duodenal mucosa, only a few duodenal epithelial cells were found to have viral DNA. Viral DNA was detected at low levels in the bursa throughout the study (Suresh and Sharma, 1996).

Intravenous inoculation with THEV results in the earliest splenic lesions. Viral antigen detected in the spleen by AGID peaked at 2 days post-IV inoculation. Antigen decreased 3 dpi, and disappeared by 4 dpi (Gross and Domermuth, 1975). During splenic THEV vaccine preparation, IV inoculation consistently results in splenomegaly peaking at 3 dpi. It is hypothesized that splenomegaly peaks earlier because the inoculum is able to travel directly to the spleen without the need for initial replication in the bursa, cecal tonsil, or gut-associated lymphoid tissue (GALT).

HE is generally associated with an acute viral infection leading to splenomegaly and duodenal hemorrhage at 3-5 dpi. Beyond that point in time, the virus is apparently cleared from the infected poults. In one of several studies documenting this, no viral antigen was detected in the spleen by ELISA at any time beyond 6 dpi (Sharma, 1994). However, there is some evidence that THEV is able to remain in infected birds after the initial acute phase. One study examined the presence of nuclear inclusions in the spleens and kidneys of field cases of non-vaccinated turkeys through 15 weeks-of-age. The study found that younger birds (6-9 weeks) were more likely to have THEV-related splenic inclusion bodies, while older birds (8-12 weeks) were more likely to have renal inclusion bodies. The oldest birds (12-15 weeks) were found to have higher antibody titers, and subsequently fewer intranuclear inclusions (Meteyer *et al*., 1992). The fact that sero-positive turkeys had THEV-specific INIs in cells in the kidney indicates the virus is capable of remaining in the host beyond the onset of significant antibody titers.

Seroconversion and Vaccination

Several studies have been performed to determine the characteristics of the antibody response to THEV infection. Precipitating antibodies were detected by AGID in infected turkey poults 14 days post-cloacal inoculation, and 11 days post-IV inoculation. The duration of the antibody response was also examined: 75% of birds were positive at

14 dpi; 100% were positive 21-98 dpi; 88% were positive 140 dpi (Domermuth *et al*., 1972; Gross and Domermuth, 1975). Another observation of serologic response detected precipitating antibody by AGID in 100% of infected poults 14-91 dpi, and 87% were positive at 336 and 476 dpi (Domermuth *et al*., 1973). The antibody response of turkey poults cloacally inoculated was also examined. Antibody was first detected by AGID in 33% of the birds at 12 dpi. 75% were positive by 14 dpi, 88% by 28 and 35 dpi (Domermuth *et al*., 1973). ELISA was used to detect antibody in IP-inoculated turkey poults. Antibodies were detectable at very low levels starting at 3-4 dpi, and titers climbed to a plateau at 14 dpi (Silim and Thorsen, 1981).

It is important for the commercial turkey industry to limit clinical HE in their flocks. Several live virus vaccines have been developed and tested in the field for the prevention of infections with THEV-V. One of the earliest vaccines was produced from a strain of THEV-A isolated from pheasants, known as the Virginia Avirulent Strain (VAS). It does not cause mortality or duodenal hemorrhage in turkey poults, but does replicate in the spleen and stimulates a protective antibody response against THEV-V (Domermuth *et al*., 1977; Domermuth and Larsen, 1984). One field test using this vaccine confirmed lower spleen:body weight ratios in vaccinated birds after challenge with THEV-V (Thorsen *et al*., 1982). This strain is still in use as a live-bird propagated crude splenic vaccine.

The first reliable cell culture system for the propagation of THEV *in vitro* was developed in the early 1980's (Nazerian *et al*., 1982). The Marek's disease virus-induced chicken lymphoblastoid cell line (MDTC RP-19) permitted serial passage and attenuation of THEV. Starting with the VAS, serial passage *in vitro* enabled production of HE vaccine without the need for live birds. This was advantageous for the vaccine makers, as it allowed them to receive licensing from the USDA for the sale of a commercial vaccine product. The tissue culture-propagated VAS (VAS-TC) was infectious to turkeys and stimulated a protective immune response. In a series of field tests, VAS-TC elicited precipitating antibody in 88-97% of orally inoculated poults by 3 weeks pi. Challenge with THEV-V resulted in 16% mortality in unvaccinated poults, whereas the vaccinated

birds showed no clinical signs. Additionally, the results of field tests indicated the average weight of vaccinated birds at market age was the same as unvaccinated birds (Fadly *et al.*, 1985). This was an important indication that vaccination did not significantly interfere with flock productivity.

Although the splenic VAS and the tissue culture propagated VAS-TC apparently originated as the same virus strain, there was observation of differences in field performance. One field comparison examined the difference in antibody response and protective ability of the two vaccines. Both had stimulated detectable antibody in 100% of vaccinated birds by 4 weeks pi, though the VAS-TC had a higher titer as determined by ELISA. Despite the difference in titer, both protected equally against THEV-V challenge (Barbour *et al.*, 1993). However, in another study the VAS vaccine induced a detectable antibody response earlier than the VAS-TC vaccine. This was attributed to a greater degree of viral proliferation in the spleen of birds receiving the VAS vaccine. These birds had a higher mean antigen titer and incidence, as well as larger spleen:body weight ratios than the birds receiving the VAS-TC vaccine. Titers had reached high levels in all vaccinated birds by 3 weeks pi, and remained high throughout the 6 week study (Sharma, 1994).

Several attempts have been made to produce a vaccine for HE that does not involve live, infectious THEV. At least one virus neutralizing epitope has been mapped to the major capsid protein, the hexon (van den Hurk, 1988). A recombinant fowlpoxvirus expressing the THEV hexon gene has been engineered for use as an HE vaccine (Cardona *et al.,* 1999). A recombinant fiber-knob protein vaccine has also been engineered and tested *in vivo* for its ability to protect against virulent challenge (Pitcovski *et al*., 2005). There are drawbacks to the use of a recombinant subunit vaccine, as there may not be complete protection against a broad range of viral strains. Most subunit vaccines cannot be administered orally, and are not suitable for mass vaccination. Moreover, it can be difficult to ensure proper dosage to individuals, resulting in failure to seroconvert and protect against virulent challenge.

Immunosuppression

A major drawback to infection with any strain of THEV is a brief period of immunosuppression following the acute stage of viral replication. The immune system is depressed during THEV infection, and permits opportunistic infection by bacteria, especially *E. coli*. This period of immunosuppression also interferes with vaccination protocols for other major avian pathogens (Sharma, 1991).

Colibacillosis is perhaps the most problematic complication resulting from vaccination with live THEV. Early case reports of HE in turkeys documented the ability to culture *E. coli* from the liver, heart, and spleen of 20% of birds who had died in HE outbreaks (Gale and Wyne, 1957). Colibacillosis was a common problem during field vaccination trials. Mortality caused by colibacillosis interfered with comparison of mortality rates between HE vaccinated and unvaccinated turkeys. One observation was that, "clinical manifestations of colibacillosis often coincide with the time when turkeys seroconvert to HE" (Fadly *et al*., 1985). Another study examined colibacillosis outbreaks in the field. One case had a spike of 3.5% mortality over a two week period, with 75% of deaths in first 3-4 days. Spleens of dead poults were negative for THEV antigen by AGID, but poults orally inoculated with the splenic material were positive at 7 dpi, with no clinical HE lesions. Observed peak colibacillosis mortality was 7 days after peak viral load in the spleen, the same time antibody titers begin to climb. Immunosuppression leading to colibacillosis may be due to macrophage depletion (Sponenberg *et al*., 1985). The timing of immunosuppression was confirmed by experimental infection soon after. Mortality caused by colibacillosis was greatly increased in birds receiving THEV 7 days prior to *E. coli* inoculation. Incidence of airsaculitis and pericarditis was also higher in birds receiving both THEV and *E. coli* than those receiving *E. coli* or THEV alone (Larsen *et al*., 1985; Newberry *et al*., 1993; van den Hurk *et al*., 1994). Birds infected with THEV were not able to clear bacteria from circulation effectively. THEV infection may inhibit the activity of phagocytes, and therefore decrease the first line of defense against bacterial infection (van den Hurk *et al*., 1994).

Synergistic relationships have been observed between avian pathogens, resulting in greater lesion incidence and severity. Turkey poults receiving *Bordatella avium* or NDV followed by THEV, then *E. coli*, had enhanced pericarditis and mortality compared to turkey poults receiving only one or two of the agents (Pierson *et al*., 1996). Turkey poults receiving NDV-B1 and THEV-A had enhanced white mottling and greater numbers of apoptotic cells in the spleen. THEV infected cells were actually decreased at 4 dpi, and the THEV-specific antibody response was decreased at 14 dpi (Rautenschlein and Sharma, 1999).

The actual mechanism of the immunosuppression is unknown. Due to its relatively short duration, cellular damage is not expected to be the primary cause of immunosuppression, despite some reports of bursal damage during THEV-V infection (Fadly and Nazerian, 1982). Lymphoblastogenesis comparisons of peripheral blood leukocytes from turkeys receiving THEV vaccine indicated immunosuppression in 70% of vaccinated birds (Cardona *et al.,* 1999). In another study, THEV-V interfered with NDV vaccination. THEV might cause, "an effect on the proliferative potential of lymphocytes that results in depressed antibody production to NDV," and it may be a result of, "two antigenic determinant competing for a population of immunocompetent cells" (Nagaraja *et al*., 1985).

1.3.3 Strain Differentiation

There is a great deal of confusion regarding the identity, origin, and characteristics of the different strains of THEV. Traditionally, viruses have been classified based on the host species from which they were isolated. This permitted the grouping of viruses based on the clinical disease, tissue tropism, and virulence caused in individual host species. This is useful, particularly when the virus in question is only capable of infecting a single host species. However, strains of THEV responsible for three distinct clinical diseases have been isolated from chickens, turkeys, and pheasants (Pierson and Fitzgerald, 2003). Most experimental evidence indicates that all three diseases are caused by the same virus species. Unfortunately, when a virus that is capable

of infecting several host species is isolated separately in association with different clinical diseases, the actual relationship between the isolates can easily be mistaken.

Marble Spleen Disease of Pheasants

THEV strains of pheasant origin have been called Marble Spleen Disease Virus (MSDV), named from the clinical disease it causes in pheasants. MSD is caused by THEV, and is characterized by splenomegaly, pulmonary congestion and edema, and mortality in 2-8 month-old pheasants (Pierson and Fitzgerald, 2003). Mortality resulting from pulmonary lesions has been described in field outbreaks of MSD, but documented experimental infections have not resulted in the same lesions or mortality. The earliest accounts of MSD are from outbreaks in farm-raised pheasants in the United States in the early 1970's. In one study, 27 birds died due to anoxia resulting from severe pulmonary edema. All of the pheasants had enlarged spleens with mottling. Lungs were firm, wet and liver colored, but no other lesions were discovered. Microscopically, there was splenitis, necrosis, and amyloidosis with numerous INIs present in the spleen. The tertiary bronchi were filled with red blood cells, and small scattered foci of necrosis were found in the lung parenchyma (Wyand *et al*., 1972). In two MSD outbreaks on the same farm, one year apart, 8-12 week-old pheasants broke with 3% daily mortality over a two week period. Gross lesions included enlarged, mottled spleens and acute pulmonary congestion and edema. Microscopically, focal necrosis of the air capillaries around tertiary bronchi was observed, along with severe congestion and hemorrhage into the tertiary bronchi. Spleens had areas of focal necrosis in the germinal follicles, and lymphoid necrosis with INIs was common. Focal necrosis and inflammation was also noted in the pancreas and intestine, with lymphocyte and heterophil infiltration (Carlson *et al*., 1973). A more recent outbreak in 3-4 month-old pheasants in Korea resulted in a spike of 12% mortality. The lungs of affected pheasants were generally congested and edematous. The spleens were enlarged and mottled, with diffusely necrotic lymphoid follicles, mild to moderate lymphoid hyperplasia, and numerous INIs verified to be THEV-specific by IPA (Lee *et al*., 2001). Pheasants experimentally infected with THEV

do not have the same severe pulmonary lesions seen in field cases of MSD. In all cases, infection of pheasants with THEV resulted only in splenomegaly with INIs (Iltis *et al*., 1975a; Fadly *et al*., 1988; Fitzgerald and Reed, 1991; Fitzgerald *et al*., 1995). One study indicated that during experimental production of MSD, "some synergistic factor was absent, since death as well as the full spectrum of naturally occurring MSD lesions was lacking" (Iltis *et al*., 1975a). It is not known whether the synergistic factor is another infectious agent, but such a relationship would not be surprising considering the multi-agent synergy observed in THEV infection of turkeys.

Aviadenovirus Type II-Associated Splenomegaly of Chickens

THEV strains of chicken origin have been called *Aviadenovirus* Type II-Associated Splenomegaly Virus (AASV), named from the clinical disease seen in chickens. The name is outdated, as THEV is no longer classified as a Type II *Aviadenovirus*. The condition was first documented in the late 1970's. Twelve chickens from a flock with unusually high numbers condemned for MDV were examined. Enlarged spleens from the 12 cases were homogenized and passaged twice in turkey poults, then inoculated back into chickens. Inoculated chickens had enlarged spleens, with mottling and hyperplasia, but there were no THEV-associated INIs. The spleens were not positive for THEV antigen by AGID until the samples were concentrated, indicating low levels of virus (Domermuth *et al*., 1979b). In another study using the same isolate, good viral replication was observed. Oral inoculation of 7 week-old broiler chickens resulted in 80% splenomegaly with hyperplasia and INIs 6 dpi. 58% of affected chickens had lymphoid degeneration or necrosis in the spleen, and 22% had pulmonary hemorrhage or edema. 44% had enlarged lymphoid nodules in the duodenum and ileum, due to hyperplasia. No hemorrhagic enteritis was evident, and there was no mortality (Veit *et al*., 1981). The only fatal case of AAS was reported in 1982. Broiler chickens 22 weeks-of-age broke with splenomegaly, pulmonary and splenic congestion, and enteritis, with a total mortality of 8.9%. Microscopically, there was necrosis of the white pulp of the spleen, mainly affecting lymphocytes, and hyperplasia with many INIs. Lungs of

fatally affected chickens were congested with massive edema, and airsacs were filled with proteinaceous material in some instances. No lesions were present in the liver or heart (Domermuth *et al*., 1982).

THEV infection is thought to be widespread among commercial chicken flocks. Nearly 50% of chicken flocks tested by AGID in 1978 were positive for THEV antibody, generally with higher numbers of positive results in older flocks (Domermuth *et al*., 1979b). The current incidence of THEV infection in chickens is unknown. THEV causes a relatively minor subclinical infection in chickens, and vaccination is not performed as with commercial turkeys. The few isolated cases with increased mortality were most likely due to synergistic multi-agent infections.

Comparative Pathology

The relationship between THEV strains isolated from different host species can be further clarified by experimental cross-infection. A field isolate of THEV-V in turkeys was inoculated cloacally into eight turkeys, nine peafowl, and ten chickens. Two of the turkeys died from HE at 6 dpi, and all birds were euthanized and examined at that time. All of the peafowl had some congestion of the intestine. Intestinal content of two chickens with slight intestinal congestion caused lesions in subsequently inoculated turkeys (Gross and Moore, 1967).

THEV strains isolated from spontaneous cases of MSD in pheasants have been studied in turkeys. Pheasant-origin strains were shown to be "readily capable of reciprocal crossing between pheasants and turkeys." Infection of turkeys and pheasants resulted in identical lesions consisting of splenomegaly and INIs. No lesions were observed in infected 5 week-old chickens (Iltis *et al.,* 1975a). In another study, pheasant-origin THEV was serially passaged four times in turkeys. The resulting virus caused splenomegaly with systemic INIs when inoculated into pen-raised wild turkeys. No signs of clinical MSD were evident (Iltis *et al*., 1975b). In eight turkeys inoculated with purified pheasant-origin THEV, seven had many INIs and splenomegaly. Passage into 25 susceptible turkeys only resulted in splenomegaly and INIs in six of the poults (Iltis *et al*.,

1977). The overall trend in these experiments is that THEV isolated from pheasants with MSD is capable of infecting turkeys, but only results in splenic lesions. The results are identical to the experimental infection of pheasants with pheasant-origin THEV.

THEV strains isolated from turkeys have been studied in experimental infections of chickens and pheasants. One study successfully passaged a turkey-origin strain eight times in leghorn chickens. Clinical HE was not observed in any of the chickens, though various gross lesions were described. The duodenal epithelium was edematous and congested, and the cecal nodes were enlarged and hemorrhagic. Microscopically, there was lymphocytic hyperplasia of the lamina propria of the intestine, and lymphoid infiltration with many INIs in large mononuclear cells. Splenic lesions were identical to those seen in turkeys (Silim *et al.*, 1978). Intramuscular injection of leghorn and broiler chickens with virulent and avirulent THEV from turkeys resulted in subclinical infection. Only splenomegaly and numerous INIs were seen in infected chickens (Beasley and Clifton, 1979). A different study inoculated chickens IV with turkey-origin THEV-V. 90% of infected chickens had splenomegaly. None of the chickens had clinical HE lesions, compared with 70% of inoculated turkeys that did (Rautenschlein *et al.*, 1998). Virulent and avirulent isolates from turkeys have also been used to infect pheasants. Both strains resulted in splenic lesions characterized by INIs in 60-75% of infected pheasants and THEV antigen in the spleen detected by AGID. There was no clinical HE observed in any of the pheasants (Fadly *et al.*, 1988).

Although the splenic lesions found in turkeys, chickens, and pheasants are the same, the lesions attributed to clinical disease differ between them. Clinical lesions associated with virulence are found in the intestine of turkeys and the lungs of pheasants and chickens. Though there are differences in the location of the lesions, the pathogenesis seems to be the same. It is hypothesized that the molecular pathway associated with clinical MSD is related to the same immune mediated pathway of intestinal hemorrhage in THEV. However, the three are distantly related enough that key differences in susceptibility and lesions can be explained by a host-specific determinant. The difference in shock organs between the two host species determines the location of the symptoms.

1.3.4 Virulent vs. Avirulent Strains

All of the THEV strains isolated to date have been capable of infecting chickens, pheasants, and turkeys. THEV infection causes primarily subclinical infection in chickens, and is not of great significance to the commercial chicken industry. THEV causes clinical HE and immunosuppression in infected turkeys, and clinical MSD in infected pheasants, both of which are economically important diseases worthy of vaccine development. Several isolates have been studied and used in experiments for decades.

The Virginia Virulent Strain (VVS) of THEV was isolated in the 1970's from spleens from turkeys that had died of clinical HE (Domermuth *et al.*, 1977). Following the development of the MDTC RP-19 lymphoblastoid cell line, the VVS was passaged *in vitro* and used as the virulent challenge strain for many vaccine trials and infection experiments. Seven passages *in vitro* were not enough to diminish virulence in turkeys (Nazerian and Fadly, 1982). However, thirty passages *in vitro* did diminish the ability of the VVS to cause clinical HE, although it was still infectious *in vivo* and stimulated a protective immune response (Sharma, 1994). Strains of THEV that cause clinical HE and mortality in turkeys (specifically the VVS) have not been shown to cause similar virulent lesions in chickens or pheasants. There are no documented strains of THEV that cause clinical disease experimentally in chickens or pheasants.

Early research revealed that naturally occurring strains of THEV-A were able to infect turkeys and pheasants without causing clinical disease. Vaccination with THEV-A protects susceptible turkeys and pheasants from infection by virulent field strains (Domermuth *et al.*, 1977, 1979a). In the late 1970's, two strains of THEV-A were isolated and tested for use as a vaccine against HE and MSD.

The first strain, known as the Virginia Avirulent Strain (VAS) was originally called "Avirulent I." It was isolated from the spleens of pheasants suspected of having MSD. The virus was passaged twice in turkey poults and stored for future experimentation. Because of its pheasant-origin, and because it didn't cause any mortality or intestinal lesions in infected turkeys, it was chosen as the primary THEV-A strain in vaccines for the prevention of HE in commercial turkeys (Domermuth *et al.*, 1977;

Domermuth and Larsen, 1984). The VAS was donated to Nazerian and Fadly during the development of the MDTC RP-19 cell line, and the tissue-culture propagated strain became the foundation of commercially available tissue-culture HE vaccines (Nazerian and Fadly, 1982; Fadly *et al*., 1985). The VAS has been used in a large number of studies over the last 30 years. Because of its pheasant origin, there is some confusion caused by the continued use of the name "MSDV vaccine" to describe the strain.

The second strain was originally called "Avirulent II." It was isolated from turkey litter at a farm in Virginia whose turkey poults exhibited a natural resistance to challenge by the VVS (Domermuth *et al*., 1977). After two passages in turkey poults, it was stored for future use. During vaccine efficacy testing, 25% of turkey poults had moderate intestinal lesions with no mortality. Field trials indicated 0.15% mortality associated with a non-hemorrhagic enteritis 6-10 dpi. Avirulent II was not used further as a vaccine against HE in turkeys (Domermuth *et al*., 1977). However, this strain was found to be useful as a vaccine against MSD in pheasants (Domermuth *et al*., 1979a). There is some confusion about the naming of the strain after this point. It is likely that the virus would have been called "MSD Vaccine" within the laboratory of Domermuth. When donating to other laboratories, it may have been called "avirulent THEV" due to its turkey origin, in order to prevent confusion with "MSDV vaccine," which is the VAS.

1.3.5 Serologic Differences

The structural similarities between each strain of THEV have been confirmed by electron microscopy and SDS-PAGE. However, it is their serological identity that allowed for their classification as Type II *Aviadenoviruses*. In addition, no serologic differences have been documented between virulent and avirulent isolates of THEV, regardless of their origin. Immunochemical similarity was observed by AGID precipitin line identity between pheasant- and turkey-origin THEV antigens (Iltis *et al*., 1975a). Antigens produced by infection with the VVS, VAS, and Avirulent II strain were indistinguishable by AGID, and all three protected from subsequent challenge with the VVS identically (Domermuth *et al*., 1977). The VAS and VVS are antigenically

indistinguishable (Nazerian *et al*., 1991). AGID confirmed THEV precipitating antibodies in commercial chickens using VVS antigen (Domermuth *et al*., 1979b, 1982). The turkey-origin Avirulent II strain protects pheasants against clinical MSD, and pheasant-origin VAS protects turkeys against clinical HE (Domermuth *et al*., 1977, 1979a; Domermuth and Larsen, 1984). A semi-virulent THEV isolated from turkeys displayed AGID cross-reactivity with pheasant and turkey isolates of THEV after infection in chickens (Silim *et al*., 1978). Monoclonal antibodies developed for use in an IPA recognized antigens from pheasant and turkey isolates of THEV (Fitzgerald *et al*., 1992). VVS and VAS antigens did not react differently when used in development of ELISA techniques. ELISA was unable to distinguish the two strains (van den Hurk, 1986). Inoculation of the VAS in pheasants protected against infection with the VVS (Fadly *et al*., 1988). One study examined a panel of monoclonal antibodies for viral neutralization and specificity. 8/10 monoclonal antibodies neutralized both the VAS and the VVS, and the other 2 did not neutralize either. All of them reacted with Type II *Aviadenovirus* isolates, and none reacted with Type I *Aviadenoviruses* (van den Hurk, 1988).

1.3.6 Sequence differences

Although there are differences in clinical lesions caused by avirulent and virulent strains of THEV, the actual reason for the observed difference is unknown. It is hypothesized that in order for two viral strains to have a reproducible phenotypic difference, they must have some key genetic differences. The same avirulent strains have been used as live-virus vaccines for decades without documented spontaneous reversion to virulence. The stability of avirulent strains used in vaccines suggests that the differences between the THEV strains are clearly defined. Attempts to genetically differentiate isolates using restriction endonuclease fingerprinting resulted in apparent confirmation of genomic differences (Zhang and Nagaraja, 1989). However, close inspection of the agar gels and comparison to partial sequence data indicates serious flaws in this study.

In the mid 1990's, it was hypothesized that the cause of intestinal lesions in turkeys infected with THEV-V was a toxic reaction to an over-expression of the viral penton protein. To discover differences in the penton of the VAS and VVS, a 2477 bp restriction fragment from each was sequenced. The putative amino acid sequence was identical in both strains, and very similar to penton sequences of other adenoviruses. An LDV amino acid motif was discovered, which may be responsible for interaction with alpha-4-beta-1 integrins on cells, including lymphocytes, monocytes, basophils, and bone marrow progenitors. This interaction has not been experimentally confirmed (Suresh *et al*., 1995). A similar sequencing project was performed around the same time, but focused on the VAS only. The overall genome size was estimated at 25.5 kb, which made the genome of THEV the shortest of all adenoviruses studied. In addition, a remarkably low G+C content of 33.8% was observed within the region sequenced. A 2643 bp fragment of the VAS genome containing the penton, pVII, pX, and part of the pVI gene was sequenced. The penton sequence had only a few bp differences when compared to the other VAS penton sequences in GenBank. Comparison of the other core protein genes with available sequences from other adenoviruses at the time confirmed that THEV is an adenovirus, and that it was no more closely related to *Aviadenoviruses* than *Mastadenoviruses*. This was confirmation that THEV belonged in a separate classification from the other aviadenoviruses (Jucker *et al*., 1996). The great differences between THEV and other aviadenoviruses were confirmed when a field strain of THEV-V was isolated from turkeys, "exposed to the virulent field strain of HEV" in Israel (IVS) and fully sequenced. The actual virulence of this strain was not documented. The sequence of the IVS mostly agreed with the partial sequences of the VVS and VAS already published. For the first time, the full length genome sequence of THEV revealed a much lower C+G content (34.93%), shorter inverted terminal repeats (39 bp), and the shortest adenovirus genome at the time. Not only was the size different from other adenoviruses, the overall genetic complement was found to be much simpler. THEV lacks the mastadenoviral pIX and pV proteins, which are capsid and core related. No ORFs with homology to E1, E3, or E4 genes in any of the aviadenoviruses or

mastadenoviruses were observed. These regions code mostly for genus-specific genes, so THEV was shown to be unique (Pitcovski *et al*., 1998). The fiber gene of the VAS was sequenced as well. Comparison of this sequence with the sequence of the IVS revealed two putative amino acid changes (McQuiston *et al*., 1997, not published; GenBank accession # AF036243). Because of its genetic difference from all of the other adenoviruses, THEV would later be removed from the genus *Aviadenovirus*. Similarity to FrAdV-1 allowed for the creation of a separate genus, *Siadenovirus*, whose name was derived from the single large E1 gene product with similarity to bacterial sialidase proteins (Davison *et al*., 2000; Davison and Harrach, 2002).

Comparison of partial sequence data from the VAS, IVS, and VVS showed extremely high sequence identity throughout the fiber, penton, and core protein genes. There does not appear to be a significant sequence difference between the strains, and while this is consistent with observed serological identities, it does not begin to account for the differences in virulence in infected turkeys.

1.3.7 Diagnostic Methods

Diagnostic tools for the detection of THEV in turkeys have changed greatly over the decades, as the progression of science and technology has extended the sensitivity and specificity of testing. HE was first seen in field outbreaks prior to the discovery of viruses. Prior to the late 1960’s, the disease was thought to be caused by a filterable agent associated with streptococci bacteria. It was not known whether this agent was a virus (phage) or a toxin (Gross and Moore, 1967). Electron microscopy of the causal virus was performed in the early 1970’s, revealing morphological characteristics similar to the virus family *Adenoviridae* (Carlson and Al-Sheikhly, 1974; Iltis *et al*., 1977). Methods of diagnosis were limited to histopathology and serological methods. The most common and simple test is the AGID test, in which lines of precipitin form between wells containing viral antigen and antiserum (Domermuth *et al*., 1972, 1973). AGID has a lower sensitivity than histopathology, and is roughly equal in sensitivity to *in vitro* virus isolation in MDTC RP-19 cells (Iltis *et al*., 1977; Nazerian and Fadly, 1986). The AGID

test may be used to test for THEV antigen in samples using a known positive serum, or serum can be tested for THEV antibodies against a positive control antigen. Immunohistochemical staining techniques have been developed for the visualization of THEV in infected tissues. Immunoperoxidase and immunofluorescent techniques, both direct and indirect, have been developed and used in the detection of viral antigens (Iltis *et al*., 1977; Silim and Thorsen, 1981; Trampel *et al*., 1992; Fitzgerald *et al*., 1992; Saunders *et al*., 1993; Rautenschlein *et al*., 1998). *In situ* hybridization has been used to detect viral DNA in infected cells (Suresh and Sharma, 1996). Enzyme-linked immunosorbent assays have been developed that allow for more sensitive quantification of viral antigen and serum titers (Ianconescue *et al*., 1984; Nazerian and Fadly, 1986; van den Hurk, 1986; Ahmad and Sharma, 1993; Cardona *et al*., 1999).

PCR assays for the detection of THEV DNA in infected birds are an important addition to the collection of diagnostic tools. PCR was used to confirm ISH results in one study (Suresh and Sharma, 1996). As PCR becomes more affordable and sensitive, it will see more widespread use. There has only been one diagnostic PCR protocol published to date (Hess, 1999). This PCR protocol is now seven years old, and an update using more modern techniques is needed.

1.4 Persistent and Latent Infection

A persistent infection may be defined as one in which infectious virus can be reproducibly and continuously recovered from the host well past the usual period of illness. Latent infections are very similar, differing only in the fact that infectious virus is only detected intermittently, usually associated with clinical recurrence of disease (Jones, 2003). Regardless of the true nature of the infection, the ability of a persistent/latent virus to avoid host defense mechanisms is an important advantage to have during replication (Roizman, 1996).

Infection of lymphocytes and other immune cells often directly results in latent/persistent infection. Some viruses are capable of surviving after ingestion by macrophages, and may be able to replicate within them. Virus shed from persistently

infected macrophages can be distributed throughout the host as they circulate. Some viruses completely avoid the host immune system by shutting down viral replication, allowing their genome to remain dormant within the host cell. These viruses then reactivate under favorable conditions and resume their infectious cycle. This strategy is true latency, where only viral nucleic acid is present in cells and infectious virions are shed only sporadically. During the period of latency, the viral genome may be covalently integrated within the host cell genome (as in retroviruses) or remain independent as episomes of circular DNA. Viral reactivation is commonly a result of immunosuppression from stress or other causes (Jones, 2003; Roizman, 1996).

Adenoviruses are non-enveloped icosahedral viruses, and are known to infect most classes of vertebrates. Genome sizes range from 26-50 kb which encode 22-45 proteins. The linear, double stranded DNA genome has 36-200 bp inverted terminal repeats used in genome replication. Adenoviruses were originally discovered in 1953 and named based on their properties of latency in human adenoidal tissue. Since their discovery, many acute diseases in humans and animals have been attributed to active adenoviral infection. Adenoviruses are known more for the acute diseases they cause than their ability to persist in the host for months or years (Shenk, 1996).

Though most details of the mechanism of adenoviral persistence are not understood, there seems to be a definite link to infection of lymphocyte and monocyte populations. Adenoviral DNA has been detected in T-lymphocytes isolated from human tonsils with no evidence of active viral replication. Viral genomic DNA was detected in cells not producing infectious virus, indicating a block of viral transcription preventing viral replication (McNees *et al*., 2004; Garnett *et al*., 2002). Infection of a human umbilical cord lymphocyte cell line resulted in production of infectious virus for months without cytopathic effect. Culture of these infected cells for months in the presence of neutralizing antibodies did not hinder the infection, indicating the ability to establish a non-lytic persistent infection (Andiman and Miller, 1982). A human monocyte cell line was reported to support a persistent adenoviral infection for a year (Chu *et al.,* 1992). Low levels of DNA have been found to persist in human B-lymphocytes (Flomenburg *et*

al., 1996). It is suspected that latent virus is responsible for the recurrence of adenoviral infection in immunocompromised hosts.

The ability of an adenovirus to enter a latent/persistent state is dependent on its ability to generate viral genomes within a cell without that cell succumbing to apoptosis. To that end, adenoviruses encode many genes that are responsible for takeover of host cellular machinery as well as evasion of the host immune response. In mastadenoviruses, the genes encoded by early region 1 (E1) are responsible for viral transcriptional activation and simultaneous repression of cellular transcription. Some E1 gene products push the cell into S phase, where viral replication is more favorable. These same gene products can cause the host cell to go into apoptosis, a cellular strategy to limit viral replication. Other E1 gene products are responsible for blocking apoptosis so viral replication can continue (Grand, 1987). When the host cell is a lymphocyte or macrophage, other viral genes responsible for limiting immune response are specifically upregulated. In mastadenoviruses, genes in the early region 3 (E3) can prevent MHC-I expression on the surface of cells. This prevents proper antigen presentation by the infected cell and subsequent neutralization by cell-mediated immunity (Ginsberg *et al*., 1989). Adenovirus's abilities to control the infected cell and prevent apoptosis are essential to its ability to persist (Shenk, 1996; McNees *et al*., 2004).

Adenoviruses persist in relatively long-lived cell types and have displayed the ability to protect the host cell from apoptosis in order to promote long-term infection. Viruses evolve to take advantage of any possible strategy for more efficient viral replication. Selective pressures are constantly exerted on viruses to develop ways to avoid host defenses, and latency is the ultimate viral strategy.

1.5 Statement of Hypothesis

PCR assays for the specific detection of THEV DNA in infected birds are very important additions to the collection of diagnostic tools. The single published PCR protocol is now seven years old, and an update using more modern techniques is needed.

The purpose of this research was to develop PCR protocols that would provide diverse strategies for the detection and quantification of THEV DNA.

The reason for the differences in virulence between strains of THEV is unknown. The same avirulent strains have been used as live-virus vaccines for decades without documented spontaneous reversion to virulence. The stability of avirulent strains used in vaccines suggests that the differences between the THEV strains are clearly defined. Comparison of partial DNA sequences has revealed that the sequences are almost identical. The first step in fully understanding the differences between the strains of THEV is to compare their complete genomic sequences. Therefore, the second purpose of this research was to locate regions of genetic diversity in virulent and avirulent isolates of THEV.

THEV infection stimulates a strong life-long protective antibody response. The pattern of viral distribution and corresponding serologic response has been studied to a certain extent in turkeys. Most of those studies focus on the acute stage of infection, the first 5-6 days post-inoculation. Intranuclear inclusions observed in the kidney tissue of seropositive turkeys indicate the virus may be capable of avoiding complete neutralization. The distribution of THEV in the host is appropriately widespread, considering it is capable of infecting macrophages and lymphocytes that travel throughout the host. Pilot PCR studies revealed the presence of THEV DNA in tissues of infected birds beyond the acute stage of infection. No evidence of infectious virus has been detected in infected birds after 1 week pi, but thus far only crude methods of detection have been used. It is possible that THEV remains in B-lymphocytes or macrophages in low numbers and continually stimulates the immune response of the host. The third purpose of this research was to determine if THEV remains in infected turkeys beyond the onset of significant antibody response, and to explore the long-term tissue distribution of the virus.

Hypotheses

Based on published research and pilot data, I hypothesize that:

1. Genetic changes are directly responsible for the difference in virulence of THEV-V and THEV-A.

2. Target cells are capable of being latently or persistently infected by THEV, leading to stimulation of a long-term immune response.

1.6 References

Ahmad, J. & Sharma, J.M. (1993). Protection against hemorrhagic enteritis and newcastle disease in turkeys by embryo vaccination with monovalent and bivalent vaccines. *Avian Dis* 37, 485-491.

Andiman, W.A., & Miller, G. (1982). Persistent infection with adenovirus types 5 and 6 in lymphoid cells from humans and woolly monkeys. *J Infect Dis* 145, 83-88.

Arnberg, N., Mei, Y., & Wadell, G. (1997). Fiber genes of adenoviruses with tropism for the eye and genital tract. *Virology* 227, 239-244.

Arnberg, N., Edlund, K., Kidd, A.H., & Wadell, G. (2000a). Adenovirus type 37 uses sialic acid as a cellular receptor. *J Virol* 74, 42-48.

Arnberg, N., Kidd, A.H., Edlund, K., Olfat, F., & Wadell, G. (2000b). Initial interactions of subgenus D adenoviruses with a549 cellular receptors: sialic acid versus alpha-v integrins. *J Virol* 74, 7691-7693.

Barbour, E.K., Poss, P.E., Brinton, M.K., Johnson, J.B., & Nabbut, N.H. (1993). Evaluation of cell culture propagated and *in vivo* propagated hemorrhagic enteritis vaccines in turkeys. *Vet Immunol Immunopathol* 35, 375-383.

Beasley, J.N., & Wisdom, J. (1978). Pathogenesis of hemorrhagic enteritis in turkeys. *Avian Dis* 22, 313-319.

Beasley, J.N., & Clifton, S.G. (1979). Experimental infection of broiler and leghorn chickens with virulent and avirulent isolates of hemorrhagic enteritis virus. *Avian Dis* 23, 616-621.

Benko, M., & Harrach, B. (1998). A proposal for a new (third) genus within the family adenoviridae. *Arch Virol* 143, 829-837.

Benko, M., Elo, P, Ursu, K., Ahne, W., Lapatra, S.E., Thomson, D., & Harrach, B. (2002). First molecular evidence for the existence of distinct fish and snake adenoviruses. *J Virol* 76, 10056-10059.

Binger, M.H., & Flint, S.J. (1984). Accumulation of early and intermediate mRNA species during subgroup C adenovirus productive infections. *Virology* 136, 387-403.

Both, G.W. (2002). Atadenovirus. In *The Springer Index of Viruses*, pp.2-8. Edited by C.A. Tidona and G. Darai. New York: Springer-Verlag.

Botting, C.H., and Hay, R.T. (2001). Role of conserved residues in the activity of adenovirus preterminal protein. *J Gen Virol* 82, 1917-1927.

Cardona, C.J., Reed, W.M., Witter, R.L., and Silva, R.F. (1999). Protection of turkeys from hemorrhagic enteritis with a recombinant fowl poxvirus expressing the native hexon of hemorrhagic enteritis virus. *Avian Dis* 43, 234-244.

Carlson, H.C., Pettit, J.R., Hemsley, R.V., and Mitchell, W.R. (1973). Marble spleen disease of pheasants in ontario. *Can J Comp Med* 37, 281-286.

Carlson, H.C., and Al-Sheikhly, F. (1974). Virus particles in spleens and intestines of turkeys with hemorrhagic enteritis. *Avian Dis* 18, 67-73.

Chang, L-S., and Shenk, T. (1990). The adenovirus DNA binding protein can stimulate the rate of transcription directed by adenovirus and aav promoters. *J Virol* 64, 2103-2109.

Chary, P., Rautenschlein, S., Sharma, J.M. (2002). Reduced efficacy of hemorrhagic enteritis virus vaccine in turkeys exposed to avian pneumovirus. *Avian Dis* 46, 353-359.

Chu, Y., Sperber, K., Mayer, L., and Hsu, M.T. (1992). Persistent infection of human adenovirus type 5 in human monocyte cell lines. *Virology* 188, 793-800.

Davison, A.J., Wright, K.M., and Harrach, B. (2000). DNA sequence of frog adenovirus 1. *J Gen Virol* 81, 2431-2439.

Davison, A.J., and Harrach, B. (2002). Siadenovirus. In *The Springer Index of Viruses,* pp.29-33. Edited by C.A. Tidona and G. Darai. New York: Springer-Verlag.

Davison, A.J., Benko, M., and Harrach, B. (2003). Genetic content and evolution of adenoviruses. *J Gen Virol* 84, 2895-2908.

Domermuth, C.H., and Gross, W.B. (1971). Effect of disinfectants and drying on the virus of hemorrhagic enteritis of turkeys. *Avian Dis* 15, 94-97.

Domermuth, C.H., Gross, W.B., and Dubose, R.T., Douglass, C.S., Reubush, C.B. (1972). Agar gel diffusion test for hemorrhagic enteritis of turkeys. *Avian Dis* 16, 852-857.

Domermuth, C. H., and Gross,W.B. (1972). Effect of chlorine on the virus of hemorrhagic enteritis of turkeys. *Avian Dis* 16, 952-953.

Domermuth, C. H., Gross, W.B., and Dubose, R.T. (1973). Microimmunodiffusion test for hemorrhagic enteritis of turkeys. *Avian Dis* 17, 439-444.

Domermuth, C.H., Gross, W.B., Douglass, C.S., Dubose, R.T., Harris, J.R., and Davis, R.B. (1977). Vaccination for hemorrhagic enteritis of turkeys. *Avian Dis* 21, 557-565.

Domermuth, C.H., Gross, W.B., Schwartz, L.D., Mallinson, E.T., and Britt, R. (1979a). Vaccination of ring-necked pheasant for marble spleen disease. *Avian Dis* 23, 30-38.

Domermuth, C.H., Harris, J.R., Gross, W.B., and Dubose, R.T. (1979b). A naturally occurring infection of chickens with a hemorrhagic enteritis/marble spleen disease type of virus. *Avian Dis* 23, 479-484.

Domermuth, C.H., Van Der Heide, L., and Faddoul, G.P. (1982). Pulmonary congestion and edema (marble spleen disease) of chicken produced by group II avian adenovirus. *Avian Dis* 26, 629-633.

Domermuth, C.H., and Larsen, C.T. (1984). Vaccination against hemorrhagic enteritis virus of turkeys: an update. *J Am Vet Med Assoc* 185, 336.

Fadly, A.M., and Nazerian, K. (1982). Evidence for bursal involvement in the pathogenesis of hemorrhagic enteritis of turkeys. *Avian Dis* 26, 525-533.

Fadly, A.M., Nazerian, K., Nagaraja, K., and Below, G. (1985). Field vaccination against hemorrhagic enteritis of turkeys by a cell-culture live-virus vaccine. *Avian Dis* 29, 768-777.

Fadly, A.M., Cowen, B.S., and Nazerian, K. (1988). Some observations on the response of ring-necked pheasants to inoculation with various strains of cell-culture-propagated type II avian adenovirus. *Avian Dis* 32, 548-552.

Fadly, A.M., Nazerian, K. (1989). Hemorrhagic enteritis of turkeys: influence of maternal antibody and age at exposure. *Avian Dis* 33, 778-786.

Farkas, S.L., Benko, M., Elo, P., Ursu, K., Dan, A., Ahne, W., and Harrach, B. (2002). Genomic and phylogenetic analyses of an adenovirus isolated from a corn snake (elaphe guttata) imply a common origin with members of the proposed new genus atadenovirus. *J Gen Virol* 83, 2403-2410.

Fax, P., Lipinski, K.S., Esche, H., and Brockmann, D. (2000). cAmp-independent activation of the adenovirus type 12 E2 promoter correlates with the recruitment of CREB-1/ATF-1, E1A(12S), and the CBP to the E2-CRE. *J Biol Chem* 275, 8911-8920.

Fessler, S.P., and Young, C.S.H. (1998). Control of adenovirus early gene expression during the late phase of infection. *J Virol* 72, 4049-4056.

Fitzgerald, S.D., and Reed, W.M. (1991). Pathogenesis of marble spleen disease in bursectomized and non-bursectomized ring-necked pheasants following oral inoculation with cell-culture-propagated virus *Avian Dis* 35, 579-584.

Fitzgerald, S.D., Reed, W.M., Burnstein, T. (1992). Detection of type II avian adenoviral antigen in tissue sections using immunohistochemical staining. *Avian Dis* 36, 341-347.

Fitzgerald SD, Reed WM, Furukawa AM, Zimels E, Fung L. (1995). Effect of T-lymphocyte depletion on the pathogenesis of marble spleen disease virus infection in ring-necked pheasants. *Avian Dis* 39, 68-73.

Flomenburg, P., Piaskowski, V., Harb, J., Segura, A., and Casper, J.T. (1996). Spontaneous, persistent infection of B-cell lymphoma with adenovirus. *J Med Virol* 48, 267-272.

Freimuth, PI, Ginsberg, H.S. (1986). Codon insertion mutants of the adenovirus terminal protein. *Proc Natl Acad Sci* 83, 7816-20.

Gale, C., and Wyne, J.W. (1957). Preliminary observations on hemorrhagic enteritis of turkeys *Poult Sci* 36, 1267-1270.

Garnett, C.T., Erdman, D., Xu, W., Gooding, L.R. (2002). Prevalence and quantitation of species C adenovirus DNA in human mucosal lymphocytes. *J Virol* 76, 10608-10616.

Ginsberg, H.S., Lundholm-Beauchamp, U., Horswood, R.L., Pernis, B., Wold, W.S.M., Chanock, R.M., and Prince, G.A. (1989). Role of early region 3 (E3) in pathogenesis of adenovirus disease. *Proc Natl Acad Sci* 86, 3823-3827.

Glasgow, J.N., Kremer, E.J., Hemminki, A., Siegal, G.P., Douglas, J.T., and Curiel, D.T. (2004). An adenovirus vector with a chimeric fiber derived from canine adenovirus type 2 displays novel tropism *Virology* 324, 103-116.

Gomez-Villamandos, J.C., Carranza, J., Sierra, M.A., Carrasco, L., Hervas, J., Blanco, A., and Fernandez, A. (1994). Hemorrhagic enteritis by adenovirus-like particles in turkeys: a possible pathogenic mechanism. *Avian Dis* 38, 647-652.

Grable, M., and Hearing, P. (1990). Adenovirus type 5 packaging domain is composed of a repeated element that is functionally redundant. *J Virol* 64, 2047-2056.

Grable, M., and Hearing, P. (1992). Cis and trans requirements for the selective packaging of adenovirus type 5 DNA. *J Virol* 66, 723-731.

Grand, R.J.A. (1987). The structure and function of the adenovirus early region 1 proteins. *Biochem J* 241, 25-38.

Greber, U.F., Suomalainen, M., Stidwell, R.P., Boucke, K., Ebersold, M.W., and Helenius, A. (1997). The role of the nuclear pore complex in adenovirus DNA entry. *EMBO J* 16, 5998-6007.

Gross, W.B., and Moore, W.E.C. (1967). Hemorrhagic enteritis of turkeys. *Avian Dis* 11, 296-307.

Gross, W.B. (1967). Lesions of hemorrhagic enteritis. *Avian Dis* 11, 684-693.

Gross, W.B., Domermuth, C.H. (1975). Spleen lesions of hemorrhagic enteritis of turkeys. *Avian Dis* 20, 455-466.

Gustin, K.E., Lutz, P., and Imperiale, M.J. (1996). Interaction of the adenovirus L1 52/55-kilodalton protein with the IVa2 gene product during infection. *J Virol* 70, 6463-7.

Gustin, K.E., and Imperiale, M.J. (1998). Encapsidation of viral DNA requires the adenovirus L1 52/55 kDa protein. *J Virol* 72, 7860-7870.

Hammarskjold, M., and Winberg, G. (1980). Encapsidation of adenovirus 16 DNA is directed by a small DNA sequence at the left end of the genome. *Cell* 20, 787-795.

Harrach, B. (2002). Aviadenovirus. In *The Springer Index of Viruses*, pp.9-18. Edited by C.A. Tidona and G. Darai. New York: Springer-Verlag.

Hearing, P., and Shenk, T. (1983). The adenovirus type 5 e1a transcriptional control region contains a duplicated enhancer element. *Cell* 33, 695-703.

Hearing, P., and Shenk, T. (1986). The adenovirus type 5 E1A enhancer contains two functionally distinct domains: one is specific for E1A and the other modulates all early units in cis. *Cell* 45, 229-236.

Henry, L.J., Xia, D., Wilke, M.E., Deisenhofer, J., and Gerard, R. (1994). Characterization of the knob domain of adenovirus type 5 fiber protein expressed in *E. coli*. *J Virol* 68, 5239-5246.

Hess, M., Raue, R., and Hafez, H.M. (1999). PCR for specific detection of haemorrhagic enteritis virus of turkeys, an avian adenovirus. *J Virol Methods* 81, 199-203.

Hong, J.S., and Engler, J.A. (1996). Domains required for the assembly of adenovirus type 2 fiber trimers. *J Virol* 70, 7071-7078.

Hong, S.S., Karayan, L., Tournier, J., Curiel, D.T., and Boulanger, P.A. (1997). Adenovirus type 5 fiber knob binds to MHC class I a2 domain at the surface of human epithelial and B lymphoblastoid cells. *EMBO J* 16, 2294-2306.

Howitt, J., Bewley, M.C., Graziano, V., Flanagan, J.M., and Freimuth, P. (2003). Structural basis for variation in adenovirus affinity for the cellular coxsackievirus and adenovirus receptor. *J Biol Chem* 278, 26208-26215.

Huang, W., Kiefer, J., Whalen, D., and Flint, S.J. (2003). DNA synthesis-dependent relief of repression of transcription from the adenovirus type 2 IVa2 promoter by a cellular protein. *Virol* 314, 394-402.

Ianconescu, M., Smith, E.J., Fadly, A.M., Nazerian, K. (1984). An enzyme-linked immunosorbent assay for detection of hemorrhagic enteritis virus and associated antibodies. *Avian Dis* 28, 677-92.

Iltis, J.P., Jakowski, R.M., and Wyand, D.S. (1975a). Transmission of marble spleen disease in turkeys and pheasants. *Am J Vet Res* 36, 97-101.

Iltis, J.P, Jakowski, R.M., and Wyand, D.S. (1975b). Experimentally transmitted marble spleen disease in pen raised wild turkeys. *J Wildl Dis* 11, 484-5.

Iltis, J.P., Daniels, S.B., and Wyand, D.S. (1977). Demonstration of an avian adenovirus as the causative agent of marble spleen disease. *Am J Vet Res* 38, 95-100.

Iltis, J.P., and Daniels, S.B. (1977). Adenovirus of ring-necked pheasants: purification and partial characterization of marble spleen disease virus. *Infection and Immunity* 16, 701-705.

Itakura, C., Carlson, H.C., Lang, G.N. (1974). Experimental transmission of haemorrhagic enteritis of turkeys. *Avian Pathol* 3, 279-292.

Itakura, C., Carlson, H.C. (1975a). Electron microscopic findings of cells with inclusion bodies in experimental hemorrhagic enteritis of turkeys. *Can J Comp Med* 39, 299-304.

Itakura, C., and Carlson, H.C. (1975b). Pathology of spontaneous hemorrhagic enteritis of turkeys. *Can J Comp Med* 39, 310-315.

Jansen-Durr, P., Boeuf, P., and Kedinger, C. (1988). Replication-induced stimulation of the major late promoter of adenovirus is correlated to the binding of a factor to sequences in the first intron. *Nucl Acids Res* 16, 3771-3786.

Jansen-Durr, P., Boeuf, P., and Kedinger, C. (1989). Replication-dependent activation of the major late promoter is mediated by the increased binding of a transcription factor to sequences in the first intron. *J Virol* 63, 5124-5132.

Jansen-Durr, P, Wintzerith M, Reimund B, Hauss C, Kedinger C. (1990). Two distinct cellular proteins interact with the E1A-responsive element of an adenovirus early promoter. *J Virol* 64, 2384-7.

Jones, C. (2003). Herpes simplex virus type 1 and bovine herpesvirus 1 latency. *Clin Microbiol Rev* 16, 79-95.

Jucker, M.T., McQuiston, J.R., van den Hurk, J.V., Boyle, S.M., and Pierson, F.W. (1996). Characterization of the haemmorhagic enteritis virus genome and the sequence of the putative penton base and core protein genes. *J Gen Virol* 77, 469-479.

Larsen, C.T., Domermuth, C.H., Sponenberg, D.P., and Gross, W.B. (1985). Colibacillosis of turkeys exacerbated by hemorrhagic enteritis virus laboratory studies. *Avian Dis* 29, 729-732.

Lee, J., Choi, J., Lee, D., Kim, S., Fitzgerald, S.D., Lee, Y., amd Kim, D. (2001). Marble spleen disease in pheasants in korea. *J Vet Med Sci* 63, 699-701.

Lin, H.J.L., and Flint, S.J. (2000). Identification of a cellular repressor of transciption of the adenoviral late IVa2 gene that is unaltered in activity in infected cells. *Virol* 277, 397-410.

Liu, H., Naismith, J.H., Hay, R.T. (2003). Adenovirus DNA replication. *Curr Top Microbiol Immunol* 272, 131-164.

Lu, H., Reach, M.D., Minaya, E., and Young, C.S.H. (1997). The initiator element of the adenovirus major late promoter has an important role in transcription initiation *in vivo. J Virol* 71, 102-109.

Lutz, P., Kedinger, C. (1996). Properties of the adenovirus IVa2 gene product, an effector of late-phase-dependent activation of the major late promoter. *J Virol* 70, 1396-1405.

Magnusson, M.K., Hong, S.S., Boulanger, P., and Lindholm, L. (2001). Genetic retargeting of adenovirus: novel strategy employing "deknobbing" of the fiber. *J Virol* 75, 7280-7289.

McNees, A.L., Mahr, J.A., Ornelles, D., and Gooding, L.R. (2004). Postinternalization inhibition of adenovirus gene expression and infectious virus production in human T-cell lines. *J Virol* 78, 6955-6966.

Meteyer, C.U., Mohammed, H.O., Chin, R.P., Bickford, A.A., Trampel, D.W., and Klein, P.N. (1992). Relationship between age of flock seroconversion of hemorrhagic enteritis virus and appearance of adenoviral inclusions in the spleen and renal tubule epithelia of turkeys. *Avian Dis* 36, 88-96.

Morrison, M.D., Onions, D.E., and Nicolson, L. (1997). Complete DNA sequence of canine adenovirus type 1. *J Gen Virol* 78, 873-878.

Nagaraja, K.V., Kang, S.Y., and Newman, J.A. (1985). Immunosuppressive effect of virulent strain of hemorrhagic enteritis virus in turkeys vaccinated against newcastle disease. *Poult Sci* 64, 588-590.

Nagy, M., Nagy, E., and Tuboly, T. (2001). The complete nucleotide sequence of porcine adenovirus serotype 5. *J Gen Virol* 82, 525-529.

Nagy, M., Nagy, E., and Tuboly, T. (2002). Sequence analysis of porcine adenovirus serotype 5 fibre gene: evidence for recombination. *Virus Genes* 24, 181-185.

Nazerian, K., Elmubarak, A., Sharma, J.M. (1982). Establishment of B-lymphoblastoid cell lines from Marek's disease virus-induced tumors in turkeys. *Int J Cancer* 29, 63-68.

Nazerian, K., and Fadly, A.M. (1982). Propagation of virulent and avirulent turkey hemorrhagic enteritis virus in cell culture. *Avian Dis* 26, 816-827.

Nazerian, K., and Fadly, A.M. (1986). Further studies on *in vitro* and *in vivo* assays of hemorrhagic enteritis virus (hev). *Avian Dis* 31, 234-240.

Nazerian, K., Lee, L.F., Payne, W.S. (1991). Structural polypeptides of type II avian adenoviruses analyzed b y monoclonal and polyclonal antibodies. *Avian Dis* 35, 572-578.

Newberry, L.A., Skeeles, J.K., Kreider, D.L., Beasley, J.N., Story, J.D., McNew, R.W., and Berridge, B.R. (1993). Use of virulent hemorrhagic enteritis virus for the induction of colibacillosis in turkeys. *Avian Dis* 37, 1-5.

Opengart, K. N., Eyre, P., and Domermuth, C.H. (1992). Increased numbers of duodenal mast cells in turkeys inoculated with hemorrhagic enteritis virus. *Am J Vet Res* **53**, 814-819.

Ossa, J.E., Bates, R.C., and Schurig, G.G. (1983a). Hemorrhagic enteritis in turkeys: purification and quantification of the virus. *Avian Dis* 27, 235-245.

Ossa, J.E., Alexander, J., and Schurig, G.G. (1983b). Role of splenectomy in prevention of hemorrhagic enteritis and death from hemorrhagic enteritis virus in turkeys. *Avian Dis* 27, 1106-1111.

Ostapchuck, P., and Hearing, P. (2003). Minimal cis-acting elements required for adenovirus genome packaging. *J Virol* 77, 5127-5135.

Pardo-Mateos, A., and Young, C.S.H. (2004a). A 40kda isoform of the type 5 adenovirus IVa2 protein is sufficient for virus viability. *Virol* 324, 151-164.

Pardo-Mateos, A., and Young, C.S.H. (2004b). Adenovirus IVa2 protein plays an important role in transcription from the major late promoter *in vivo*. *Virol* 327, 50-59.

Parker, S.F., Felzien, L.K., Perkins, N.D., Imperiale, M.J., Nabel, G.J. (1997). Distinct domains of adenovirus E1A interact with specific cellular factors to differentially modulate human immunodeficiency virus transcription. *J Virol* 71, 2004-2012.

Pierson, F.W., Larsen, C.T., and Domermuth, C.H. (1996). The production of colibacillosis in turkeys following sequential exposure to newcastle disease virus or *bordatella avium*, avirulent hemorrhagic enteritis virus, and *E. coli*. *Avian Dis* 40, 837-840.

Pierson, F.W., and Fitzgerald, S.D. (2003). Hemorrhagic enteritis and related infections. In *Diseases of Poultry,* 11th ed., pp. 237-247. Edited by Y.M. Saif. , H.J. Barnes, J.R. Glisson, A.M. Fadly. L.R. McDougald, and D.E. Swayne. Ames: Iowa State University Press.

Pitcovski, J., Mualem, M., Rei-Koren, Z., Krispel, S., Shmueli, E., Peretz, Y., Gutter, B., Gallili, G.E., Michael, A., and Goldberg, D. (1998). The complete DNA sequence and genome organization of the avian adenovirus, hemorrhagic enteritis virus. *Virology* 249, 307-315.

Pitcovski, J., Fingerut, E., Gallili, G., Eliahu, D., Finger, A., Gutter, B. (2005). A subunit vaccine against hemorrhagic enteritis adenovirus. *Vaccine* 23, 4697-4702.

Pomeroy, B.S., and Fenstermacher, R. (1937). Hemorrhagic enteritis in turkeys. *Poult Sci* 16, 378-383.

Rautenschlein, S., Suresh, M., Neumann, U., and Sharma, J.M. (1998). Comparative pathogenesis of haemorrhagic enteritis virus (hev) infection in turkeys and chickens. *J Comp Path* 119, 251-261.

Rautenschlein, S., and Sharma, J.M. (1999). Response of turkeys to simultaneous vaccination with hemorrhagic enteritis and newcastle disease viruses. *Avian Dis* 43, 286-292.

Rautenschlein, S., Suresh, M., and Sharma, J.M. (2000). Pathogenic avian adenovirus type II induces apoptosis in turkey spleen cells. *Arch Virol* 145, 1671-1683.

Rautenschlein, S., and Sharma, J.M. (2000). Immunopathogenesis of haemorrhagic enteritis virus in turkeys. *Dev Comp Immunol* 24, 237-246.

Reddy, P.S., Idamakanti, N., Zakhartchouk, A.N., Baxi, M.K., Bok Lee, J., Pyne, C., Babiuk, L.A., and Tikoo, S.K. (1998). Nucleotide sequence, genome organization, and transcription map of bovine adenovirus type 3. *J Virol* 72, 1394-1402.

Roelvink, P.W., Lee, G.M., Einfeld, D.A., Kovesdi, I., and Wickham, T.J. (1999). Identification of a conserved receptor-binding site on the fiber proteins of car-recognizing adenoviridae. *Science* 286, 1568-1571.

Roizman, B. (1996). Herpesviridae. In *Fields Virology, 3rd ed,* pp. 2111-2148. Edited by Fields, B.N., Knipe, D.M., and Howley, P.M. Philadelphia: Lippincott – Raven.

Roy, S., Gao, G., Clawson, D.S., Vandenberghe, L.H., Farina, S.F., and Wilson, J.M. (2004). Complete nucleotide sequences and genome organization of four chimpanzee adenoviruses. *Virology* 324, 361-372.

Ruzindana-Umunyana, A., Imbeault, L., Weber, J.M. (2002). Substrate specificity of adenovirus protease. *Virus Res* 89, 41-52.

Saunders, G.K., Pierson, F.W., and van den Hurk, J.V. (1993). Haemorrhagic enteritis virus infection in turkeys: a comparison of virulent and avirulent virus infections, and a proposed pathogenesis. *Avian Pathol* 22, 47-58.

Schmid, S.I., and Hearing, P. (1997). Bipartite structure and functional independence of adenovirus type 5 packaging elements. *J Virol* 72, 6339-6347.

Sharma, J.M. (1991). Hemorrhagic enteritis of turkeys. *Vet Immunol Immunopathol* 30, 67-71.

Sharma, J.M. (1994). Response of specific-pathogen-free turkeys to vaccines derived from marble spleen disease virus and hemorrhagic enteritis virus. *Avian Dis* 38, 523-30.

Shenk, T. (1996). Adenoviridae: the viruses and their replication. In *Fields Virology,* 3rd edn, pp. 2111-2148. Edited by Fields, B.N., Knipe, D.M., and Howley, P.M. Philadelphia: Lippincott – Raven.

Sheppard, M., Werner, W., McCoy, R.J., and Johnson, M.A. (1998b). The major late promoter and bipartite leader sequence of fowl adenovirus. *Arch Virol* 143, 537-548.

Silim, A., Thorsen, J., and Carlson, H. (1978). Experimental infection of chickens with hemorrhagic enteritis virus. *Avian Dis* 22, 106-114.

Silim, A., and Thorsen, J. (1981). Hemorrhagic enteritis: virus distribution and sequential development of antibody in turkeys. *Avian Dis* 25, 444-453.

Singh, M., Shmulevitz, M., Tikoo, S.K. (2005). A newly identified interaction between IVa2 and pVIII proteins during porcine adenovirus type 3 infection. *Virology* 336, 60-69.

Song, B., Hu, S.L., Darai, G., Spindler, K.R., and Young, C.S. (1996). Conservation of DNA sequences in the predicted major late promoter regions of selected mastadenoviruses. *Virology* 220, 390-401.

Song, B., Young, C.S.H. (1998). Functional analysis of the CAAT box in the major late promoter of the subgroup C human adenoviruses. *J Virol* 72, 3213-3220.

Sponenberg, D.P., Domermuth, C.H., and Larsen, C.T. (1985). Field outbreaks of colibacillosis of turkeys associated with hemorrhagic enteritis virus. *Avian Dis* 29, 838-842.

Stevenson, S.C., Rollence, M., White, B., Weaver, L., and McClelland, A. (1995). Human adenovirus serotypes 3 and 5 bind to two different cellular receptors via the fiber head domain. *J Virol* 69, 2850-2857.

Stouten, P.F.W., and Sander, C. (1992). New triple-helical model for the shaft of the adenovirus fibre. *J Mol Biol* 226, 1073-1084.

Suresh, M., and Sharma, J.M. (1995). Hemorrhagic enteritis virus induced changes in the lymphocyte sub-populations in turkeys and the effect of experimental immunodeficiency on viral pathogenesis. *Vet Immunol Immunopathol* 45, 139-150.

Suresh, M., Cyr, S.St., and Sharma, J.M. (1995). Molecular cloning and sequence analysis of the penton base genes of type II avian adenoviruses. *Virus Res* 39, 289-297.

Suresh, M., and Sharma, J. M. (1996). Pathogenesis of type II avian adenovirus infection in turkeys: *in vivo* immune cell tropism and tissue distribution of the virus. *J Virol* 70, 30-36.

Tan, P.K., Michou, A., Bergelson, J.M., and Cotton, M. (2001). Defining CAR as a cellular resceptor for the avian adenovirus CELO using a genetic analysis of the two viral fibre proteins. *J Gen Virol* 82, 1465-1472.

Thorsen, J., Weninger, N., Weber, L., Van Dijk, C. (1982). Field trials of an immunization procedure against hemorrhagic enteritis virus of turkeys. *Avian Dis* 26, 473-477.

Tolin, S.A., and Domermuth, C.H. (1975). Hemorrhagic enteritis of turkeys: electron microscopy of the causal virus. *Avian Dis* 19, 118-125.

Trampel, D.W., Meteyer, C.U., Bickford, A.A. (1992). Hemorrhagic enteritis virus inclusions in turkey renal tubular epithelium. *Avian Dis* 36, 1086-1091.

Tribouley, C., Lutz, P., Staub, A., Kedinger, C. (1994). The product of the adenovirus intermediate gene IVa2 is a transcriptional activator of the major late promoter. *J Virol* 68, 4450-4457.

van den Hurk, J.V. (1986). Quantitation of hemorrhagic enteritis virus antigen and antibody using enzyme-linked immunosorbent assays. *Avian Dis* 30, 662-71.

van den Hurk, J.V. (1988). Characterization of group II avian adenoviruses with a panel of monoclonal antibodies. *Can J Vet Res* 52, 458-467.

van den Hurk, J.V. (1990). Propagation of group II avian adenoviruses in turkey and chicken leukocytes. *Avian Dis* 34, 12-25.

van den Hurk, J.V. (1992). Characterization of the structural proteins of hemorrhagic enteritis virus. *Arch Virol* 126, 195-213.

van den Hurk, J.V., Allan, B.J., Riddell, C., Watts, T., Potter, A.A. (1994). Effect of infection with hemorrhagic enteritis virus on susceptibility of turkeys to *E. coli*. *Avian Dis* 38, 708-716.

van Raaij, M.J., Mitraki, A., Lavigne, G., Cusack, S. (1999). A triple B-spiral in the adenovirus fibre shaft reveals a new structural motif for a fibrous protein. *Nature* 401, 935-938.

Veit, H.P., Domermuth, C.H., and Gross, W.B. (1981). Histopathology of avian adenovirus group II splenomegaly of chickens. *Avian Dis* 25, 866-873.

Wadell, G. (2002). Mastadenovirus. In *The Springer Index of Viruses*, pp.19-28. Edited by C.A. Tidona and G. Darai. New York: Springer-Verlag.

Webster, A., Leith, I.R., and Hay, R.T. (1997). Domain organization of the adenovirus preterminal protein. *J Virol* 71, 539-547.

Wellehan, J.F.X., Johnson, A.J., Harrach, B., Benko, M., Pessier, A.P., Johnson, C.M., Garner, M.M., Childress, A., Jacobsen, E.R. (2004). Detection and analysis of six lizard

adenoviruses by consensus primer PCR provides further evidence of a reptilian origin for the atadenoviruses. *J Virol* 78, 13366-13369.

Wilcock, B.P., and Thacker, H.L. (1975). Focal hepatic necrosis in turkeys with hemorrhagic enteritis. *Avian Dis* 20, 205-208.

Wyand, D.S., Jakowski, R.M., and Burke, C.N. (1972). Marble spleen disease in ring-necked pheasants: histology and ultrastructure. *Avian Dis* 16, 320-329.

Young, C.S.H. (2003). Structure and function of the adenovirus major late promoter. *Curr Top Microbiol Immunol* 272, 213-249.

Zain, S., Sambrook, J., Roberts, R.J., Keller, W., Fried, M., and Dunn, A.R. (1979). Nucleotide sequence analysis of the leader segments in a cloned copy of adenovirus 2 fiber mRNA. *Cell* 16, 851-861.

Zhang, C., and Nagaraja, K.V. (1989). Differentiation of avian adenovirus type-II strains by restriction endonuclease fingerprinting. *Am J Vet Res* 50, 1466-1470.

Zhang, C.L., Nagaraja, K.V., Sivanandan, V., and Newman, J.A. (1991). Identification and characterization of viral polypeptides from type-II avian adenoviruses. *Am J Vet Res* 52, 1137-1141.

Zhang, W., and Imperiale, M.J. (2000). Interaction of the adenovirus IVa2 protein with viral packaging sequences. *J Virol* 74, 2687-2693.

Zhang, W., Low, J.A., Christensen, J.B., and Imperiale, M.J. (2001). Role for the adenovirus IVa2 protein in packaging of viral DNA. *J Virol* 75, 10454-10466.

Zhang, W., and Imperiale, M.J. (2003). Requirement of the adenovirus IVa2 protein for virus assembly. *J Virol* 77, 3586-3594.

Zhang, W., and Arcos, R. (2005). Interaction of the adenovirus major core protein precursor, pVII, with the viral DNA packaging machinery. *Virology* 334, 194-202.