DISSERTATION

PATHOGENESIS AND IMMUNITY OF RABIES VIRUS INFECTION IN BATS

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY APRIL DAWN DAVIS ENTITLED PATHOGENESIS AND IMMUNITY TO RABIES VIRUS INFECTION IN BATS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

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ABSTRACT OF DISSERTATION

PATHOGENESIS AND IMMUNITY TO RABIES
VIRUS INFECTION IN BATS

Rabies is one of the oldest known viral diseases and, with a >99% fatality rate, it is also one of the most deadly. Although a major public health concern, human deaths due to rabies in the developed world are rare. Worldwide, statistics are very different as rabies kills in excess of 50,000 humans per year, most often the result of canine rabies variants. During the 1950's, canine rabies cases began decreasing in the United States as a result of vaccination efforts, followed by a decrease in human rabies cases. Rabies in insectivorous bats was first reported in the US in the 1950's and has now been reported in most North American bat species. Over the last 20 years, 92% of the human rabies cases have been the result of a bat variant.

This purpose of this work was to expand our knowledge of rabies variants associated with silver hair bats, Mexican free-tailed bats, and big brown bats. The goal of the first study was to characterize disease progression between two closely related big brown bat rabies variants. The data from this experiment indicated that changes in the rabies virus genome may have profound effects on infectivity and virulence.

The second study was designed to determine if rabies virus could be transmitted to bats through the aerosol route. Outbred mice and two species of bats were exposed to three variants of rabies virus by aerosol exposure. Although all bats survived the aerosol exposure, 44% of the mice died of rabies. Following exposure, anti-rabies virus

neutralizing antibodies were demonstrated in all bats. Following an intramuscular inoculation of rabies virus six months after the aerosol exposure, the number of seropositive bats that developed rabies was equal to the control bats.

The third study examined the dynamics of rabies virus infection in bats from colonies in Texas and Colorado. The ability of healthy wild bats incubating rabies to transmit rabies virus was also examined. Rabies virus antigen was found in 50% of the salivary glands from rabid bats, yet infectious rabies virus was isolated from less than 35% of rabid bats. Evaluation of of bats from both the field and in captive colonies demonstrated that approximately 0.5-2% of clinically healthy bats were in the incubation phase of rabies virus infection.

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CHAPTER I

REVIEW OF LITERATURE

1.1 INTRODUCTION

History of Rabies

Rabies is one of the oldest known viral diseases in history, emerging approximately 11,000 years ago (Badrain and Tordo, 2001). The earliest descriptions of human deaths from rabies virus infection were documented around 2300 BC. These initial cases from Mesopotamia were the first to associate the bite of a mad dog with clinical manifestations of rabies. The treatment of rabies over the years has been quite variable, including "going to the sea and making nine waves", "widening the wounds", and "sucking out the venom", along with various herbal remedies (cited in Wilkinson, 2001). In Europe, nineteen Saints were celebrated for their ability to prevent or cure rabies, including St. Denis and St. Hubert who often employed cautery with St. Hubert's key (Baer et al.,1996). Until the 1970's cautery remained part of the first aid treatment following potential rabies exposure. Since the fatality rate of the disease nears 100%, euthanizing humans strongly suspected to be infected with rabies virus was acceptable until the early eighteenth century (Wilkinson, 2001). Successful treatment with Pasteur's vaccine regimen did not begin until 1885 (Wilkinson, 2001).

The number of human rabies cases in the United States is very low, typically resulting in two or three deaths per year. With few exceptions, human rabies cases in the U.S. are attributed to rabies variants of chiropteran origin.

Worldwide the statistics are considerably different: approximately 40,000-100,000 humans die of rabies each year, or one every fifteen minutes (Rupprecht et al., 1995; Neil, 2003; Miranda, 2003). Ninety-five percent of these rabies cases are the result of a bite from a rabid dog in Africa, India, and underdeveloped nations in Asia (CDC, 2006a). All too often such patients either do not seek treatment following the bite of a wild or pet dog because of the cost, receive inadequate treatment, or do not believe they need treatment at all. Worldwide, more than half of the human rabies cases are in children under 14 due to their interactions with wild and pet dogs and the inability to afford or seek treatment (Miranda, 2003).

Several countries in which rabies once killed thousands of people every year have begun affordable vaccination programs for stray and pet dogs. This has drastically decreased the number of human deaths in Mexico and other Latin American countries to less than 100 per year. Millions of dollars and thousands of hours made these results possible (USDA, 2002). Epidemiologists and rabies experts believe that as the number of dog rabies cases decreases, human rabies cases attributed to bats will become more apparent. Most rabies cases in developed nations are the result of bat rabies variants. Typically these are classified as "cryptic" rabies cases, as no known bite can be associated with the infection. Much attention has been focused on cryptic cases in an attempt to determine if the rabies variant is more infectious and/ or virulent, if the bite of the animal vector goes unnoticed, or both.

Besides canine rabies, several regions in the world are affected by other terrestrial rabies variants associated with mongooses, foxes, skunks, raccoons, and a variety of wildlife in Africa. Although rabies in red foxes has been endemic in Europe since the 1940's and continues to be a problem, wide-scale use of oral rabies vaccines has been relatively successful in reducing the numbers of rabies cases in red foxes (Nizgoda et al, 2001). In the Caribbean, rabies in mongooses has been documented since the 1950s. The introduction of rabies into mongooses and red foxes has been attributed to dogs, as demonstrated by genetic analysis of the viruses (Smith, 1992, Childs, 2001). In Africa, maintenance of rabies viruses has been documented in animal species belonging *to Viverridae, Canidae, Mustelidae,* and *Felidae* (Childs, 2001). The first report of rabies in South Africa occurred in 1893 and was ascribed to a dog imported from England (Childs, 2001). An alternative hypothesis proposed by King et al. (1994) suggests rabies was already endemic prior to 1893.

In the Americas, rabies is maintained in wild terrestrial animals including raccoons, foxes, coyotes, and skunks as described in Childs' (2001) detailed review. Documented instances of rabies were reported in raccoons and skunks within the U.S. prior to becoming endemic in Canada (CDC, 2000) and countries south of the United States. Reports of rabid foxes in the mid-Atlantic British colonies in the 1700s were likely the result of translocation of rabid foxes and dogs from Europe and may have been present long before large scale outbreaks were recorded (Niezgoda et al., 2001). In the 1940s, rabies in foxes was documented in arctic and red foxes in northern Canada. Twenty years later the outbreak progressed south to the United States.

The rabies variant infecting coyotes in the United States is closely related to the rabies variant infecting domestic dogs in Latin America. The original source of this variant is under debate; did rabid dogs infect coyotes or were rabid coyotes the source of rabies in domestic dogs? The variant of rabies infecting coyotes in southern Texas in recent years is believed to have originated with rabid Mexican dogs as demonstrated by antigenic and genetic analysis of the infecting viruses (Childs, 2001; Clark et al., 1994). Currently there are eight terrestrial rabies variants that are endemic in the United States: three variants of skunk rabies, three variants of fox rabies, and one variant of both coyote and raccoon rabies.

Rabies vaccines have been one of the most important tools in fighting terrestrial rabies. Prior to the 1940's, approximately 100 humans contracted and died of rabies every year in the United States, typically the result of exposure to rabid dogs (Childs, 2001). Since the inception of large scale vaccination programs in the United States during the 1950's, the number of human deaths due to canine rabies has dropped dramatically and are practically non-existent in the US today (Childs, 2002). During the early1950's, approximately 10 to 20 humans died every year as a result of canine rabies. By the 1960's, an average of one human died yearly of canine rabies and during the last fifteen years, two humans have died of canine rabies. This decrease in human rabies cases directly attributed to dogs is a result of education and dog vaccination (Childs, 2001).

The raccoon rabies epidemic in the eastern U.S. began in Florida during the 1940's and in the year 2000, raccoons were deemed the most important rabies reservoir in the U.S. (Niezgoda, 2002). During the past 60 years, during which time raccoon rabies spread throughout the Eastern-Atlantic states, no human cases were ascribed to this

variant, possibly due to its less virulent character (Jones, 2002). In 2003 a young man died of raccoon rabies but denied any exposure to raccoons. Although initially suspected as a "cryptic" rabies case, it is believed he remained outdoors all night following his bachelor party in a rugged mountain setting where he could have been exposed to a rabid raccoon with no memory of the incident (Robert Rudd, personal communication 2003).

The current status of terrestrial rabies in the United States is depicted in Figure 1. The spread of raccoon rabies continues northward into Canada and westward into Ohio. Control of raccoon rabies has been attempted by oral rabies vaccine with variable success. The oral rabies vaccine has also been somewhat successful in controlling fox and coyote rabies. The current oral rabies vaccine has had little impact on controlling rabies in skunks (Childs, 2001) and research is currently underway to develop efficacious oral rabies vaccine for these animals (USDA, 2002).

Although the incidence of rabid dogs in the US has decreased substantially in the last forty years, rabies in domestic cats has been increasing (Niezgoda et al., 2002). Most states require that dogs are vaccinated against rabies where as half the states surveyed require vaccination for domestic cats (Briggs, 2001). Between 1992 and 1998, approximately 272 cats were diagnosed with rabies, most often the result of terrestrial rabies (Niezgoda et al., 2001). In 11% of affected cats, the infecting virus was typed as a chiropteran variant. With little evidence, Tuttle (2003) suggested that cats are the vectors involved in "cryptic" cases of human rabies infections, playing a "middleman" role between bats and humans. McQuisten et al. (2001) tested 78 rabid dogs and 230 rabid cats submitted to public health laboratories during 1999. All dogs and all but one of the cats were infected with a terrestrial rabies variant. One cat was infected with the big

brown bat variant. Their work suggests that rabid cats are an unlikely rabies vector in the human cryptic rabies cases.

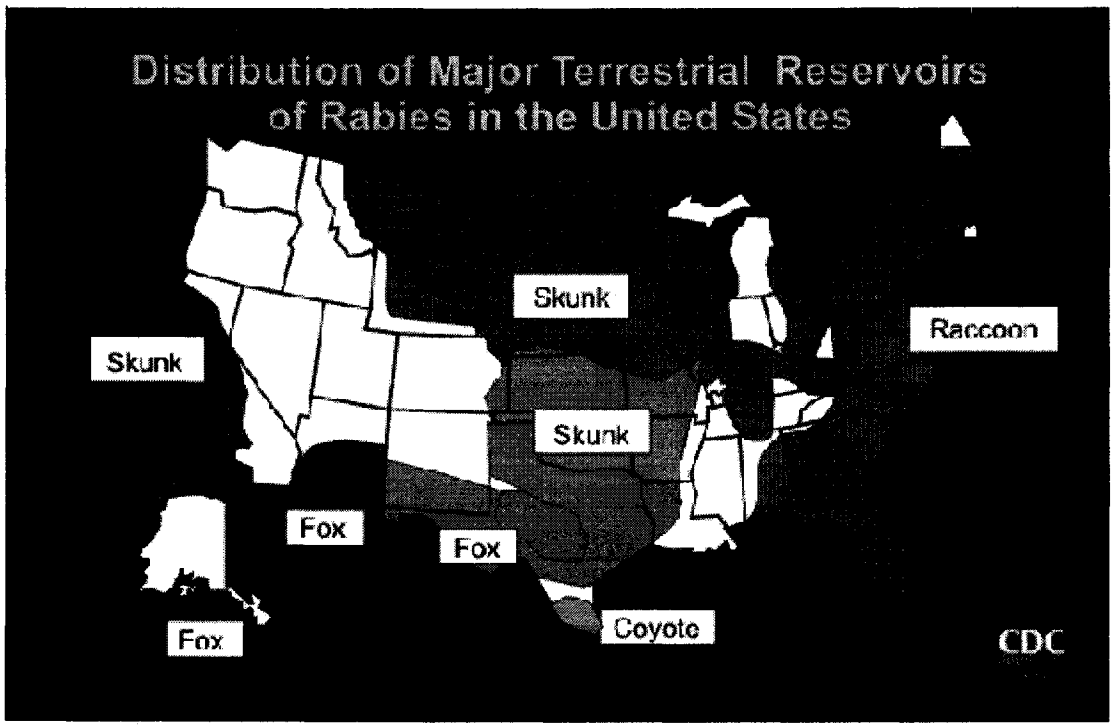

Figure 1. Map of terrestrial reservoirs within the U.S. courtesy of the CDC Rabies Laboratory (www.cdc.gov).

Spillover rabies infections were seen in 2001 during an outbreak of skunk rabies in Arizona (Leslie et al., 2006). Since Arizona is not known to have skunk rabies, the CDC became involved and began typing the variant circulating in the rabid skunks (Smith, 2001). The variant found in the skunks was determined to be the same variant associated with the southwestern big brown bats (*Eptescus fuscus*), but demonstrated different patterns of viral excretion and tissue tropism in skunks than in bats. Hughes and Rupprecht (2002) found the amount of virus in the brains of skunks infected with the *E. fuscus* variant to be similar to skunks infected with a skunk variant. Conversely, they

found the amount of virus in the saliva and salivary glands of skunks to be much less when the skunks were infected with an *E. fuscus* variant than with the skunk variant. Hughes and Rupprecht (2002) also found more nucleotide sequence diversity in the rabies genome from skunks infected with the *E. fuscus* variant, suggesting the beginning of adaptation to a new host or a bottleneck.

Rabies in hematophagus bats was first documented in the 16th century and up until 1953, it was believed that only hematophagus bats were capable of transmitting rabies (Brass, 1994). In 1953, a child was bitten by a yellow bat, the bat was killed, and submitted to the Florida Department of Health where it tested positive for rabies. This was the first incidence of rabies virus documented in insectivorous bats. Since then, samples from 30 of the 39 species of bats in the US have tested positive for rabies.

Five bat variants have been documented in human infection in the U.S. over the last fifteen years. The variant associated with the eastern pipistrelle bat, *Pipistrellus subflavus* , has been implicated in 14 human deaths, the Mexican free-tail bat, *Tadarida brasiliensis,* variant in ten, and the silver hair bat, *Lasionycteris noctivagan,s* variant in six. The eastern pipistrelle *(Pipistrellus subflavus)* is a solitary tree dwelling bat and the number of deaths caused by this variant is a conundrum since humans are highly unlikely to come into contact with this species (Messenger et al., 2003 and McQuisten et al., 2001) Conversely, contact between *Tadarida brasiliensis* and humans is common as they may be found in large numbers in and around human dwellings throughout the year, yet the *T. brasiliensis* variant has been implicated in fewer deaths than the *P. subflavus* variant.

Lyssavirus infections in chiroptera have been documented in several countries over the last fifty years (Childs, 2002). Two lyssaviruses, European bat lyssavirus (EBLV) I and II are found in western and eastern European countries, most frequently in *Eptescus serotinus*. Australian bat lyssavirus (ABLV) is found only in Australian fruit bats. Lagos bat virus has been found in *Epomorphorus* spp. in several African countries. Duvenhage virus has been isolated from *Nycteris* species and *Miniopterus schreibersii* bats in South Africa (Childs, 2002, Smith 2002).

1.2 THE VIRUS

Classification

Rabies virus is classified in the order mononegavirales and is a member of the Rhabdoviridae family. Rhabdo, Greek for "rod shaped", describes the bullet shape of all rhabdoviruses. There are over 200 rhabdoviruses that can infect humans, non-human vertebrates, plants, and insects including vesticular stomatitis virus (VSV), bovine ephemeral fever virus, and potato yellow dwarf virus. Lyssaviruses are one genus in the family Rhabdoviridae. Lyssa, Greek for "frenzied" or "madness", describes a manifestation often displayed during clinical illness of rabies. In Sanskrit rabhas means "violence" and subsequently may have been translated into the Latin term rabhere which means "to rage or rave" (Wilkinson, 2002). Today we often associate clinical rabies infection with a "furious" illness and have seperated the clincial signs of rabies infection into two categories, furious rabies and paralytic rabies.

Within the genus Lyssavirus there are seven well documented genotypes: (1) rabies virus, (2) Lagos bat virus, (3) Mokola virus, (4) Duvenhage virus, (5) European bat

lyssavirus 1, (6) European bat lyssavirus 2, and (7) Australian bat lyssavirus. Each genotype can be distinguished by nucleotide sequence analysis. More recently, it has been proposed that some of the newly discovered lyssaviruses, including Aravan and Khujad, be placed in a novel lyssavirus genotype (Kuzmin et al., 2005, Arai et al., 2003, Kuzmin et al., 2003). The current rabies vaccination protocol is effective in protecting against all the major genotypes except Mokola virus and Lagos bat virus. Currently, no effective vaccine exists for Mokola and Lagos bat virus and it is unknown if the rabies vaccines are effective against the Aravan virus or Khujad virus.

Virus Structure and Genome

Lyssaviruses are bullet shaped viruses approximately 180 nm long and 75 nm wide (reviewed by Wunner, 2002 and Koprowski, 1991). Lyssavirus virions are composed of a helical nucleocapsid encased in a lipid membrane derived from the host cell and embedded with viral glycoprotein (Wunner, 2002). The ribonucleocapsid consists of the non-segmented, negative-sense, single-stranded RNA genome surrounded by nucleocapsid proteins, phosphoproteins, and the viral RNA-dependent RNA polymerase. The glycoprotein trimers are transmembrane proteins that interact to matrix proteins lying directly beneath the viral envelope. The lyssavirus genome encodes five proteins: nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and polymerase (L) all in negative-sense RNA polarity ~11 kb in length (reviewed by Wunner, 2002 and Koprowski, 1991) (Figure 2). There is a 3' leader region, a 5' trailer and between each of the coding regions are intergenic non-coding regions. There are two bases between the N and P genes, and five separating the P, M, and G genes. The G and L genes are separated by over 400 nucleotides including a pseudogene that lacks

an open reading frame. The pseudogene is the most divergent area of the lyssavirus genome and at one point may have encoded a protein (Wunner, 2002).

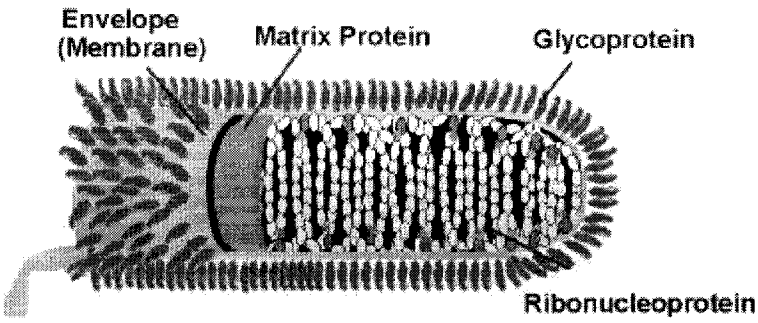

Figure 2. Lyssavirus structure and proteins. Courtesy of CDC rabies laboratory. www.cdc.gov/rabies

Rabies virus proteins

The G proteins are synthesized on membrane-bound ribosomes while N, P, M, and L are synthesized on ribosomes free in the cytoplasm. The anti-genome serves as the template for the production of viral progeny RNA that are encapsidated by N and P proteins. The L protein is incorporated into this nascent structure forming the RNP followed by the addition of the M protein. The trimeric G protein becomes embedded in a host cell membrane and extends from the plasma membrane of the infected cell during budding to form virions (Gaudin, 1992).

The N gene of lyssaviruses is composed of 1350 nucleotides. The N protein, composed of 450 amino acids, is the virus group-specific core antigen. The N protein, an integral protein in the internal helical nucleocapsid, is the most conserved of all the structural genes (reviewed by Wunner, 2002, Bourhy et al., 1993, 1992). This important protein protects the RNA template from ribonuclease activity, regulates RNA

transcription, and is believed to be the only known viral super antigen (Lafon, 1992). Large amounts of N protein become concentrated in infected cells and complex with P protein to form (Negri) bodies (Wunner , 2002)

The phosphoprotein interacts with N protein and functions as a regulatory protein within the RNA polymerase complex. This protein is composed of 297 amino acids and is well conserved, although not to the same extent as N. Phosphoprotein acts as a chaperone of soluble nascent N protein, plays a role as a cofactor in transcription and replication, may be integral in forming a protein-dynein LC8 complex for transportation along the microtubules in neurons, and stabilizes the L protein. Oligomerization of rabies virus P does not require phosphorylation (Wunner 2002, Curran et al., 1995).

The matrix protein (M) is the smallest protein. The M protein is composed of 303 amino acids and interacts with other viral proteins to give rabies virus its bullet shaped appearance (Wunner, 2002 , Lyles, 1996). This multifunctional protein interacts with the RNP, down-regulates transcription, is associated with the membrane bilayers, and is involved with the cytopathology of rabies infected cells (Ahmed and Lyles, 1998). The M protein is associated with cell to cell transmission; it initiates budding from the plasma membrane of the infected cell and is integral in exocytosis of the virion from the cell (Harty et al., 1999). Recently, the M region of the genome was implicated as a virulence factor, although not to the same degree as the G protein (Dietzschold et al., 2005; Morimoto et al., 2000).

The large protein (L) is also known as virion-associated RNA polymerase (Wunner, 2002). It is encoded by the fifth and largest gene comprising 54% of the coding genome. The L protein is composed of approximately 2135 amino acids but

varies among viral variants. The L protein is the catalytic component of the polymerase complex and, in complex with the P protein, is implicated in many enzymatic activities involved in viral RNA transcription and replication. This protein is less conserved than N or P but does contain domains with a high level of conservation in areas which are involved in catalyzing nucleotide addition (Wunner 2002, Fu et al., 1994).

The G protein has been shown to be the most important protein in conferring virulence of rabies virus (Dietzschold et al., 2005; Takayama-Ito et al., 2005; Faber et al., 2004; Wunner 2002; Gaudin et al., 1996). The glycoprotein is the second largest protein at 505 amino acids in length and is not highly conserved (reviewed by Wunner, 2002). The glycoprotein is integral for viral entry into the cell by serving as the ligand for host cell receptors and is the protein that neutralizing antibodies recognize. It also interacts with the endosomal membrane causing release of the virus into the cytoplasm. The trimeric glycoprotein spikes that extend beyond the viral envelope, are the only proteins on the viral surface, and interact with host cell receptors.

Experimentally, amino acid substitutions in the glycoprotein in street rabies viruses have been shown to result in apathogenic rabies viruses (Dietzschold et al., 2005, Faber et al., 2005). The glycoprotein is involved in the cellular uptake of the virus can affect the neuronal pathways the virus takes to reach the CNS, trans-synaptic movement, and cell-to-cell spread (Dietzschold et al., 2005, Takayama-Ito et al., 2005, Prosniak, et al., 2001, Dietzschold, 1985, Kucera, et al., 1985) Direct cell-to-cell spread is important for rabies to evade viral neutralizing antibodies. Viral movements through interstitial spaces are perilous as virus neutralizing antibodies will prevent the virus from attaching to its receptors (Wunner, 2002, Flamand et al., 1993, Dietzschold et al., 1985). Morimoto

et al. (1996) found the *L. noctivagans* rabies variant to be more virulent than a coyote variant and hypothesized the amino acid in the 333 position conferred the increased virulence. Furthermore, it has been discovered that the less glycoprotein produced, the more virulent the variant, and it appears that down-regulating the production of glycoprotein may have beneficial effects for the virus (Morimoto et al.,1999, Hooper et al., 2001).

Attachment and Entry

Rabies virus typically enters the host though a bite and is deposited into the muscle tissue where it begins to replicate or directly enter neurons. Murphy (1977) identified viral replication in striated muscle near the location of intramuscular inoculation. Using immunoflouresence and transmission electron microscopy with infected hamsters as an animal model, he found single infected muscle cells that quickly progressed to infected foci of muscle cells. Virus was observed budding from the plasma membrane of infected, yet undamaged, muscle cells. Although rabies virus is a neurotropic virus, muscle tissue has been demonstrated to be highly susceptible to infection and permissive to rabies virus replication. It is believed that replication or sequestration in muscle cells accounts for the variation in incubation time (Murphy, 1977, Charlton, et al., 1996, Charlton, et al., 1997).

Following muscle cell infection, the second location of rabies antigen detection was in unmyelinated neuromuscular spindles as well as the extracellular spaces of the neuromuscular junction. Both sensory and motor nerves can become infected with rabies virus, some anecdotal evidence indicates preference towards motor neurons (Jackson, 2002).

Once the rabies virus glycoprotein attaches to the cell membrane receptor, it is internalized by endocytosis. Following entry into the neuron, fusion of the glycoprotein to the endocytic vesicle releases the nucleocapsid into the cytoplasm where replication takes place. The endocytosed virus may remain in the vesicle and be transported along the axons to the soma prior to release and replication (Lafon, 2005).

Nicotinic acetylcholine receptors (AChR) are thought to essential in rabies virus infection. In vitro studies by Murphy (1977) reported the binding of rabies virus to AChR at neuromuscular and synaptic junctions. There are different types of AChR (nicotinic and muscarinic) that can be found on skeletal muscle tissue and neurons but most accumulate at the postsynaptic membrane of neuromuscular junctions.

More recent research has demonstrated that rabies virus is capable of infecting cells lacking AChR. Two other putative rabies virus receptors have now been identified based on the ability to infect cells that lack AChR: the neural cell adhesion molecule (NCAM) and p75NTR (Thoulose et al., 1998 and Tufferuau et al., 1998).

Neural cell adhesion molecule (NCAM) is a receptor in the immunogloubulin family found on cell surfaces and concentrated in synaptic regions of the presynaptic membrane of neuromuscular junctions. Thoulouse et al. (1998) found that cells expressing NCAM were capable of being infected with rabies virus, whereas cells lacking NCAM were not. Furthermore, inducing NCAM expression on rabies virus-resistant cell lines rendered cells susceptible to infection. Neuronal cell cultures from NCAM deficient mice are highly resistant to rabies virus infection (Thoulouse et al. 1998). A third receptor, p75NTR, has a high affinity for rabies virus and data from numerous studies indicate it is a ligand for the rabies virus glycoprotein (Tufferuau et al.,

1998) Unlike ACh and NCAM, this receptor is specific for certain lyssavirus glycoproteins in lyssavirus genotypes 1 and 6. Research by Tufferuau et al, (1998) demonstrated that p75NTR is important for virus binding to the cell membrane but not involved in cell entry. Lee et al (1992) reported that mice lacking p75 were equally susceptible to rabies virus infection as wild type mice (Langevin and Tufferuau, 2002)

Replication, Assembly, and Budding

The post-entry steps in rabies virus replication have been reviewed in Jackson, (2002) and Murphy, (1977). Following release from the vesicle, the ribonucleoprotein core is released into the cytoplasm and the tightly coiled nucleocapsid relaxes to allow viral replication. Rabies virus contains a virion-associated RNA polymerase (L protein). Transcription is initiated at the 3' end of the genome RNA. The structural genes are transcribed in sequential order, N, P, M, G, and L, producing five monocistronic mRNA transcripts. A full-length, positive-sense RNA strand termed the replicative intermediate (antigenome), is transcribed and used as a template for the production of the negative-stranded genome.

Viral proteins are produced in unequal amounts. The viral polymerase pauses as it moves downstream and it is not uncommon for the polymerase to dissociate from the viral genome. Consequently, genes nearest the 3' end are transcribed with higher frequency with in the production of more N protein (Jackson, 2002). Vesicles containing the trimeric G proteins are transported to the plasma membrane to await assembly of the RNP core structure. Accumulation of N-P or N-N complexes produce Negri bodies, the diagnostic inclusion body seen in rabies virus infected tissue. Some N-P complexes are necessary to encapsudate the positive and negative RNA strands. Following the addition

of the N-P complexes onto the progeny RNA, L protein is incorporated followed by M protein that condenses the RNP into the tightly coiled viral core structure. The M protein is believed to be important to viral morphology by interacting with the G trimers on the plasma membrane and stabilizing the glycoproteins on the virion surface (Dietzschold, et al., 1983, Wunner, 2002, Faber et al., 2004, Dietzschold, et al., 2005).

Cell-to-cell transmission and movement through the nervous system

Direct cell-to-cell transmission may be the most reliable route of rabies virus dissemination within the host. The relative lack of exposure to extracellular fluid allows the virus to move unimpeded between neurons even in the presence of neutralizing antibody (Lafon, 2001). Flamand et al., (1993) and Dietzschold et al., (1985) demonstrated viral budding from the plasma membrane into the extracellular space where it may come into contact with anti-rabies viral neutralizing antibodies (VNA). This type of spread is potentially deleterious for the virus as VNA present in the interstitial space can prevent binding to another cell. Rabies virus is also capable of moving within the neuron via the microtubule network. This process transports the virus to the synapse and facilitates the transmission from the synapse to the dendrite of a recipient neuron (Wunner, 2002 Coulon et al., 1989, Kucera et al., 1985). During centripetal spread, mature virions bud off the pre-synaptic membrane, cross the synaptic cleft, and attach and enter the post-synaptic membrane of the recipient dendrite (Jackson, 2001 and Murphy, 1977). Some studies have found that rabies virus can move between neurons without entry into the synaptic cleft through fusion of the pre- and post-synaptic membranes. This is advantageous to the virus as it decreases the potential contact with VNA (Murphy, 1977, Charlton and Casey, 1979, Jackson, 2002).

Several types of studies have been used to examine the movement of rabies virus, such as limb amputation and colchicine application to cell culture systems (Tsiang et al., 1991 and Murphy, 1977). The rate of movement within the axons is between 5 and 10 mm/day (Wunner, 2002, Tsiang et al., 1991), yet it is possible that as the virus moves centrifugally, the number of infectious particles may decrease, resulting in an aborted infection (Wunner, 2002). Clinical illness following the inoculation of a limb with cell culture adapted rabies virus was averted if the limb was amputated 4-10 hours after inoculation (Baer et, al, 1968 and 1965). The incubation time may be greatly extended following inoculation with a wild type virus since these viruses may remain at the inoculation site for a longer period of time (Murphy, 1977, Jackson, 2002).

Early after entry of rabies virus into the brain, the brainstem tegmentum and deep cerebellar nuclei become infected. Infection then spreads to the cerebellar Purkinje cells, basal ganglia, cerebral cortex, and neurons in the diencephalon (Jackson, 2002). Infection of the hippocampus occurs late in experimental peripheral infection. Lesions in the hippocampal area of the brain can result in severe clinical manifestations including hallucinations, aggression, ataxia, and seizures, all of which are often seen late in rabies infection (Wunner, 2002).

Following brain infection, rabies virus spreads centrifugally from the brain to peripheral sites along neural pathways. The most important event required for rabies transmission is infection of the salivary glands. Several nerves innervate the salivary glands (Wunner, 2002). Although uncommon, it has been reported that viral titers in the salivary glands may reach higher levels than in brain (Wunner, 2002). Dean et al (1963) found that unilateral removal of the lingual nerve and cervical ganglion in experimental

animals substantially decreased the viral titers in the denervated salivary gland as compared to the innervated salivary gland.

In 1983, Charlton et al. reported that salivary gland infection in skunks occurred via numerous axon terminals, not through epithelial cell spread. It is unknown if virus titers reach high levels in the salivary glands of animals though infection and replication within the epithelial cells or if the virus present in the infected epithelium is solely the result of neuronal transport (Charles E Rupprecht, personal communication 2003).

As virus disseminates from the CNS, some organs are more likely to be infected than others: the adrenal glands, cardiac ganglia, tongue, and nerve plexuses in the gastrointestinal tract often become infected during the later stages of infection. Rabies virus antigen may also be present in the lungs, pancreas, liver, bladder, skeletal muscle, and hair follicles. In these non-neural tissues, antigen may be more associated with innervating fibers than the cells themselves. (A. Davis 2000, unpublished data and Trimarchi et al, 1986). Transmission of rabies virus has been associated with corneal transplantation on several occasions between 1978 and 1994 (Jackson, 2002, Gode and Bhide, 1988). More recently, there have been two incidences where rabies virus was transmitted though solid organ transplant. The first event occurred in May 2004, when four individuals who received organs from the same donor died of rabies. The donor was a young man diagnosed with subarachnoid hemorrhage (Srinivasan, et al., 2005). One recipient received a liver, two received kidneys, and one received an arterial segment. All developed clinical symptoms compatible with rabies infection, subsequently died, and rabies was confirmed by immunofluorescence. The donor was diagnosed following the rabies diagnosis of the four recipients. During the initial stages of clinical rabies virus

infection, the donor reported a history of a bat bite to friends. Genetic analysis implicated a Mexican free-tailed bat-like variant.

The lack of viremia in rabies pathogenesis has presented a number of questions concerning the transmission of rabies via solid organs. It is likely solid organs became infected with rabies virus via centrifugal dissemination as nerves associated with the organs are commonly infected (Srinivasan et al., 2005). Virus from the donors' organs or nerves likely came into contact with susceptible tissues in the recipient.

Transmission of rabies virus

With few exceptions, rabies virus is transmitted through the bite of a rabid animal. Some references state simplistically that "virus laden saliva" is introduced into the muscle after a bite wound, but the probability of transmission occurring is complex and dependent on several factors. The dose of virus inoculated through a bite must be important, but the actual amount of virus delivered is not known with any precision for any species. The amount of virus would likely differ substantially between the bites of different animals; say between a bat and a raccoon. Another factor that affects the dose delivered through a bite is the degree of virus replication that has occurred or is occurring within salivary glands. Another factor that influences the dose delivered thorough a bite is the characteristic of the bite and whether it is an isolated "nip" or involves deep penetration with prolonged chewing (Jackson, 2002).

A factor more important to transmission than absolute dose may be the variant of the inoculated virus. By comparing a variant associated with coyotes to a silver hair associated bat variant, Morimoto et al., (1996) found that not all rabies variants are of similar virulence. Indeed, one of the most important factors in viral transmission may be

the rabies variant to which the animal is exposed (Jackson, 2005, Willoughby et al., 2005). Messenger et al., (2003) reported a large number of terrestrial animals infected with the silver hair bat (*L. noctivagans)* and Eastern pipistrelle (*P. subflavus)* variants when compared to the number of terrestrial animals infected with a terrestrial. One may expect that since terrestrial animals would have more contact with other terrestrial wild animals rather than solitary tree dwelling bats, a terrestrial variant would be the more reasonable culprit to result in rabies virus infection. This pattern is similar to the pattern demonstrated in the human rabies cases in the US as the variant associated with the *L. noctivagans* and *P. subflavus* bats were the infecting variant in 56% of the human cases.

Current guidelines dealing with a bite from an unvaccinated dog state that if healthy at the time of exposure, the biting animal should remain in strict quarantine for 10 days (Rabies Compendium, 2006). If the dog remains healthy throughout the 10 days, the assumption is that the animal was not excreting rabies virus in saliva at the time of the bite. This assumption may seem problematic because of variations in the incubation periods and the period of virus shed in saliva varies relative to the onset of clinical signs. The bite may have occurred during the incubation period and the dog could develop clinical illness at a later point, and viral excretion may begin 10 days prior to symptoms. The research supporting these guidelines was published in 1961 and indicated that excretion of live virus in the saliva of dogs does not occur more than seven days prior to clinical illness (Reviewed by Vaughn, 1965, Vaughn et al., 1961). More recent studies with experimentally infected dogs demonstrated excretion of virus 13 days prior to clinical illness (Fekadu et al., 1982). This later study also provided additional information on the dynamics of rabies virus transmission. First, Fekadu et al., (1982)

found that inoculation of large amounts of virus was more likely to result in clinical rabies virus infection and a short incubation time as compared to a lower dose that lengthens the incubation time. Second, they reported that inoculation with a lower dose of virus decreased the mortality rate but increased the percentage of infected animals with infectious saliva suggesting that inoculation with a higher dose of rabies virus may result in the death of the host prior to centrifugal spread to the salivary glands (Fekadu et al., 1992)

Aghomo and Rupprecht (1990) isolated rabies virus from the oral swabs taken from 4 of 1500 (0.03%) healthy, previously unvaccinated dogs presented for routine veterinary examinations in Nigeria. However, no follow-up exam was performed on any of the 1500 dogs and it is unknown if any these dogs developed clinical rabies after leaving the clinic. Although this paper reports the presence of infectious virus in saliva in healthy dogs, no conclusions can be drawn as these animals were not followed for 10 subsequent days.

Niezgoda et al., (1998) published their findings on the relationship between the experimental dose and excretion of rabies in the saliva. They were unable to isolate virus from oral swabs of three ferrets with viral antigen in their salivary glands. They also found that animals that developed rabies following inoculation with the lowest dose of rabies virus failed to have virus in their saliva or salivary glands, whereas animals inoculated with higher doses produced more viral antigen and higher amount (LD_{50}) of virus are excreted in saliva.

Sikes (1962) determined that the amount of rabies virus in the saliva of skunks and foxes varied substantially. Following experimental intracranial inoculation of a fox rabies virus variant into foxes and skunks, he measured the amount of virus in the saliva. Five of nine

foxes and one of thirteen skunks produced less than 10^2 50% mouse lethal doses (MLD_{50})/30µl, and two of nine foxes and three of thirteen skunks produced between 10^2 and 10^3 MLD_{50}/30µl. Two of the nine foxes and nine of the thirteen skunks produced between 10^3 and 10^6 MLD_{50} /30µl in their saliva. In foxes, higher inoculation doses resulted in less infectious virus in the saliva: lower inoculation doses resulted in more infectious saliva. Conversely, in skunks, the lowest inoculated dose produced no illness or infectious saliva.. Furthermore, increasing the dose of virus in the inoculum did not increase the amount of virus in the saliva, as dosages between 1.4x 10^3 to 1.4x 10^5 MLD_{50}/30µl resulted in approximately the same number of skunks excreting infectious rabies virus in their saliva. This paper illustrates the number of variables involved in rabies transmission includes the variant type, infected host species, "target" host species, and duration of excretion of virus in the saliva. Another important finding of this work was that skunks were 100 times less susceptible to rabies virus infection when a fox variant was employed as compared to a skunk variant which supports the hypothesis of a host species barrier which as been demonstrated in other studies (Sikes, 1962).

Sikes (1962) reported that a large inoculum of the fox rabies variant is more likely to result in the death of experimentally inoculated foxes prior to centrifugal spread to the salivary glands. Conversely, when the virus was inoculated into a non-host species, more virus was demonstrated in the salivary glands rather than resulting in the death of the animal. The amount of fox virus variant inoculated into skunks was proportional to the number of skunks with virus in their saliva. The possibility of host death prior to centrifugal spread to the salivary glands suggest that host species exposed to large

amounts of virus may become dead end hosts, although this may not be true for non-host species. This demonstrates different viral characteristics may be partially host dependent. Hughes and Rupprecht (2001) illustrated the concept of selective pressure on quasispecies evolution. During a 2001 outbreak of skunk rabies demonstrated to be the result of an infection with a big brown bat (*Eptescus fuscus)* variant, the virus exhibited different characteristics when comparing the host species (*E. fuscus* bats) to the spillover cases (skunks). The concentration of virus isolated from the salivary glands of skunks was much less than the amount of virus in the brain. The amount of virus isolated from the brain of non-host species was similar to that of the host species. The results of this research are in contrast to those reported by Sikes (1962), which demonstrated a higher rabies virus titer in the salivary glands of the non-host species than in the host species. Furthermore, Hughes and Rupprecht (2001) reported an increase in nucleotide sequence diversity within the virus found in the salivary glands of the skunks as compared to the bats. They suggested this diversity may indicate spillover from bats to skunks followed by adaptation by this virus to the new host.

Spillover of rabies virus from vampire bats to cattle occurs in Latin America with some frequency. This variant has never established itself within the cattle population, as it is unlikely the anatomy and physiology of cattle could sustain a transmission cycle. Unlike in the study by Sikes (1962), infectious saliva has rarely been reported in cattle infected with vampire bat rabies virus.

In Nicaragua alone, more than 10,000 cattle die of vampire bat rabies virus each year. The economic impact of infected cattle exceeds a million dollar and the number of humans that come into contact with rabid cattle makes it a potential national and

international public health problem (Brass, 1994). These numbers are considerably higher when including other Latin American countries. Despite the large number of cattle that die of rabies and the humans exposed to infectious saliva only one human death has been attributed to this type of exposure (Delpietro et al., 2000). Two hypotheses have been suggested for the lack of human deaths attributed to cattle. First, many of the individuals exposed to rabid cattle are farmers who are uneducated about the risks of rabies, unable to afford the health care, or both. Regardless of the reason, it has been suggested these individuals are unlikely to receive medical care following exposure, and it would be unlikely their death would be confirmed in the laboratory and officially recognized as a case of rabies.

Alternatively, vampire bat rabies in cattle may result in a rapid death; not allowing time for centrifugal spread to the salivary glands thus not exposing farmers to infectious virus. Delpietro et al. (2000) tested 87 pairs of salivary glands from naturally infected cattle and four (5%) contained rabies antigen, yet out of the 62 saliva samples tested, virus could be isolated from only one of the oral swabs (<2%).

Bell et al. (1965) reported isolating rabies virus from the salivary glands of 86% of naturally infected bats and virus was isolated from the saliva of 74% of clinically rabid bats. Fifty-two percent of the bats bitten by clinically rabid bats developed clinical rabies virus infection. Furthermore, Bell et al. (1965) reported that some rabid bats survived for more than 10 days after first shedding infectious virus. This finding is important as a 10 day quarantine period established for dogs may not be adequate for other animal species, including bats.

There have been reports suggested testing saliva for viable rabies virus or viral RNA as an alternative to traditional rabies diagnostics although rabies virus is not found in the saliva regularly during the course of clinical infection (Echevarria et al., 2001). Intermittent excretion of virus in the saliva of infected animals has been documented with dogs by use of the mouse inoculation test (Fekadu, 1981, 1980). Viral excretion in the saliva may terminate any time prior to death, occur intermittently during the course of infection, and continue until the animal succumbs to infection, or may not occur at all. Antibody clearance of virus from the salivary glands has been a proposed reason for the lack of continuous viral excretion in the saliva (Vos, et al., 2004, Fekadu, 1981, 1980) and may explain the termination of excretion prior to death. In a study by Baer and Bales (1966), rabies virus was detected in the saliva of experimentally infected Mexican free-tail (*Tadarida brasiliensis*) bats between 0 and 15 days prior to clinical illness. They reported a lack of virus in the salivary glands upon death of three bats that had secreted virus in their saliva 5 to 8 days prior to clinical illness. In these animals, virus was detected in the saliva the day the animals were observed to be clinically rabid.

Vaughn et al. (1965) inoculated dogs with either a dog or fox rabies virus variant in the masseter or deltoid muscle. Forty-nine percent of the 110 dogs developed clinical rabies virus infection and rabies virus antigen was found in the salivary glands of 61% of the rabid dogs. Rabies virus was isolated from the salivary glands of 48% of dogs that developed clinical rabies. The presence of rabies virus antigen in salivary glands was much greater in dogs that developed furious rabies (90%) as compared to dumb rabies (50%). Furthermore, they found that rabies virus could not be isolated from all of the salivary glands in which rabies antigen was detected, but they were able to isolate virus

from several salivary glands that did not contain rabies virus antigen by the use of direct immunoflorescence test. Other studies have reported the presence of Negri bodies in the salivary glands of 70% of naturally infected dogs tested (Vaughn, et al., 1965).

Fekadu (1982) reported that 65-70% of salivary glands from rabid animals were positive for rabies antigen, 13% of which had asymmetrical infection. Moreover, as the period post-inoculation increased, so did the titer of virus in the salivary glands.

Balachardran and Charlton (1994) examined the infection of non-nervous tissue in skunks and foxes. Through the use of immunohistochemical techniques and electron microscopy, they reported viral antigen in salivary glands, adrenal glands, and the cornea. They also report viral replication producing large amount of viral nucleocapsid in infected cells of the adrenal and nasal gland but the production of low to moderate amounts of infectious virus. Conversely, in the salivary glands a lesser amount of nucleocapsid is produced and higher viral titers are reported. Their conclusion was that tissues, which accumulated less nucleocapsid, were more likely to have high viral titers.

The onset of viral shedding in the saliva has been determined in certain species of experimental animals, including dogs. Vaughn et al. (1956) infected numerous dogs and documented the presence of live virus in the saliva by virus isolation. They found that virus shedding began within 7 days prior to the onset of clinical illness, and the 10-day quarantine period was developed following this research. The idea was that if a clinically normal dog was incubating rabies and shedding virus in the saliva, it would become clinically rabid within 10 days. If the animal did not become clinically ill within 10 days, the animal was not shedding virus when the bite was inflicted. This does not mean that the animal was not incubating rabies when the bite was inflicted, just that virus was not

being shed in the saliva; but possibly developing clinical rabies at a later date. Although Fekadu et al. (1982) demonstrated that in rare instances virus may first appear in the saliva after the 10 day quarantine period; it is much more likely to occur within the 10 day window and this continues to be the recommended quarantine period by the Compendium of Animal Rabies Prevention and Control (2006).

In two studies, rabies virus RNA has been detected in oral swabs of non-infected seropositive animals (East et al., 2001, Echevarria et al., 2001). This has led to some speculation of a carrier state. Detecting virus in oral swabs has also been suggested as an antemortem diagnostic test in endangered animals (Echevarria et al., 2001), yet live virus could not be isolated from the saliva specimens in which RNA was detected. East et al. (2001) was able to isolate rabies virus from a small percentage of the RNA containing samples. The inability to isolate virus in cell culture demonstrates a lack of infectious virus in the saliva or difficulties in laboratory techniques. Without the presence of live virus in the saliva, transmission would not occur. An explanation for this observation may be that the animals may have been exposed to rabies via ingestion or grooming, including oral contact with a rabies virus infected animal (Vos et al., 2004). Alternately, it may indicate viral clearance in a previously exposed animal (Rupprecht, 2004 personal communication). It may also represent a false positive test, possibly resulting from laboratory error. Although interesting, this study does not provide convincing evidence of a carrier state.

1.3 EVOLUTION OF RABIES VIRUS

Viral Evolution

It has been hypothesized that rabies virus was originally a pathogen of bats that spilled over into terrestrial mammals thousands of years ago (Niezgoda et al., 2002; Brisbane and Tordo, 2001). Evolutionary research on currently circulating rabies virus variants is based on molecular clocks and genetic analysis. According to work by Badrain and Tordo (2001), lyssavirus genotypes 1,4,5,6,and 7 originated from genotype 2, the Lagos bat virus. This initial spillover incident occurred around 5,000 years ago. Approximately 4,000 years ago classical rabies virus began circulating within the Americas, presumably in carnivores. The first rabies virus was documented in carnivores over 4000 years ago in Mesopotamia but this is not the same variant identified today. Badrain and Tordo (2001) suggested this original genotype 1 lyssavirus became extinct. Approximately 1,500 years ago, a spillover of chiropteran rabies into carnivores produced the current canine variant. The origin of the rabies variant infecting dogs in Mesopotamia is unknown, but is presumed to be of bat origin (Badrain and Tordo, 2001).

Some researchers taking a less technical route contend there is a very remote chance a dog would transmit rabies to a bat though a bite since a dog bite is likely to be lethal to a bat. It is much more conceivable that a bat would inflict a non-traumatic bite on a dog, at the same time exposing the dog to rabies virus. The debate concerning this "chicken and egg" origin of rabies virus has not been fully elucidated.

Lyssaviruses have also been hypothesized to be arboviral in origin. Three rhabdoviral arboviruses, Obodhiang, Rochambeau and Kotonkan, are closely related to rabies viruses. Aitken, et al. (1984) reported the ability of Mokola virus to replicate in *Aedes aegypti* mosquitoes. By the use of fluorescent antibodies, they found Mokola virus antigen in salivary glands and nervous tissue of *Ae. aegypti*. Although documented

transmission of Mokola virus to baby mice via *Ae. aegypti* was also reported, as was transovarial transmission; Mokola virus replicates much more slowly in mosquitoes than traditional arboviruses (Aitken et al., 1984). It has been hypothesized that the virus preceding current genotypes 1-7 was an arbovirus that became adapted to bats. The incipient chiropteran arbovirus may have evolved into Mokola, from which Lagos bat virus arose (Badrain and Tordo, 2001, Aitken et al., 1984).

In the US, almost all cases of human rabies are attributed to a bat variant (Rabies Compendium, 2006 Willoughby et al., 2005,). Rabies in vampire bats dates back to 1911 when vampire bats were linked to rabies epidemics in terrestrial mammals such as Brazilian cattle (Hughes et al., 2005 Childs, 2002 Brass, 1996). The first reported cases of human rabies resulting from vampire bat variant came two decades later (Pawan, 1936). The first case of rabies in an insectivorous bat was reported from Florida during 1953 (Irons, 1957). It was believed that transmission to insectivorous bats occurred though exposures from a rabid vampire bat. Subsequently, hypotheses were made linking the lineages of all US insectivorous bat rabies variants to vampire bat rabies but genetic analysis does not support this theory (Hughes et al., 2005, Smith 2002). In fact, the vampire bat lineage is more distant than the variants currently circulating within the US, with the exception of the Mexican free-tailed variant (Smith, 2002). The Mexican free-tailed variant is closely related to the vampire bat variant as well as to viruses from other species of South American free-tailed bats. Constantine (1966) reported cohabitation between vampire bats and Mexican free-tailed bats and documented vampire bats occasionally feeding on Mexican free-tailed bats. It is quite possible that rabid vampire bats biting Mexican free-tailed bats led to the origin of the virus variant now found in

Mexican free-tailed bats. Like the vampire bat variant, the Mexican free-tailed variant is not thought to be the origin of the rabies variants circulating in North American insectivorous bats (Hughes et al., 2005, Smith, 2002). The original source of insectivorous bat rabies in the US is unknown.

Evolution of more virulent bat viruses has been proposed by several investigators (Dietzschold et al., 2005, Messenger et al., 2004, Morimoto et al., 1996). As mentioned previously, the majority of human rabies deaths in the US are the result of three bat variants, the silver hair bat, (*L. noctivagans*), eastern pipistrelle, *(P. subflavus)*, and Mexican free-tailed (*T. brasiliensis)* variants. Morimoto et al., (1996) found the *L. noctivagans* variant capable of replicating at lower temperatures as compared with a coyote variant. They also reported the ability of this virus to replicate robustly in epithelial and fibroblast cell lines, a characteristic not seen in the coyote variant. Conversely, the coyote variant was more neurovirulent when inoculated intranasally or intramuscularly but the coyote and *L. noctivagans* variants were similar in neurovirulence when inoculated intracranially or intradermally. This suggests the *L. noctivagans* variant has adapted to subcutaneous or intradermal transmission routes but studies comparing the *L. noctivagans* variant to either the *P. subflavus* or the *T. brasiliensis* have not been done.

Messenger et al. (2004) examined 98 rabies viruses from terrestrial animals in areas where human rabies cases could be attributed to the silver hair bat (*L. noctivagans)* variant. Human rabies cases due to the variant associated with *L. noctivagans* were most often found in the northern part of the U.S. Additionally, 57% (n=10) of the rabid terrestrial animals in the northern U.S. infected with a bat variant were infected with the *L. noctivagans* variant. In the eastern US, most human rabies cases are associated with

the eastern pipistrelle (*P. subflavus)* variant, which accounts for 63% (n=18) of the terrestrial animals infected with a bat variant in the eastern part of the U.S.. The number of terrestrial animals infected with the Mexican free-tailed (*T. brasiliensis)* variant was similar to that of the *L. noctivagans* variant. They found that approximately 61 of the 98 rabid terrestrial animals were infected with a variant not commonly identified in human cases. Rabies virus variants associated with big brown bat (*Eptescus fuscus*) and the small brown bat (*Myotis)* species were the most commonly identified rabies variants in terrestrial animals. This is not surprising as these are among the most commonly diagnosed rabid bat species in diagnostic laboratories. It is noteworthy that in the United States these variants are rarely associated with human infection (Willoughby et al., 2005).

Most commonly, animals are infected with a strain of rabies virus adapted to their species (Smith, 2002). This is true for both terrestrial and non-terrestrial animals. There have been notable recent incidents of spillovers, including a 2001 outbreak of bat rabies in Arizona skunks (Hanlon et al., 2002). Following a spillover event, selective pressures exerted on the virus may result in a more or less virulent virus. It has been proposed that the variant associated with *L. noctivagans* may be the origin of the more virulent *P. subflavus* virus, employing *Myotis* species as an intermediate host (Messenger et al., 2004). There is some support for this hypothesis as the number of *Myotis* infected with *P. subflavus* virus is much higher than any other bat species. In one study, *Myotis* sp. were infected with the *P. subflavus* virus more often than with a *Myotis* variant (A. Davis unpublished data) but more work is required employing molecular techniques to substantiate the hypothesis of *Myotis* as an intermediate host.

1.4 PATHOGENESIS

Characteristics of Virulence

The pathogenesis of rabies is unique to this fatal disease. Clinical signs of rabies virus are often severe, including aggression, ataxia, hypersalivation, and paralysis. Based on these signs, one may expect widespread cellular damage, necrosis, and apoptosis, but histopathologic lesions seen in animals infected with this virus are often quite mild.

Research has demonstrated that the amount of cellular damage induced by rabies virus is related to the infecting variant. The viral glycoprotein plays an important role in the tropism, movement, evolution, infectivity, and pathogenesis of rabies virus. The antigenic site III of the rabies glycoprotein is composed of amino acids 330-338 and is reported to an important determinant of virulence (Coulon et al., 1989). Two important amino acids are found in antigenic site III, amino acids 330 and 333. In virulent rabies variants, the position 333 amino acid is an arginine or lysine. (Dietzschold et al., 1983, Tuffereau et al., 1989, and Coulon et al., 1998). Substitution of amino acid 333 with a glutamine or methionine or alteration of lysine to asparagine renders the virus nonvirulent or non-invasive (Dietzschold et al.,1983, Coulon, et al., 1989).

Nonvirulent rabies viruses may remain neuroinvasive, although less so than virulent viruses. Conversely, some nonvirulent variants do not demonstrate the limited cellular tropism exhibited by virulent rabies variants as was demonstrated by Kucera et al., (1985). The characteristics of avirulent rabies viruses vary, from abortive infection to altered cellular tropism (Kucera et al., 1986, Strict and Kelly, 2000). By employing ocular inoculation of two noninvasive viruses, AVO1 and 2, Kucera et al, (1986) found the nonvirulent virus moved more slowly than the challenge virus standard (CVS),

infected different structures in the brain, and that infection with AVO1 and 2 cleared completely within three weeks. When comparing intramuscular inoculation of AVO1 and CVS, both were neuroinvasive and the movement of the nonvirulent virus was not impeded during transit to the CNS. However, the avirulent variant may use more restricted pathways to gain entry to the brain than CVS. A mutation in the glycoprotein is purported to be responsible for the change in viral characteristics in avirulent rabies viruses (Strict and Kelly, 2000)

The amount of glycoprotein produced during a rabies virus infection inversely correlates with the virulence of the virus. To elucidate the impact of glycoprotein production on virulence, Morimoto et al., (1999) compared two different "subvariants" of CVS-24, a mouse brain adapted variant. The two "subvariants" of CVS-24 were specified as CVS-B2c and CVS-N2c, and the N2c virus was 50% more virulent than B2c when employing the mouse inoculation test. The N gene sequences were identical between the two subvariants; analysis of the glycoprotein identified 10 amino acid differences between the two subvariants outside antigenic site III. The proportion of the two variants in CVS-24 differed depending on the cells they were passaged in; when passaged in neuroblastoma cells or mouse brain, the dominant subvariant was N2c, when passaged in BHK cells, B2c became the dominant variant. Both subvariants produced similar amounts of N proteins whereas B2c produced four-fold higher amounts of glycoprotein. Furthermore, within 24 hours, high levels of annexin V binding, indicating apoptosis, was demonstrated in B2c infected neurons; this was not seen in neurons infected with the N2c virus. Morimoto et al. (1999) proposed that the increased amount of glycoprotein expression resulted in enhanced levels of apoptosis. Increased

presentation of glycoprotein on neurons and their subsequent apoptosis impedes the spread of rabies virus to neighboring neurons, hindering viral dissemination (Morimoto et al., 1999). Lafon (2005) rejected the hypothesis that neuronal death prevents or slows the spread of rabies virus. Rabies viruses regulate the presentation of glycoprotein on neurons via proteolytic degradation of the glycoprotein (Morimoto et al., 1999). Indeed, the amount of glycoprotein produced during rabies virus infection is inversely correlated with the virulence of the virus (Takayama-Ito, M , 2006, Morimoto et al., 2000). Thus, the more virulent the variant, the less glycoprotein is expressed on the neuronal membrane. Furthermore, Jackson (2002) reported that experimental infection with a "fixed" rabies virus variant resulted in more severe cytopathic effects in infected neurons.

Morimoto et al., (2000), substituted the glycoprotein gene in an avirulent rabies virus with one from a pathogenic variant, and found that the resulting virus demonstrated a decreased level of viral virulence when compared to the parent pathogenic variant. Furthermore, the amount of viral glycoprotein produced was much greater in recombinants possessing the glycoprotein from the virulent variant than the wild type virulent variant. They concluded the rabies glycoprotein is important for neurotropism but suggested that non-glycoprotein regions of the rabies genome are also essential for virulence.

To further elucidate the viral proteins involved in virulence, Faber et al. (2004) employed reverse genetics to determine viral genomic elements responsible for neuroinvasiveness. By replacing virulent proteins with proteins from nonvirulent rabies viruses they found that the L and G proteins are integral in rabies virus neuroinvasivenss. Although the M gene was important in cell entry, it did not appear to be as important as L

and G proteins. The N and P genes were found to be essential in various functions of rabies virus but their impact on neurovirulence was minimal. It is important to note that the gold standard in genetically typing rabies viruses is by examining the N protein (Smith, 2002). By comparing the N genes between two rabies viruses, it would be difficult to make any type of comparison of virulence. Furthermore, since the N gene is the most conserved area of the rabies genome, genetic differences that may impact virulence are unlikely to be detected in this region of the virus (Smith, 2002). Conversely, the G gene is one of the most variable regions of the genome. To date, there is not a tremendous amount of information on genetic sequences of the L and G genes as well as a non coding region of the genome, Psi.

Although typically believed to be inevitably fatal once clinical signs begin, rabies virus *exposure* is not uniformly fatal. An accidental study commenced following the attack of 32 individuals by a rabid wolf in Iran in the 1950's (Gremliza, 1953). Of the 32 exposed individuals, none of whom were given treatment, 24 were bitten on the face and neck. Twelve of the 24 (50%) bite victims survived. The other eight individuals were bitten on the extremities and six (75%) survived. Laboratory animal studies have confirmed bites in the head region are more likely to result in a productive rabies infection (Shah and Jaswal, 1976).

There are a number of reasons why the 18 individuals might have survived without treatment. Exposure does not constitute infection, infection does not constitute clinical illness, and clinical illness does not always result in death. Some possible reasons accounting for the survival of exposed individuals include exposure to insufficient amounts of virus to produce clinical infection, viral variant, exposure to high

amounts of defective interfering (DI) viral particles, depth of wound, and immunocompetence of the host.

Pathologic changes during rabies virus infection

Macroscopic changes in brains acutely infected with rabies virus are typically unremarkable. Hemorrhage and tissue necrosis are not typical features of rabies virus infections, but congestion may be seen in parenchymal vessels resulting from terminal respiratory and cardiac functions (Iwasaki and Tobita, 2002). Although the incubation period of rabies may be days to months to years, the lack of reaction may be due to acute encephalitis with an abbreviated clinical course (Iwasaki and Tobita, 2002, Smith, 2002). Viral persistence in extraneural organs or infection with a non-immunogenic virus could allow the virus to remain undetected during incubation, thus not inducing a destructive immune response (Iwasaki and Tobita, 2002). Microscopic changes are more dramatic but do not correlate to the severity of clinical illness. Negri bodies are diagnostic but other histopathological changes may be present. Neuronal degeneration is often found throughout the CNS, particularly in the brainstem. Inflammatory cells are often present, typically clustered around degenerating neurons or in perivascular cuffs. Rabies virus does not affect all cell types or neurological structures the same. In a review article, Fu and Jackson (2005) reported different neuronal cell types may respond differently to the same variant. Guigoni and Coulon (2002) reported apoptosis in 90% of rabies infected purified hippocampal neurons as compared to rat spinal motor neurons that showed no major evidence of apoptosis. Furthermore, there is debate concerning the importance of the immune response in pathology of rabies virus infected cells. Lafon, (2004) found much less apoptosis in nude mice than in wild type mice.

The role of nitric oxide (NO) in rabies virus infection may be both beneficial and detrimental. The inducible form of NO, iNO, exhibits antiviral activity and has been found to increase in mice experimentally infected with street rabies variants (Jackson, 2002). The amount of iNO in the brain has been reported to increase 30-fold as clinical illness progresses when compared to non-infected controls (Fu and Jackson, 2005). However, iNO produced during rabies infection is believed to directly kill neurons by combining with O_2- producing peroxynitrate. By inhibiting iNO, death was delayed in CVS infected animals (Fu and Jacskon, 2005).

Tissue Tropism

Infection of the brain typically requires movement through the peripheral nervous system (PNS). Although rabies virus infection is typically associated with neurons, the presence of viral antigen in astrocytes, oligodendroglia, ependymal cells, and neurtropil has been reported and the type of cell infected may be a function of the route of inoculation (Jackson, 2002). Motor neurons in the spinal cord and primary sensory neurons in the dorsal root ganglia may be involved during both the early or late stages of infection (Jackson, 2002). Myelin is required for normal neuronal functioning. The infection of Schwann cells with rabies virus and demyelination of neurons has been reported (Atanasiu, 1965). Virions budding from myelinated neurons may become trapped in the space between the myelin and the neuron or may accumulate at the nodes or Ranvier (Murphy, 1977).

Experimental inoculation of rabies virus has been done employing several routes. The site of inoculation may play a role in the cell type involved during rabies virus infection, duration of incubation, and possibly the route of excretion. Inoculation via oral,

intranasal, aerosol, and intracranial administration may result in viral infection bypassing the PNS altogether, allowing direct infection of cells in the CNS (Murphy, 1977). Fischman and Schaeffer (1971) found that animals were capable of becoming infected following the ingestion of rabid mouse brain and through direct inoculation to the stomach. Rabies viral antigen was found in several different cell types following consumption of rabid material including cheek mucosa, mucosal cells of the tongue, filiform papilla and fungiform papilla of the tongue, and several nerve bundles traversing the tongue. Correa-Giron et al. (1970) reported finding virus in intestinal mucosal cells, and nervous tissue associated with the stomach and intestine contained rabies virus antigen. Nervous tissue associated with the esophagus, lungs, trachea contained virus, epithelial cells of the esophagus, lungs, and trachea did not (Correa-Giron et al., 1970). Following intranasal instillation of rabies virus, the mucous membranes in the nasal cavities were infected one day prior to brain infection. Subsequently, rabies viral antigen was detected in the olfactory bulb (Murphy, 1977). Infection of the retina occurred following nasal inoculation and resulted in a densely infected optic nerve. Following Murphy's 1977 findings, it has been suggested that rabies virus could be spread in the aerosols exiting the noses of rabid animals (Brass 1994).

Virus has also been demonstrated in the smooth muscle of the bladder but was confined to nerve cells (Sitprija et al., 2003, Jackson et al., 1999, Murphy, 1977). The detection of rabies virus antigen in the nerve cells innervating the bladder has led to some suspicion that rabies virus may be present in the urine and this may be a route of exposure. Indeed, Trimarchi and Debbie (1970) and Blancou et al., (1979) reported finding trace amounts of virus in the urine. In 1972 an outbreak of rabies in a captive

colony of skunks was suspected to be through the aerosolization of the virus from the urine, feces, and saliva yet this was never proven (Winkler et al., 1972).

The route of inoculation may also play a role in host survival and immunity. Charlton and Casey (1979) inoculated skunks to rabies virus though oral, nasal, tracheal, and intestinal routes. They found the amount of virus inoculated orally did not correlate to mortality and that less than 50% of the animals died after inoculation. Intraduodenal inoculation did not result in disease. Intratracheal inoculation produced disease in 40% of the animals. Intranasal inoculation killed 100% of inoculated animals. Following intranasal inoculation, the neuro-epithelial cells are directly exposed resulting in heavy infection of the olfactory end organ. All surviving animals were challenged with a street virus of which 33% of orally inoculated animals and 25% animals inoculated orally via forced feeding survived the challenge (Charlton and Casey, 1979). None of the animals inoculated though intratracheal, intraduodenal, or oral routes via ad libitum feeding survived challenged. These experiments indicate an effective immune response against rabies is not mounted during mucosal inoculation. Although oral inoculation typically results in low mortality, this may be correlated to the variant to which the animal is inoculated. Bell and Moore (1971) reported skunks that consumed mice infected with the *L. noctivagans* variant developed rabies; no other rabies variants tested produced clinical infection.

Neuronal dysfunction

As demonstrated in animals clinically affected by rabies virus, neuronal dysfunction is severe. The basis for neuronal dysfunction in rabies virus infection has been examined for decades but remains somewhat of a mystery. Fu et al. (1993) found a

decrease in the amount of host mRNA within infected cells as infection progressed. The lack of production of host mRNA and the suppression of host protein synthesis is likely to interfere with typical host activities. Decreased expression of proenkephalin in brain of rats infected with CVS was reported as rabies clinical infection advanced (Fu et al., 1993).

Alterations in the amounts of neurotransmitters are believed to occur in rabies virus infection (reviewed in Jackson, 2002). The amount of serotonin released and the ability of serotonin to bind to the proper receptor is impaired. In studies examining rabies virus-infected rat neuronal cell cultures, the release and uptake of GABA was impaired, and a 45% decrease in uptake observed three days following infection corresponded to peak viral growth in cultures and a decrease in active GABA transport sites (Jackson, 2002).

A recent study in association with clinically rabid human patients examined the electrophysiological changes accompanying rabies virus infection. Mirabhakdi et al. (2005) reported that axonal degeneration or peripheral nerve demyelination may occur in paralytic rabies, resulting in the observed weakness. Progressive focal denervation beginning at the location of the bite was also seen in furious rabies cases but did appear to result in weakness (Mitrabhakdi, 2005). Both showed similar lesions when employing MRI to detect differences between paralytic and furious rabies (Laothamatas et al., 2003).

Individuals infected with rabies virus typically report insomnia. Fu and Jackson (2005) reported a decrease of REM sleep cycle in mice infected with CVS virus but not in mice infected with street virus. Electroencephalography of infected mice revealed a decrease in brain activity that continued as the disease progressed. Interestingly, brain

activity stopped approximately thirty minutes prior to cardiac arrest in both CVS and street virus infected mice (Fu and Jackson, 2005).

Iwata et al. (1999) reported a decrease in the resting membrane potential in neurons infected with rabies virus. Alterations in action potentials and synaptic potentials were associated with a decrease in sodium channels and inward rectifier potassium channels. Like all organ systems, the nervous system is highly specialized and any change affecting the membrane potential is likely to result in severe dysfunction. It was suggested by Iwata et al.(1999) that infection of the CNS with rabies virus may suppress neuronal action potentials, thereby interfering in nerve-to-nerve conduction. Alterations in the ability of noradrenaline to bind to voltage dependent calcium ion channels may occur. This decrease in binding may result in some of the dramatic clinical signs seen in rabies infection, including aggressive behavior and hyper-excitability (Jackson, 2002).

Behavioral changes that accompany clinical rabies virus infection have been associated with infection of the limbic system (Johnson, 1971) although rodent models do not support this theory (Jackson 2002). Kalin (1999) reported aggressive behavior in humans to be associated with low CNS serotonergic activity and increased levels of testosterone (cited by Jackson, 2002). The behavioral changes seen during clinical rabies virus infection is often attributed to the species of the animal. For many years it was erroneously believed that rabies infection in carnivores resulted in the furious form whereas paralytic rabies infection was believed to occur in all other species, including bats. A study done by Hudson et al (1996a) reported that 70% of experimentally infected cattle and 80% of experimentally infected sheep demonstrated clinical signs associated

with furious rabies infection. Experimental rabies inoculation in horses resulted in 43% developing furious rabies (Hudson et al., 1996b). Frequently, both the furious and paralytic forms of rabies are seen in any species of animal and may be dependent on the time course of the infection. For decades, bats were believed to only demonstrate the paralytic form of rabies, yet this does not appear to be a valid assumption. Clinically rabid bats of several different species have demonstrated the aggressive form of rabies, clinical signs include attacking other bats in the colony, charging and attacking a human hand, and loud, excessive vocalization (A. Davis, unpublished data).

Early behavioral changes in bats may go unnoticed by humans whereas other bats within the colony may pick up on early subtle changes, such as alterations in vocalizations or movements. During a 2002 study examining the effects of West Nile virus inoculation in bats, one bat became isolated from its cagemates approximately 20 weeks after being brought into captivity. This bat frequently remained at the top of the roost in close proximity to food and water and appeared healthy. Other bats in the colony would not approach the food or water until this bat had moved to another area of the cage. Three days after this bat was isolated, she began vocalizing loudly, became increasingly aggressive and attacked a human hand. This bat was euthanized and found to be rabid (A. Davis, 2002, personal observation)

1.5 IMMUNOLOGY

Immune Response

The highly neurotropic nature of rabies virus provides some ability to evade the immune system during infection. Certain rabies variants lack the ability to induce a

major immune response in the host and some more virulent variants may kill the host before the immune system is able to mount a response against the virus (Hooper, 2005, Lafon, 2002). Combined, these characteristics may help the virus escape detection by the host altogether. Indeed, it is not unheard of for an animal or human to die of rabies and have no circulating anti-rabies VNAs (Lafon, 2005, 2002).

The immune component of the nervous system is unlike that of most other organ systems in the body (Steinman, 2004, Tambur and Roitberg, 2005). Although it is commonly described as immunoprivileged, the immune system does function in the CNS, but typically to a lesser degree, since immune responses often do considerable damage to the tissues (Steinman, 2004, Tambur and Roitberg, 2005, Lafon, 2002). Repressed expression of MHC class I on neurons is believed to prevent neurons from presenting foreign antigens (reviewed by Lafon 2002). Healthy neurons and glial cells downregulate the expression of MHC class I and II on their membranes. Glial cells may become antigen-presenting cells following injury or stress but cannot stimulate a primary immune response since they do not migrate to secondary lymphoid organs. Dendritic cells present in the meninges, CSF, and choroid plexus are migratory and may migrate to secondary lymphoid organs, stimulating the development of a primary immune response to rabies virus (Lafon, 2002).

Rabies virus exploits the unique relationship between the immune system and the CNS, often enabling the virus to remain undetected for a considerable period of time or to evade detection completely. Following a bite, rabies virus may or may not replicate within the muscle prior to entering the nervous system (Shankar et al., 1991). If the immune system of the host detects rabies antigen, it can mount an immune response and

clear the virus prior to entry into the nervous system (Lafon, 2004, 2002). If the virus evades the host's immune system, it may stay in the muscle for varying amounts of time prior to entering the nervous system (Jackson, 2002). Incubation times range from days to several years, but are typically between four to six weeks (Smith, 2002). Once the virus is transported into the nervous system, it is less likely to stimulate an immune response or be responsive to therapeutic measures (Lafon, 2005, 2004, and 2002). As the virus enters the CNS and brain, it replicates and is transmitted though synaptic transmission, direct cell to cell contact, or budding into the intracellular space (Fu and Jackson, 2005, Iwasaki and Tobita, 2002, Charlton and Casey, 1979). T cells play an important role in rabies virus infections. Their role has been demonstrated by studies employing various animal models including nude, immunosuppressed and T cell reconstituted mouse models. In nude mice, the lack of T cells renders them unable to resist a challenge with rabies virus or mount an antibody response following vaccination; the effect of immunosuppression with corticosteroids supports these findings (Lafon, 2002 Smith, 1981). CD4+ T lymphocytes, otherwise known as T helper cells, are necessary for the production of antibodies. Depletion of CD4+ lymphocytes in a mouse strain naturally resistant to a street rabies virus infection resulted in the inability to mount an antibody response after vaccination and death following peripheral challenge (Lafon, 2002). Furthermore, Lodmell (1983) and Dietzschold (1992) demonstrated IgG was responsible for a protective immune response whereas IgM and IgA were not.

Anti-rabies viral neutralizing antibodies (VNA) are the most important component of the immune system to protect against rabies virus infection. The VNAs are produced in response to the glycoprotein of rabies virus. The ability of the host to resist a

challenge is dependent on the quantity and quality of the VNAs. Hooper et al. (1998) demonstrated that mice lacking B cells succumbed to a challenge with an attenuated strain of rabies virus. Clearance of the virus from the nervous system via VNA has been demonstrated following infection with an attenuated virus, but the resulting paralysis was irreversible (Hooper, 1998). The presence of VNA does not always correlate with protection nor does the absence of antibodies result in lack of protection (Fekadu et al., 1992, Lafon, 1994, 2002). The lack of demonstrable VNAs usually indicates the host has had no previous exposure to the antigen. As in most viral diseases, a seropositive animal may, after varying lengths of time, revert to a seronegative state.

CD8+ T cells, also known as cytotoxic T cells, are involved in the clearance of viruses by lysing infected cells and stimulating the production of various cytokines. The importance of CD8+ T cells in rabies virus infection has been debated for years. Perry and Lodmell (1991) reported that mice lacking CD8+ T cells were not more susceptible than control mice when exposed to a street rabies virus (cited in Lafon, 2002). Moreover, the lack of CD8+ cells did not alter the survival rate of vaccinated mice following challenge. Rabies virus specific cytotoxic T cells did not protect animals from rabies virus challenge if they were deficient in neutralizing anti-rabies antibodies (Celis, 1990). In some cases, cytotoxic immunity was induced by nonvirulent variants, whereas virulent variants did not induce cytotoxic T cells (Lafon, 1994). Following infection with an attenuated rabies virus, control mice were able to clear the infection within 21 days post infection (Hooper et al., 1998). Conversely, mice lacking B cells, or B and T cells, were unable to clear the CNS infection (Hooper et al., 1998). Mice lacking CD8+ T cells were able to clear the virus but more slowly than control mice, suggesting that CD8+ T cells

accelerate viral clearance (Hooper et al., 1998). Finally, activation of CD8+ T cells following immunization is undesirable as cytotoxic T cells are associated with paralysis via neuronal apoptosis; thus the use of live vaccines for postexposure therapy is discouraged (Hooper, 2005, Lafon, 2004, 2002).

The presence of VNA alone did not result in rapid clearance from the CNS as recovery from disease required a rabies specific antibody response (Lafon, 2002). A timely antibody response requires other factors, such as CD+8 T cells, which hasten viral clearance. Hooper et al., (1998) reported that cytotoxic T cells enhanced the production of IFN-γ and stimulated an inflammatory response within the CNS. Interferons may play a role in clearing rabies virus infections and have been studied in mouse models employing IFN-α/β and IFN-γ receptor knockouts. Hooper et al., (1998) reported a delayed production of VNAs in mice lacking IFN-α/β and IFN-γ. Although VNAs are the most important component of the immune system in clearing rabies virus infection, CD+8 T cells and inflammatory cytokines are essential in hastening the production of VNA and clearing the virus before the infection is unable to be controlled or prior to irreversible neurological damage (Lafon, 2002, Hooper et al., 1998).

Lafon (2002) reviews the immune response following rabies virus infection. Chemokines and inflammatory cytokines released following neural infection stimulate the migration of activated lymphocytes of unrelated specificity though the blood brain barrier (Lafon, 2002, Hooper, 1998). Due to the lack of rabies antigen outside the CNS, these migrating lymphocytes were never activated in the periphery. T cells entering the nervous system are inefficient at controlling acute rabies infections (reviewed in Lafon, 2002). These T cells may not be rabies specific, may be anergic, or destroyed by

apoptosis following entry into the CNS. Lafon (2002) reported that the destruction of migratory T cells occurs when activated T cells expressing Fas moved across the blood brain barrier and interacted with the infected neurons, upregulating their expression of Fas-L. Interaction between Fas and Fas-L resulted in T-cell apoptosis and allowed the virus to replicate in the nervous system (Lafon, 2002). Rabies virus infection is immunosuppressing in its ability to impair lymphocyte response and decrease cellular immunity. Immunosuppression has been reported to occur following infection with a pathogenic variant by interfering with the spleen's ability to act as a lymphocytic organ (Lafon, 2002). Finally, recent research has demonstrated that the P protein plays a role in inhibiting the production of IFN within rabies infected cells (Fink, 2005).

As discussed previously, healthy neurons typically repress the expression of MHC class I, preventing the presentation of antigen in a healthy CNS (Lafon, 2002). During infection with an abortive variant of rabies, the unique relationship between the immune system and nervous system is not maintained. Rabies virus antigen is presented to the immune system in the periphery and rabies specific activated lymphocytes move across the blood brain barrier. (Lafon, 2002).

Vaccinology

Rabies virus is typically inoculated into muscle tissue where it replicates prior to entering the nervous system (Wunner, 2002). Rabies virus has the ability to quickly enter the CNS before the individual is able to receive post exposure treatment (Hooper, 2001). Individuals exposed to rabies virus do not always seek prompt treatment, in fact, the time between exposure and treatment is typically 10 days following exposure (Rudd, R.J. personal communication) and rabies vaccine failure continues to be very rare. Vaccine

failures are typically associated with improper administration or employing biologicals with equivocal efficacy (Smith, 2002).

For decades it was unknown how rabies vaccination was able to prevent infection following parental exposure to rabies virus. By examining the humoral response to other neurotropic agents, hypotheses have been proposed as to why rabies vaccination is capable of preventing clinical illness, even after CNS infection (Lafon, 2004, 2002). Like most viral infections, rabies results in an inflammatory response that will stimulate the hosts' innate and acquired immune response. The inflammation resulting from viral infection and injury attracts these components allowing anti-rabies viral neutralizing antibodies though the blood brain barrier into the CNS. Because the blood barrier that surrounds peripheral nerves is more permeable than the blood barrier that surrounds the CNS, it is more accessible to anti-rabies VNA and may be capable of clearing early infection of the nervous system (Lafon, 2002).

In developed and most developing countries, rabies pre-exposure vaccination in humans consists of immunization with three inoculations using whole, killed virus grown in cell culture. These vaccinations are highly efficacious with a low incidence of side effects. Current rabies biologics are far superior to the nerve tissue-derived vaccinations employed in underdeveloped countries. Nerve tissue derived vaccines are much less expensive to produce than cell tissue vaccines but the potency is questionable, their shelf life is short, and there is a high incidence of side effects. Side effects and sequelae of these nerve tissue vaccines can be severe, including paralysis and death. Newer nerve tissue derived vaccines have fewer side effects due to lack of myelin in the vaccines.

They still fall short of the safety and efficacy of cell culture vaccines and WHO has called for the replacement of nerve tissue vaccines with modern cell culture vaccines.

Post-exposure rabies vaccination is highly efficacious, consisting of injections of vaccine on days 0, 3,7,14, and 28, and rabies immunoglobulin (RIG) which is infiltrated into the bite wound on day 0. RIG is not to be administered following the initial dose on day 0 as this has been found to decrease the efficacy of the active vaccination (Lang et al., 1998). The decreased immune response to active vaccination when RIG is administered incorrectly cannot be compensated for by additional vaccination. Proper administration of post-exposure prophylaxis is of the utmost importance for the individual to mount an effective immune response following rabies exposure (Rabies Compendium, 2006, CDC, 1999, Gacouin et al., 1999).

Currently, there are several vaccines marketed for domestic animals including dogs, cats, ferrets, horses, cattle, and sheep, most of which are killed virus vaccines (Rabies compendium, 2006). More recently, a recombinant rabies vaccine has been approved for use in cats. Although there has been great debate over vaccinating wolf hybrids, no approved vaccine is currently available (Rabies compendium, 2006).

Rabies vaccine failures are rare, and in humans, is usually the result of improper administration or handling. There have been published reports of vaccine failures in humans that often lead to a better understanding of rabies immunology (Childs, 2002, Gacouin et al., 1999). In the 1980's a woman in the Peace Corps was vaccinated for rabies prior to leaving for her post in Africa. While there, she was bitten by a dog and later died of rabies. Upon review of her case, public health officials found that following vaccination, blood was not drawn to determine if she had an appropriate response to the

vaccine. Second, she had been taking antimalarials while undergoing vaccination for rabies virus. The antimalarials rendered her immunosuppressed and she apparently never developed an adequate immune response against rabies (Gacouin et al., 1999, Pappaioanou, et al., 1986, Bernard et al., 1985).

Two cases of vaccine failure, one in a 9-year-old boy and the other in a 72-year-old woman, were reported in Thailand in 1999 (Hemachudha et al., 1999). Two theories have been proposed as to why this occurred. First, rabies virus may have been directly inoculated into the nerve endings of these individuals (Shankar et al., 1991). Alternatively, the rabies vaccine and RIG administered to these individuals could have been handled improperly. Freeze-dried rabies vaccines, Vero cell vaccines, the Human Diploid Cell Vaccine (HDCV), and RIG all require storage at $4^{o}C$. Arya (1999) questioned the efficacy of these vaccine products as substandard biologicals are frequently provided to patients in Thailand. Arya's 1999 paper concluded that it was possible, if not probable, the vaccine products were improperly handled.

In the United States, vaccine failure in animals is uncommon but does occur. Immune responses can be overwhelmed with the introduction of large amounts of virus, which may be the case in animals interacting with downed rabid bats or fighting with rabid terrestrial wildlife. From the years 1994 –1999 a mean of 280 cats and 127 dogs were diagnosed with rabies in the US every year (McQuiston et al., 2001). McQuiston et al.(2001) typed the rabies viruses infecting 78 dogs and 230 cats, to identify the original host reservoir. Approximately 53% of the animals in this study were owned. Of these animals, 57% were infected with a raccoon variant, 22% with the north-central skunk variant, 14% the south-central skunk variant, 2% Texas fox variant, 2% Texas dog

variant, 1% California skunk variant, 1% Arctic fox variant, and <1% (1 cat) with the *E. fuscus* variant. Of the owned animals, 94% of the owners reported an unknown or unvaccinated history for the animal, 3% had past but not current vaccination, and 3% had been vaccinated less than 30 days prior to onset of illness. One of the rabid animals with a current history of vaccination was not given a booster following fighting with a wild animal.

Early death syndrome

Antibody enhancement has been documented in several viral infections, including dengue virus, West Nile virus, and St. Louis encephalitis virus (Ludwig et al., 1986, King et al., 1984, and Peiris and Porterfield, 1979). In rabies, the "early death syndrome" has been reported in mice, primates, and humans (Andral and Blancou, 1981, Prabhakarm and Nathanson, 1981). Experimental animals improperly immunized against rabies die more rapidly following challenge than unimmunized experimental animals, although all challenged animals succumbed. Porterfield (1981) proposed an increased uptake of rabies virus into macrophages though opsonization of antibody complexes. Rabies has been found to infect macrophage cells and Ray et al., (1995) suggested that macrophages can become persistently infected with rabies virus, sequestering the virus until later activation, at which time rabies virus is released potentially resulting in a productive infection. King et al., (1984) reported that at low levels, rabies viral neutralization correlated with the antibody titer, whereas at higher titers, the viral neutralization plateaued and neutralization began to decrease to levels demonstrated in control animals. King et al., (1984) also reports the ability of rabies virus to replicate in a macrophage cell line. Prabhakar and Nathanson (1981) reported that irradiated mice subsequently

exposed to rabies died within 72 hours after inoculation with a high-egg passage vaccine (HEPV). The same results were demonstrated after irradiated mice exposed to rabies virus were reconstituted with B cells or antibodies from mice immunized with HEPV. Mice reconstituted with normal mouse sera or T cells did not experience the early death syndrome. In these experiments, all animals eventually succumbed to rabies virus infection yet antibody enhancement resulted in a more rapid progression.

Recovery

Recovery following a rabies infection is very rare but has been documented in a number of species, including humans. As of 2006, six humans have been reported to have survived clinical rabies virus infection. Of these, two died four years following infection due to sequelae and one reportedly has severe neurological sequelae. The first two individuals known to survive clinical rabies infections are the only ones without neurological sequelae. In a well-publicized case in 2005, a young woman survived clinical rabies infection (Willougbhy et al., 2005). She was bitten or scratched by a bat and never received post exposure prophylaxis. Her physicians decided against post exposure prophylaxis, placed her in a drug-induced coma with ketamine and midazolam, and administered amantadine and ribaviran in hopes her immune system would clear the infection. She recovered and at the beginning of the year had severe neurological sequelae described as palsy-like. Her brain function is believed to be normal and she is expected to make a full recovery (Willoughby et al., 2005). This treatment was initially praised as a novel and promising treatment for clinical rabies infection, yet recent attempts employing these techniques have failed (Willoughby, 2007, Geldermann, 2005).

There are several classical rabies papers describing the recovery of dogs, cats, bats, and agricultural animals (Brass, 1994, Fekadu et al., 1992, Fekadu et al., 1980). These reports are rare, suggesting recovery following clinical infection with rabies virus does occur but infrequently. Most of the animals were in experimental conditions where they were hand fed, unavailable to predators, and their ability to survive in the wild was unlikely. It is conceivable that recovery in wildlife occurs, but is likely to be extremely rare.

1.6 RABIES IN BATS

Epidemiology

Worldwide, tens of thousands of humans die each year due to rabies virus infection, most commonly the result of a dog bite (CDC, 2006). In the US and most developed countries, less than five people die of rabies each year, almost always following infection with a bat variant (CDC, 2006). As described previously, variants from the silver hair bat (*L. noctavagans),* Mexican free-tailed bat *(T. brasiliensis)*, and eastern pipistrelle bat (*P. subflavus*) are most often linked with human rabies cases.

There are numerous variants of bat rabies and each variant is typically associated with one host species (Smith, 2002) and several variants may be found within a species. For example, there are currently three identified big brown bat (*Eptescus fuscus)* rabies virus variants that are associated with the geographic range of the *Eptescus fuscus* subspecies (Smith, 2002). Although there may be virulence differences among bat variants, these differences have not been adequately studied. What has been reported is the lack of association with bats in humans who develop bat-associated rabies (Gibbons,

2002). "Cryptic" rabies cases in humans have spawned a new fear of rabies via aerosol as well as the implementation of new public health guidelines including testing bats that are in rooms with sleeping individuals, or living areas with children, mentally disabled individuals, or intoxicated individuals (Rabies Compendium, 2006). Cryptic rabies is a misnomer since further investigation into these cases has revealed some past association with bats such as bats living in the house. A better way to describe these cases would be as rabies virus infection resulting from an unnoticed bite (Gibbons, 2002). Bat bites are typically inconspicuous, they are very small, often microscopic, may not bleed, and may not have the pain associated to wake a sleeping individual. Some bat bites may be hardly recognizable to a fully cognizant individual (Gibbons, 2001).

Chiroptera are believed to be among the more resistant animals to clinical rabies virus infection presumably because of their long history and possible coevolution with the virus (Constantine, 1988). In studies of rabies in wild bat populations, approximately 0.5% -2.5% of wild bats are diagnosed as positive (Trimarchi and Debbie, 1977). The low frequency of rabies in bat populations may allow the virus to maintain itself without decimating host populations. Constantine (1988) suggested that clinical rabies is a disease of immunodeficient bats. Employing theoretical immune response models, Dimitrov et al (2006) examined the immune response of bats when exposed to various rabies virus challenge. Their data demonstrate a low level of clinical infection and a high prevalence of VNA allow rabies virus to circulate in low levels within the population.

It is not uncommon to detect rabies virus antigen in the brain of a rabid animal without detectable viral antigen in its salivary glands (Brass, 1994). This may result in a

dead end infection and could help explain the low level of rabies virus constantly circulating within bat colonies. Constantine (1966) inoculated *T. brasiliensis* bats intramuscularly with a *T. brasiliensis* salivary gland suspension. Five of nineteen (26%) developed clinical rabies virus infection following an incubation of 21-36 days. Rabies virus antigen was found in the brain of all five but not in the salivary glands. This variant was also inoculated into several terrestrial species including dogs, cats, foxes, coyotes, raccoons, skunks, ringtails, and opossums. Nine of the eighteen animals had rabies antigen in their brains, and rabies antigen was found in the salivary glands of only the two positive foxes. In a different experiment, Constantine (1966b) inoculated 32 Mexican free-tailed bats (*T. brasiliensis*) with a *T. brasiliensis* salivary gland brain homogenate. Twenty-four of the bats developed rabies and rabies antigen was found in the salivary glands of 13 (54%) animals (Constantine, 1966). Conversely, Constantine and Woodall (1966) inoculated Red bats (*Lasiurus borealis*) in the deltoid muscle with a salivary glands suspension of the *L. borealis* rabies variant. The brains and salivary glands of all four bats contained rabies antigen (Constantine and Woodall, 1966).

Anti-rabies virus antibodies in bats

There have been a number of published reports of anti-rabies VNA in wild bats (O'Shea, 2004, Steech and Altenback, 1989, Trimarchi and Debbie, 1977, Baer, 1975 and Constantine, 1968). Steech and Altenbach (1989) reported 65-80% of clinically normal *T. brasiliensis* tested were seropositive and 58% of the common vampire bats (*D. rotundus)* tested in Latin America were seropositive. The mechanisms behind the large percentage of bats with naturally occurring antibodies and low rate of clinical rabies virus infections remains a mystery but some hypotheses are as follows: 1. low dose exposure,

2. exposure to a variant of low virulence, 3. exposure to aerosol, or 4. exposure to low concentrations, which could occur during mutual grooming (Constantine, 1968, O'Shea, 2004, and Steech and Altenback, 1989). It is currently unknown what level of protection these naturally acquired antibodies provide.

Maternal antibodies against rabies virus are transmitted through the placenta to fetal bats. These antibodies are thought to be protective as the young bats develop. As antibody levels diminish the young bats become susceptible to rabies virus infection (Steech and Altenback, 1989). IgM has been found in young *T. brasiliensis* bats, typically from May through September (Steech and Altenback, 1989). It is possible that as maternal antibodies wane, the level of VNA may be protective enough to prevent a clinical infection but in low enough concentration to allow the bat to develop active immunity following an exposure (Steech and Altenback, 1989).

There are two very different hypotheses concerning maternal antibodies and active immunization. Some studies done in wild terrestrial animals reported that maternal antibodies prevented active immunization following vaccination in foxes while other studies reported no maternal interference (Blasco et al., 2001 & Muller et al., 2001). Van Kampen, (1999) reported no interference from maternal antibodies following rabies vaccination of domestic.

Rabies carrier state in bats

There have been reports of recovery in clinically rabid common vampire bat (*D. rotundus*) and Mexican free-tailed bat (*T. brasiliensis*) (Constantine, 1966, Pawan, 1936). Constantine (1966) reported that seven bats developed clinical illness compatible to rabies virus infection after which five returned to normal health 181 days following

infection. Following a 103-day incubation, one of the seven bats was euthanized and necropsied. No rabies antigen was detected in the brain but rabies antigen was found in the salivary glands. This interesting finding was not addressed further in the paper. Older papers report a carrier state in *D. rotundus* and *T. brasiliensis* bats (Pawan, 1936). The reports of a carrier state in vampire and insectivorous bats are likely to be the result of outdated laboratory techniques (Brass, 1994, Moreno and Baer, 1980). Pawan (1936) reported that mice inoculated with vampire bat saliva died with clinical manifestations typically seen in rabies virus infected mice whereas all the inoculated vampire bats remained asymptomatic (Pawan, 1936). It is believed the mice were actually infected with Rio Bravo, aka the salivary gland virus. Rio Bravo virus, a flavivirus transmitted via aerosol and bites, is frequently found in the saliva and salivary glands of asymptomatic *D. rotundus* and *T. brasiliensis* (Brass, 1994, Moreno and Baer, 1980). This virus has been reported to produce clinical signs similar to rabies virus in mice and has been reported to infect humans, typically producing flu like symptoms (Brass, 1994). Every paper attempting to replicate this carrier state study has produced rabies in all exposed bats (Brass, 1994, Moreno and Baer, 1980). Some later studies employing insectivorous bats also reported subclinical carrier states. Similar to the Pawan (1936) paper, saliva from healthy insectivorous bats was inoculated into mice. While the bats remained healthy, the mice developed clinical signs of rabies virus infection (Moreno and Baer, 1980, Constantine, 1975, Baer and Bales, 1967, Girard, 1965, and Bell et al., 1962). It is believed the saliva contained Rio Bravo virus or one of a number of other bat viruses that are found in bat saliva. Today, a carrier state in bats, or any mammal, is not believed to exist (Brass, 1994).

Aerosolized rabies virus

There have been four reported cases of human rabies associated with aerosol transmission, all of which have more plausible explanation. Two cases occurred when individuals entered caves inhabited by an estimated 30 million bats (Childs, 2002, Gibbons, 2002, Brass, 2004, Humphrey, et al., and Irons et al, 1957) One unimmunized individual who entered numerous caves in Texas during 1956 developed clinical rabies after working with bats at Frio Cave (Irons et al., 1957). The initial publication did not suggest aerosol transmission and hypothesized his exposure was through touching a rash on his neck with gloves contaminated with virus (Irons et al., 1957). This individual was known to handle bats and inoculated them with rabies virus. The second case was another unimmunized individual who entered several Texas caves in 1959 and subsequently developed rabies (Constantine 1962). On one occasion after emerging from a cave, his coworkers noticed a bleeding wound on his head. When asked he reported that "bats were so numerous he had to raise his hands over his head to protect his face." (Gibbons, 2002, Constantine 1962). He also reported that he had been nicked by a bat (Gibbons, 2002, Constantine 1962). When he became clinically rabid, he denied any contact with bats (Gibbons, 2002). For unknown reasons, this case was reported as transmission via aerosol. In 1972 an immunized individual, J.A., who worked in the New York State rabies lab processing rabies specimens, developed clinical rabies (CDC, 1977). He and others from the rabies laboratory were working on a project aerosolizing high titered rabies virus to coat small particles. (R. Rudd, personal communication, 2006). Several of the individuals working on the project rotated though each station, including where the aerosolization was taking place. J.A. survived but since

virus was never isolated it is unknown if he was infected with the virus being aerosolized or by one of the many rabies specimens handled for diagnostic purposes. Furthermore, none of the others involved in the aerosol experiment developed rabies nor did they demonstrate an increase in antibody titer.

The fourth case was in an individual who was immunized with an experimental vaccine but did not develop VNA (Conomy et al., 1977). Following the homogenization of rabid goat brains in a kitchen like blender, he mouth pipetteted the homogenate into a different container. Although the blender was found to produce aerosols, it is possible mucous membrane inoculation was the route of transmission (Gibbons, 2002, Conomy, 1977).

Bats, caves, and rabies

After the rabies deaths of the two individuals who were believed to have been exposed to rabies aerosols in caves, Constantine (1967) and Winkler (1968) documented the presence of rabies virus in the air and the feasibility of non-bite rabies transmission in Frio Cave. Frio Cave is a large cave on privately owned land in Texas. Approximately 30 million *T. brasiliensis* and considerably fewer *Myotis* spp occupy this large maternity colony (McCracken, 1996, Constantine, 1962). Constantine placed several species of animals in Frio cave for varying lengths of time. The duration of exposure, type of exposure, species of animals, and location within the 4 designated rooms within the cave were correlated to the development of rabies (Constantine, 1967, 1962). Experimental animals were placed in Frio Cave with varying caging: (1) contact with all cave fauna and excreta, (2) contact with all arthropods and excreta (3) contact with small arthropods

and excreta (4) contact with air and dermestid contaminants, and (5) contact with air only (Constantine, 1967).

During the July 1960's Frio cave experiment, all coyotes, 50% of the gray foxes, and 50% ringtails placed in one room of the cave, designated as room 2, developed rabies following exposure to the entire cave fauna, including bats. Incubation times ranged from 31d to 113d. None of the surviving animals, including the striped skunk, raccoons, ringtails, dogs, and cats developed anti-rabies VNA's. Rabies virus was detected in the salivary glands of the gray fox; none of the salivary glands from other animals contained detectable antigen. Albino Swiss mice did not develop rabies following a 5-day exposure to air and arthropods. During the September study, 28 individually caged carnivores were placed in the cave. One group had no exposure to vertebrates but all cave arthropods. The second group was caged more modestly protecting against exposure to large arthropods; neither group developed rabies and they remained seronegative (Constantine, 1967).

During the July 1961 experiment, all coyotes and gray foxes exposed only to air in room 2 developed rabies following a 24-day exposure. Virus was not detected in the salivary glands of any of the animals. The same results were demonstrated in coyotes and gray foxes following a 30-day exposure to air and dermestid contaminants. One of the gray foxes had detectable rabies vial antigen in the salivary glands. All coyotes, gray foxes, silver foxes and opossums exposed to arthropods and excreta developed rabies following a 27-day exposure. One gray fox and one coyote had detectable viral antigen in their salivary glands. None of striped skunks, spotted skunks, raccoons, ringtails, dogs, and cats developed rabies. One of the eighteen golden hamsters held in the cave for a 20

day exposure to all arthropods and excreta developed rabies. None of the 1-year-old *T. brasiliensis* exposed to small arthropods and excreta for 29 days developed rabies. With the exception of the coyotes, none of the carnivores, including the ones that developed clinical rabies, developed anti-rabies VNAs.

Experiments were also done in Lava Cave located in Texas and home to approximately one million bats. In these experiments, half of the coyotes and gray foxes exposed to all arthropods and excreta for 27 days developed clinical rabies but none of the kit foxes, striped skunks, spotted skunks, raccoons, ringtails, dogs, and cats developed rabies. Viral antigen was detectable in two coyotes and one gray fox in their salivary glands. Surviving animals did not develop anti-rabies VNA, the serological response of rabid animals was not described (Constantine, 1967).

In Frio Cave, seven of the eight foxes held in room 2 and exposed to all arthropods, excreta, and cave atmosphere for 18 days developed rabies; none of the bats, mice, and rats in this study developed rabies. Rabies antigen was present in the salivary glands of one gray fox and one red fox. To examine the presence of rabies virus in wild bats living in caves, two hundred asymptomatic suckling bats were tested though direct fluorescent antibody test (dFAT) and all were found to be rabies virus negative. They also tested 701 moribund or dead bats including symptomatic suckling bats and found 130 (19%) were rabies positive on dFAT. Antigen was found in several non neural tissues (Table 2).

Table 2. Tissue tropism results from bats.

Tissue	Adults (n=23)	Juveniles (n=107)
Brain and SG	39%	48%
Brain only	8.7%	23.6%
Brain, SG and lung	21.7%	20.7%
Brain, SG and kidney	8.7%	<2%
Brain, SG, lung and kidney	21.7%	5.7%

Following a 15-day exposure to air, excreta, and all arthropods in room 2 of Frio cave, 75% of exposed opossums died of rabies. None of the exposed bobcats, ferrets, rock squirrels, squirrel monkey, owl monkey, turkey vulture, red tailed hawk or rat snakes developed rabies. Predators captured near Texas bat caves were examined for the presence of rabies and anti-rabies VNAs during a 6-month holding period. Of these captured predators, one of the eleven raccoons and two of the nine striped skunks developed rabies. None of the wild caught gray foxes or opossums died of rabies. Viral antigen was found in the salivary glands of one trapped raccoon and one trapped skunk. One of the surviving raccoons was seropositive (Constantine, 1967).

The cave experiments performed by Constantine in the 1960s were important in demonstrating the presence of transmissible rabies virus in the air of caves occupied by large numbers of bats. Winkler (1968) was able to isolate virus from the atmosphere of Frio Cave during the July, 1960 cave experiment. Air was sampled though mechanical sampling devices and rabies virus was demonstrated though IM inoculation into red foxes (Winkler, 1968). Samples of the cave atmosphere were taken for 10 to 30 minutes. Mouse inoculation was attempted but did not result in infection. Of the twelve samples, four produced clinical rabies in foxes (Winkler, 1968). The cave experiments demonstrated the animals that developed clinical rabies following exposure only to the

cave atmosphere are typically species known to be more susceptible to rabies virus infection. Opossums, know to be highly resistant to rabies virus infection, were readily infected though exposure to the cave atmosphere. Coyotes and gray foxes held in room 2 for 1 hour or a different room for up to 29 days with exposure to arthropods and excreta did not develop rabies. In fact, the only room in Frio Cave in which animals developed rabies was in room 2. This room is home to suckling bats whose mothers attend to their pups in the mornings and afternoons. The high incidence of rabies antigen in the salivary glands of rabid suckling bats may be significant in the production of aerosolized rabies virus.

One downfall of these cave experiments is the lack of shorter exposure time to only the cave atmosphere. The shortest experiment exposed rodents to the cave atmosphere for 15 minutes or 5 days; no animals developed rabies in these experiments. The shortest exposure a carnivore had in the cave was a one-week exposure to the entire cave fauna, including a possible bat bite. One cannot extrapolate the impact of the cave atmosphere on carnivores placed in the caves for less than 24 days. Although the cave experiments demonstrate viable virus in the air within Frio Cave and the ability of this virus to produce clinical rabies in some experimental animals, the infectious aerosol dose is unknown.

1.7 OBJECTIVES

Over the last 17 years, the majority of human rabies cases in the US were attributed to a bat rabies variant. Most of these cases have been defined as 'cryptic' as the infected individual has no memory of a bat bite. It has been postulated that bat rabies

variants may be more pathogenic than terrestrial rabies variants, yet studies comparing bat rabies variants have not been published. The route of inoculation in these cryptic cases has been questioned with some speculation that aerosol exposure, imperceptible bite wound, or subcutaneous inoculation may be associated with these cases.

The studies presented in this dissertation were designed to increase our knowledge of bat rabies by examining the pathogenesis and transmission of several bat rabies variants. The **first aim** was to 1. Characterize the pathogenesis of two bat rabies viruses. 2. Follow the immune response of inoculated bats and mice, and 3. Compare the genomes of these variants and identify changes that may be involved in virulence. The **second aim** was to 1. Determine if rabies virus could be aerosolized, 2 Determine if an exposure to aerosolized rabies was infectious to bats and mice, and 3. Compare the immune response of bats and mice following exposure to different bat rabies variants. The **third aim** was to 1. Establish an accurate percentage of healthy wild bats that are incubating rabies, 2. Ascertain the percentage of rabid bats with infectious saliva 3. Examine the immune response of naturally infected rabid bats.

CHAPTER II

A Tale of Two Rabies Viruses: Unique Characteristics of Rabies viruses in Big Brown Bats (*Eptesicus fuscus)*

2.1 INTRODUCTION

There are approximately forty different species of bats found in the United States and rabies virus infection has been diagnosed in at least thirty of these species (Hughes et al., 2005; Childs, 2002; Smith, 2002; Constantine, 1979). The number of rabies variants circulating within bat populations is unknown but they are typically host specific (Shankar et al., 2005, Smith, 2002). Some rabies virus variants appear to be host specific to the level of subspecies, as postulated in big brown bat (*Eptesicus fuscus)* populations (Shankar et al., 2005, Smith, 2002). There are three known subspecies of big brown bats (*Eptesicus fuscus)* in the United States (Vaughan, 1954). The Southwestern big brown bat, *E. f. pallidus*, is found in the Southwestern U.S. including California, Colorado, Arizona, New Mexico, and the western regions of Kansas and Texas. The Western big brown bat, *E. f. bernardinus*, is found in California. The Eastern big brown bat, *E. f. fuscus*, is found in the eastern U.S. including the eastern regions of Kansas and Texas to the Atlantic coastal states (Vaughan, 1954).

There are at least two different rabies virus variants circulating within *E. fuscus* species, one associated with the Southwestern big brown bat (*E. f. pallidus)* and a second with the Eastern big brown bat (*E. f. fuscus*) (Rudd et al., 2005). The differences between

these variants have not been widely studied. It has been proposed that viral epitopes from the *E. f. pallidus* and *E. f fuscus* variants differ enough to result in diagnostic problems with one of the most commonly employed diagnostic fluorescent antibody conjugates (Rudd et al., 2005).

Differences in virulence between rabies viruses are believed to be the result of changes in the viral glycoprotein (Faber et al., 2005, Sarmento et al., 2005, Takayama et al., 2005, Mebatsion, 1999). To date, there are few studies directly comparing the virulence of different rabies virus variants. One study by Morimoto et al. (1999) evaluated the virulence of a silver hair bat (*Lasionycteris* noctivagans) variant, yet this report compared the *L. noctivagans* variant to a coyote rabies variant instead of another bat rabies virus variant. Comparing rabies variants occurring in subspecies or closely related species could help determine potential differences in virulence and may also be valuable in viral evolutionary studies.

Big brown bats are one of the species most commonly diagnosed as rabid, and frequently live in close proximity to humans, yet this variant has been implicated in only one human case of rabies virus infection. Conversely, the *L. noctivagans* and Eastern pipistrelle *(Pipistrelle subflavus)* bat species are somewhat solitary, tree-dwelling bats that have infrequent contact with humans. These two bat species make up <5% of the bats diagnosed with rabies virus. The purpose of this study was to characterize the differences between two *E. fuscus* isolates of rabies virus, designated *Efv1* and *Efv2,* with respect to the progression of the disease, dissemination of the virus, and genetic differences between the variants. We undertook this study with the expectation that the viral pathogenesis would be comparable between viruses with similar genomes.

The timeline for rabies studies can be lengthy due to the incubation time following experimental inoculation and the requirement to quarantine wild caught bats. Whenever possible, wild caught bats should remain quarantine for six months. The duration of quarantine is important to the experimental results since the incubation time following natural infection with rabies in bats has not been well studied.

The viruses *Efv1* and *Efv2* were both obtained from Colorado *E. fuscus* bats. The gold standard for genetic analysis of this gene employs primers that amplify 320 base pairs of the N gene (Shankar et al., 2004). This portion of the genome was identical between the two viruses and it was anticipated that the viruses would behave similarly when inoculated into bats and mice. Two experiments were performed: Experiment 1 was done in the spring of 2003 with virus isolated from the salivary glands of bats without passage in cell culture. The results obtained from Experiment 1 were unexpected and consequently a second experiment, Experiment 2, was performed in 2004. In the second experiment, viruses were passed in cell culture three times in order to obtain adequate amounts for animal and cell culture inoculation.

2.2 GENERAL METHODS AND MATERIALS

Rabies viruses

All rabies viruses were isolated from bats provided by the Colorado Department of Public Health and Environment (CDPHE). All bats tested positive for rabies antigen by direct fluorescent antibody test (dFAT) at the CDPHE and were re-tested when bats were necropsied at Colorado State University (NASPHV, 2006). Virus was isolated from the salivary glands as described in Rudd and Trimarchi (1989, 1987). The salivary

glands were removed and homogenates (10% w/v) were made with a Ten-Broeck tissue grinder using 1.0 ml of growth medium (minimal essential medium supplemented with 10% fetal bovine serum and 2.0 mM glutamate, with 100 IU penicillin G, 50 μg streptomycin, and 2.5 μg amphotericin B per ml) as a diluent. Salivary gland homogenates were frozen at -80^0C until further processing could take place. Viral titration was done in duplicate using neuroblastoma cells (NA-1300) in 96-well plates, as described in Trimarchi et al. (1996). Briefly, 50 μl of test inoculum was added to 50μl neuroblastoma cells and 150 μl growth medium. Test inoculua were diluted 1:5 in each individual well. The plates were incubated in 5% CO_2 for 72 hours, after which fluid was aspirated from the wells. The wells were washed twice in phosphate buffered saline (pH 7.6) for 5 min, air-dried, and fixed with 75% at -20^0C for at least 1hour. After one hour, the acetone was removed and the wells were allowed to air dry. Further processing of slides for the direct fluorescent antibody (dFA) testing followed the rabies compendium protocol (CDC, 2006) employing the Chemicon anti-rabies conjugate (Chemicon International, Temecula, CA) or Centocor anti-rabies conjugate (Centocor Inc. Horsham, PA). Both the Chemicon and Centocor products are mixtures of FITC-labeled monoclonal antibodies of murine origin. (CDC, 2006). Following application of the anti-rabies conjugate, the plates were placed in an incubator at 5% CO_2 and 37^0C for 1 hour. The conjugate was decanted and the wells were washed twice as described above and allowed to air dry. One or two drops of mountant media (Rudd et al., 2005) were placed in each well and the cells were viewed with a Nikon fluorescent microscope. Titers were calculated with the Reed and Munch equation.

Although the initial plan of the project was to employ one rabies isolate, *Efv1,* the amount of salivary gland homogenate from one bat was not enough to perform the planned experiments. Since the amount obtained from a single salivary gland homogenate was inadequate for the experiment, two more variants were included: *Efv2,* in addition to *Efv1*, for the primary challenge, and *Efv3* for the second challenge in Experiment 1. All isolates were chosen due to their high titer in cell culture. The titer of *Efv1* in cell culture was 2x 10^5 $TCID_{50}$/ml; The titer in cell culture of both *Efv 2* and *Efv 3* was 1x 10^4 $TCID_{50}$/ml.

Two viral isolates, *Efv1* and *Efv2*, were employed in the primary inoculation in both experiments. Prior to the experiment, both viruses were molecularly typed using the N gene primers 1087Deg and 304, and the resulting sequences of the 320 nt amplicon were identical. The secondary challenge virus in Experiment 1 was *Efv3*, which was identical to *Efv1* and *Efv2* when typed with 1087 Deg and 304 primers.

During experiment 1, differences in the pathogenesis between *Efv1* and *Efv2* became apparent and consequently we decided to explore the genome of both viruses further. Four

Table 2.1. Primers employed in PCR reactions and optimal annealing temperatures. "Name" lists the common name of the primer often reported in scientific literature.

NAME	LOCATION ON GENOME	FORWARD SEQUENCE	NAME	LOCATION ON GENOME	SEQUENCE	SIZE	TEMP
N GENE							
L5	Leader seq	CTACAATGGATGCCGAC	304	1514	ATGAGCAAGATCTTTGTCAA	1447	55^{0}C
21g	57	ATGTAACACCTCTACAATG				1457	
1087 Deg	1157	GAGAARGAACTTCARGAAT				357	
P GENE							
1330N	1400	AAGACATACTCGAGTGATTC	36M	2150	CCTACARTTCTTCACTATCT	1110	51^{0}C
M GENE							
828 P	2401	TTAGTTGAGCCYGACAAGCT	213 G	3520	TATCCCACCTTTAGTTCCAT	1119	51^{0}C
G GENE							
828P	2401	TTAGTTGAGCCYGACAAGCT	213G	3250	TATCCCACCTTTAGTTCCAT	1119	55^{0}C
828 P	2401	TTAGTTGAGCCYGACAAGCT	989G	5742	CTGAGACGTCTGAAACTCAC	3341	55^{0}C
199 G	3520	ATGGAACTAAAGGTGGGATA	310Ψ	5260	GTGTCCTTCATAGGAGGCAA	1740	48^{0}C
952 G	4975	CTAGATGCACTGGAGTCCAT	310 Ψ	5260	GTGTCCTTCATAGGAGGCAA	285	55^{0}C

genes (N, M, P, and G) were amplified and sequenced to help identify regions in the genome that may account for the differences in pathogenesis.

Amplification of cDNA and Nucleotide Sequencing

The primers used for reverse transcription, polymerase chain reaction (PCR) and nucleotide sequencing are listed in Table 2.1. All primers were made by Invitrogen, Carlsbad, CA.

Reverse transcription employed the SuperScript^{TM} First-Strand Synthesis System (Invitrogen, Carlsbad, CA) according to manufacturer recommendations with minor modifications. The amount of RNA in the reaction was increased from 5 µl to 6 µl, while decreasing the amount of water. A negative control consisting of 5ul of water was done in unison with the rabies virus (RV) positive samples. The primers used in the synthesis of the cDNA varied with the gene of interest. Random hexamers from the SuperScript First Strand Synthesis System^{TM} were employed in the cDNA synthesis of the M gene and P gene. Gene specific forward primers were employed to synthesize the N and G genes. The cDNA was stored at –20 until processed further.

The optimal PCR reaction employing the TURBO^{TM} system (Stratagene, LaJolla, CA) contained 5 µl cDNA, 33 µl water, 5 µl 10X (-Mg) Turbo cloned pfu Buffer, µl 50mM Mg, 4µl dNTP, 1µl of each primer (20mM) and 1µl 500 pfu TURBO DNA polymerase. Two negative controls were run with each PCR, one substituting 5µl water for cDNA and a second substituting 5 µl cDNA from the RT negative control. The positive control consisted of 5 µl CVS-11 cDNA, generously provided by the New York State Department of Health Rabies Laboratory. The PCR reaction cycles were as follows: 1

cycle at 94^{0}C for 1 minute followed by 40 cycles at 94^{0}C for 1 min., 48-55^{0}C for 1 min, 2 min at 72^{0}C followed by one 10 min incubation at 72^{0}C.

The amplicons were separated by electrophoresis on a 1% SeaKem agarose gel and 1X TE buffer (BMA Products, Rockland, Maine, USA.). To verify the size of the amplicon, a 1Kb ladder was included on each gel next to the negative control. The appropriate band from each reaction mixture was excised with a sterile razor blade and placed in a sterile, labeled centrifuge tube. Electrophoresed cDNA was purified using the Qiagen QIAquick Gel Extraction Kit following manufacturer's recommendations (Qiagen, Valencia, CA). The purified PCR products along with 8µl of 3 mM primers were sent to Davis Sequencing for processing. Initial sequence analysis was done with CHROMAS version 1.45 (School of Health and Science, Griffith University, Australia). Sequence results were compared to other rabies sequences from the NCBI web site (www.ncbi.nlm.hih.gov/blast). The sequences were compared employing CLUSTAL-W (European Molecular Biological Laboratory, European Bioinformatics Institute, UK). The RNA sequences were entered into ExPasy (NCUS US, http://us.expasy.org/tools/dna/html) to ascertain the amino acid sequences of the encoded proteins virus. The resultant amino acid sequences for N, P, M, and G proteins were compared in CLUSTAL-W.

Antigen detection in brains and salivary glands

To examine tissues for rabies virus infection, approximately 30 mg of brain or approximately 50 of macerated salivary gland were placed on each slide for rabies antigen detection. Slides were dried and fixed in 100% acetone at –20 C overnight. Further processing of slides for the direct fluorescent antibody (dFA) testing followed the

rabies compendium protocol (CDC, 2006) employing the Chemicon or Centocor anti-rabies conjugate, as described previously (CDC, 2006). Slides were viewed on a Nikon fluorescent microscope at 150X and 450X.

Bats

During the 2003-2004 summers, *E. fuscus* were captured from roosts in Fort Collins, Colorado. Bats were held in quarantine within a biosaftey level 3 facility in groups of 4-5 for at least 6 months. All bats entering the captive colony were bled from the uropatagial vein, weighed, and an oral swab and a wing biopsy were taken; serum was tested for the presence of anti-rabies viral neutralizing antibodies (VNAs). To identify individual bats, a passive integrated transponder (PIT) tag (Avid, Inc. Norco, CA) was inserted subdermally. Bats had free access to water and were fed supplemented mealworms, and were occasionally provided beef baby food. The room temperature was maintained at 85-90^{0}F and the humidity was approximately 20-30%. Bats were weighed twice a week at which time an oral swab was taken and they were given a brief physical exam. Any bat that lost more than 2 grams between weightings was placed in a smaller isolation cage, monitored more closely, hand-fed meal-worms and beef baby food, and if necessary, administered lactated Ringers saline subcutaneously. Any bat that did not improve within 24 hours was euthanized, necropsied, and tested for rabies. All bats were bled monthly during the quarantine period to test for rabies virus neutralizing antibodies. Two weeks prior to and two weeks post-rabies virus inoculation, bats were bled from the uropatagial vein, and serum was tested for rabies VNA employing the RFFIT.

2.3 EXPERIMENT I

MATERIALS AND METHODS

For the primary rabies virus challenge in Experiment 1, bats were inoculated in the right deltoid muscle with either 10^2, 10^3 or 10^4 $TCID_{50}$ of *Efv1* or 10^2 or 10^3 $TCID_{50}$ *Efv2* in a volume of 50 µl (Table 2.2). The deltoid muscle was chosen for inoculation as it is large and easily accessible in the bat and has been the site of experimental chiropteran vaccination in previous studies. Following virus inoculation, bats were weighed and oral swabs were obtained twice weekly. Bats were checked at least twice daily for changes in behavior. Animals with a weight loss of >2 g were placed in an isolation cage, hand-fed beef baby food, and given subcutaneous lactated ringers solution. Any animal that continued to deteriorate was sedated with pentobarbital (80mg/kg body weight) and exsanguated though cardiac puncture. Blood was placed into sterile 1.7ml collection tubes, and serum was separated by centrifugation for 5 minutes and stored at –80°C. All bats were necropsied and a brain smear was made to identify the presence or absence of rabies antigen as described in the rabies compendium (CDC, 2006).

Table 2.2 Experiment 1, 2003. Animals were inoculated in the right deltoid with 10^2 or 10^3 $TCID_{50}$ *Efv2* or 10^2 , 10^3 , or 10^4 $TCID_{50}$ *Efv1* rabies virus variant. Number in parentheses indicates the number of bats in each group.

Viral dose $TCID_{50}$/ml	Rabies virus inoculated		
	Efv1	*Efv2*	*Efv2*[a]
10^4	Group 1 (6)	Not Done	Not Done
10^3	Group 3 (6)	Group 4 (6)	Group 2 (5)
10^2	Group 5 (6)	Group 6 (5)	Not Done

[a] Bats in this group had naturally acquired VNA prior to entering the colony.

All animals were bled two weeks after the first inoculation then at one month intervals for the next five months. Five months after the initial inoculation, all bats except *Efu 2* and *Efu 3* (Group 4) were inoculated in the right deltoid with 10^4 $TCID_{50}$ *E. fuscus* rabies virus *Efv3*. Following the first rabies inoculation, bats *Efu 2* and *Efu 3* had developed signs compatible with clinical rabies virus infection, survived, and were not re-challenged. Bats were maintained for one month following the second inoculation, then euthanized as previously described.

Mice

Fourteen four to eight week-old laboratory mice (*Mus musculus,* ICR strain) were received from Taconic Farms. Mice were lightly anesthetized using an intraperitoneal injection of ketamine-xylazine, and inoculated in the right deltoid with either 10^2 or 10^3 $TCID_{50}$/ml of *Efv 2* or 10^4 $TCID_{50}$/ml *Efv 1* rabies virus (Table 2.3).

Table 2.3 Experiment 1. Mice were inoculated in the right deltoid with 10^2 or 10^3 $TCID_{50}$ *Evf 2* or 10^4 $TCID_{50}$ *Efv 1* Number represents the number of mice in each group.

Viral dose ($TCID_{50}$)	Number of mice inoculated with *Efv1*	Number of mice inoculated with *Efv2*
10^4	4	Not Done
10^3	Not Done	5
10^2	Not Done	5

Mice were monitored at least twice daily for signs of behavioral changes. Any mouse displaying clinical signs compatible with rabies virus infection was immediately euthanized and necropsied. Mice were bled from the tail vein two weeks following inoculation then monthly for the next five months. Five months following the first rabies

inoculation, mice were inoculated in the right deltoid with 10^4 $TCID_{50}$ *Efv3*. Mice were maintained for one month, then euthanized and processed as previously described.

Serology

Blood was collected in heparinized microcapillary tubes following veinpuncture with a sterile 26-gauge needle, as described in Shankar et al. (2004). Blood was centrifuged for 5 minutes; serum was separated and stored at –80°C until processed further. Virus neutralizing antibodies (VNA) were measured by the rapid fluorescent focus inhibition test (RFFIT) (Shankar et al., 2004, Smith et al., 1996). To inactivate complement, serum was heated at 56°C for 40 minutes. Five-fold dilutions of serum were mixed with a constant dose of challenge virus standard-11 (CVS-11) rabies virus in the Lab-Tek Chamber Slide ™ system (Nalge, Nunc Rochester, NY,USA). CVS-11 was obtained from the New York State Department of Health Rabies Laboratory. Infection of mouse neuroblastomas (Neuroblastomas C-1300, compliments of New York State Department of Health) was demonstrated by the use of Chemicon anti-rabies conjugate. The CVS-11 virus was diluted as recommended by Smith et al. (1996) and Trimarchi et al. (1996). The U.S. Standard Rabies Immunoglobulin (R-3), a human derived rabies immunoglobulin (Bethesda Maryland, Food and Drug Administration, Office of Biologics Research and Review) was employed as the positive control in each RFFIT. Antibody titers are reported in International Units (IU) unless otherwise specified. A serum titer of $\geq$ 0.5 IU is considered acceptable for individuals working with rabies virus (World Health Organization, 2005, Briggs, 2002).

Because only a small amount of blood can be collected from bats, the volume of serum per sample assayed ranged from 3 µl to 50 µl. Approximately 90% of the sample

volumes were between 20-30 µl. The RFFIT detected anti- rabies antibodies in serum samples containing a volume of 5µl or greater and with a limit of sensitivity of 0.0625 IU or greater.

Virus isolation from oral swabs

Oral swabs were collected twice weekly by swabbing the oral cavity with a cotton tipped applicator stick moistened with BA-1 (MEM salts, 1% bovine serum albumin, 250 mg/L sodium bicarbonate, 50 µg/ml gentamycin, 1 µg /ml amphotericin B in 50mM Tris, pH 7.6). The cotton applicator was placed in a sterile 1.5 ml tube containing 1.0 ml BA-1 and stored at -80^0C until further processing. Freshly trypsinized murine neuroblastoma cells were inoculated into a T-25 flask at a concentration of $1x10^5$-$3x10^5$ cells/ml. Oral swabs were thawed and vortexed for 5 minutes. Virus isolation from the oral swabs was multiplexed: 500 µl of 10 individual samples were added to the T-25 flask. Cells were grown for 3 days and passed at a concentration of $1x10^5$ to $3x10^5$ cells/ml. Each time cells were passed, approximately 10^3 cells were grown out overnight on Gold seal Rite-on fluorescent antibody microscope slides (Portsmouth, NH). Excess medium was removed and slides were briefly washed once in PBS, dried, and fixed in 100% acetone overnight at $-20C^0$. Fixed slides were removed from acetone, processed as previously described, and viewed with a Nikon fluorescent microscope at 200X. All samples from a T-25 flask positive for rabies virus isolation were re-inoculated separately to determine which samples were positive. If samples remained negative after 3 passages, flasks were discarded.

Virus strains

The three viruses employed in this experiment were *Eptesicus fuscus* CDPHE accession #15100 (*Efv1*), *Eptescus fuscus* CDPHE accession #14357 (*Efv2*), and *E. fuscus* CDPHE accession # 15636 (*Efv3*). An amplicon of 320 nucleotides from the N gene 1178 to 1494 on the rabies virus genome, had identical sequences among all three variants. Although these regions of the genome were identical, the pathogenesis of *Efv1* and *Efv2* appeared to have notable differences. To determine the regions responsible for the differences in virulence, the N, P, M, and G genes of the *Efv1* and *Efv2* virus RNA were sequenced. There were several nucleotide differences throughout the genome; 2 nt changes were found in the N gene, 3 in the P gene, none in the M gene, and 6 in the G gene. There were four substitutions that resulted in amino acid changes, 1 in the N protein and 3 in the G protein.

Table 2.4 Sequence comparison between *Efv1*(V1) and *Efv2*(V2) genomes. The sequence begins at the N gene and continues through the G gene. Differences in nucleotide sequence are highlighted.

```
          N->
V2        TTTCTCTGAAGCCTGAAATTATAGTAGATCAATATGAGTACAAGTACCCGGCTATCAAAG 60
V1        TTTCTCTGAAGCCTGAAATTATAGTAGATCAATATGAGTACAAGTACCCGGCTATCAAAG 60
          ************************************************************

V2        ATTTGAAGAAGCCCAGTATAACCTTAGGAAAGGCCCCTGACTTGAACAAGGCATACAAGT 120
V1        ATTTGAAGAAGCCCAGTATAACCTTAGGAAAGGCCCCTGACTTGAACAAGGCATACAAGT 120
          ************************************************************

V2        CAGTCTTATCCGGCATGAATGCAGCCAAGCTTGACCCTGATGATGTATGCTCTTATCTGG 180
V1        CAGTCTTATCCGGCATGAATGCAGCCAAGCTTGACCCTGATGATGTATGCTCTTATCTGG 180
          ************************************************************

V2        CAGCTGCAATGCAGTTCTTTGAAGGGACATGTCCTGATGACTGGACCAGCTATGGAATCC 240
V1        CAGCTGCAATGCAGTTCTTTGAAGGGACATGTCCTGATGACTGGACCAGCTATGGAATCC 240
          ************************************************************

V2        TGATTGCACGGAAGGGAGACAAGATCACTCCAAATTCTCTTGTGGACATCAAACGTACTA 300
V1        TGATTGCACGGAAGGGAGACAAGATCACTCCAAATTCTCTTGTGGACATCAAACGTACTA 300
          ************************************************************

V2        ATGTGGAAGGGAACTGGGCTCTAACAGGGGGTATGGAGTTGACAAGAGACCCCACCGTTC 360
V1        ATGTGGAAGGGAACTGGGCTCTAACAGGGGGTATGGAGTTGACAAGAGACCCCACCGTTC 360
          ************************************************************

V2        CGGAGCATGCATCGTTAGTTGGTCTTCTCTTGAGTCTGTATAGATTGAGCAAAATATCTG 420
V1        CGGAGCATGCATCGTTAGTTGGTCTTCTCTTGAGTCTGTATAGATTGAGCAAAATATCTG 420
          ************************************************************

V2        GACAGAACACTGGCAATTATAAAACAAACATCGCTGATAGAATAGAGCAAATTTTCGAGA 480
V1        GACAGAACACTGGCAATTATAAAACAAACATCGCTGATAGAATAGAGCAAATTTTCGAGA 480
          ************************************************************

V2        CGGCCCCCTTTGTAAAGATCGTAGAACATCACACCTTGATGACAACCCACAAAATGTGCG 540
V1        CGGCCCCCTTTGTAAAGATCGTAGAACATCATACCTTGATGACAACCCACAAAATGTGCG 540
          ****************************** ***************************

V2        CTAACTGGAGCACCATACCGAATTTCAGATTTCTAGCCGGAACCTACGACATGTTTTTCT 600
V1        CTAACTGGAGCACCATACCGAATTTCAGATTTCTAGCCGGAACCTACGACATGTTTTTCT 600
          ************************************************************

V2        CCCGGATCGAACATCTGTATTCAGCAATTAGAGTGGGCACAGTTGTCACTGCTTATGAGG 660
V1        CCCGGATCGAACATCTGTATTCAGCAATTAGAGTGGGCACAGTTGTCACTGCTTATGAGG 660
          ************************************************************

V2        ACTGCTCAGGATTGGTGTCGTTCACCGGGTTTATAAAGCAAACAAATCTCACCGCAAGAG 720
V1        ACTGCTCAGGATTGGTGTCGTTCACCGGGTTTATAAAGCAAACAAATCTCACCGCAAGAG 720
          ************************************************************

V2        AAGCAATATTATATTTCTTCCATAAGAACTTTGAAGAAGAGATAAGAAGAATGTTTGAGC 780
V1        AAGCAATATTATATCTCTTCCATAAGAACTTTGAAGAAGAGATAAGAAGAATGTTTGAGC 780
          ************** *********************************************

V2        CTGGGCAGGAAACCGCAGTTCCTCACTCCTATTTCATCCATTTTCGTTCATTGGGCCTGA 840
V1        CTGGGCAGGAAACCGCAGTTCCTCACTCCTATTTCATCCATTTTCGTTCATTGGGCCTGA 840
          ************************************************************

V2        GTGGGAAATCTCCATATTCATCAAATGCAGTGGGTCACGTGTTCAATCTCATTCACTTTG 900
V1        GTGGGAAATCTCCATATTCATCAAATGCAGTGGGTCACGTGTTCAATCTCATTCACTTTG 900
          ************************************************************
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V2      TGGGATGTTATATGGGTCAAGTAAGATCTTTAAATGCAACGGTTATTGCCACATGTGCCC 960
V1      TGGGATGTTATATGGGTCAAGTAAGATCTTTAAATGCAACGGTTATTGCCACATGTGCCC 960
        ************************************************************

V2      CGCATGAGATGTCTGTTCTCGGGGGTTATCTGGGGGAGGAGTTTTTTGGAAAGGGGACTT 1020
V1      CGCATGAGATGTCTGTTCTCGGGGGTTATCTGGGGGAGGAGTTTTTTGGAAAGGGGACTT 1020
        ************************************************************

V2      TTGAGAGAAGATTCTTTAGGGACGAGAAAGAACTGCAGGAATATGAGGCAGCTGAGTCAA 1080
V1      TTGAGAGAAGATTCTTTAGGGACGAGAAAGAACTGCAGGAATATGAGGCAGCTGAGTCAA 1080
        ************************************************************

V2      CAAAGACTGATGTGGCCTTGGCAGATGACGGAACAGTCAATTCTGATGACGAGGACTACT 1140
V1      CAAAGACTGATGTGGCCTTGGCAGATGACGGAACAGTCAATTCTGATGACGAGGACTACT 1140
        ************************************************************

V2      TCTCTGGTGAAACCAGGAGTCCGGAGGCAGTTTATACTCGGATCATGATAAATGGGGGTA 1200
V1      TCTCTGGTGAAACCAGGAGTCCGGAGGCAGTTTATACTCGGATCATGATAAATGGGGGTA 1200
        ************************************************************

V2      GATTGAAAAGATCACACATAAGGAGATATGTCTCAGTAAGTTCCAATCATCAAGCTCGCC 1260
V1      GATTGAAAAGATCACACATAAGGAGATATGTCTCAGTAAGTTCCAATCATCAAGCTCGCC 1260
        ************************************************************

V2      CTAATTCATTCGCTGAGTTTCTAAACAAGACATACTCTAATGATTCATAAAGAATTGACC 1320
V1      CTAATTCATTCGCTGAGTTTCTAAACAAGACATACTCTAATGATTCATAAAGAATTGACC 1320
        ************************************************************
                                                          <- N
V2      AACAGGATTGTAAACAATAATAAATTGTGTACATCCTTCATGAAAAAAACTAACACCCCT 1380
V1      AACAGGATTGTAAACAATAATAAATTGTGTACATCCTTCATGAAAAAAACTAACACCCCT 1380
        ************************************************************
                          P->
V2      CCTCTTGAACCATCTCAGAC ATGAGCAAGATTTTTGTCAACCCCAGTGCGATCCGGGCGG 1440
V1      CCTCTTGAACCATCTCAGAC ATGAGCAAGATTTTTGTCAACCCCAGTGCGATCCGGGCGG 1440
        ************************************************************

V2      GCCTAGCTAACCTAGAGATGGCAGAAGAAACTGTGGATTTGATCGCTAAGAACATAGAAG 1500
V1      GCCTAGCTAACCTAGAGATGGCAGAAGAAACTGTGGATTTGATCGCTAAGAACATAGAAG 1500
        ************************************************************

V2      ATAACCAGGCTCATCTCCAAGGAGAACCCATAGAGGTGGACAGTCTCCCCGAAGACATGA 1560
V1      ATAACCAGGCTCATCTCCAAGGAGAACCCATAGAGGTGGACAGTCTCCCCGAAGACATGA 1560
        ************************************************************

V2      AACGGCTTCAATTGGAAGACGAGAAACCTTCTGGCCTCGGCGAGATGGCCAAATCAGGGG 1620
V1      AACGGCTTCAATTGGAAGACGAGAAACCTTCTGGCCTCGGCGAGATGGCCAAATCAGGGG 1620
        ************************************************************

V2      AGAGCAAATGTCAGGAAGACTTTCAGATGGATGAGGGGGAAGATCCCGCCCTCTTGTTCC 1680
V1      AGAGCAAATGTCAGGAAGACTTTCAGATGGATGAGGGGGAAGATCCCGCCCTCTTGTTCC 1680
        ************************************************************

V2      AGTCGTACCTGGATAATGTTGGAGTTCATATGGTCAGACAAATGAGGTCAGGAGAGAGGT 1740
V1      AGTCGTACCTGGATAATGTTGGAGTTCATATGGTCAGACAAATGAGGTCAGGAGAGAGGT 1740
        ************************************************************

V2      TCCTCAAGATATGGTCTCATACCGTCGAGGAGATCATATCCTATGTCATGGTCAATTTTC 1800
V1      TCCTCAAGATATGGTCTCATACCGTCGAGGAGATCATATCCTATGTCATGGTCAATTTTC 1800
        ************************************************************

V2      CCAGTCTGCCGAGGAAGTCTTCCGAAGACAAAGCTACCCAGACTGTCAACCGGGAGCTCA 1860
V1      CCAGTCTGCCGAGGAAGTCTTCCGAAGACAAAGCTACCCAGACTGTCAACCGGGAGCTCA 1860
        ************************************************************

V2      AGAAGGGGACAGTGTCTGTTTCTTCTCAGCGAGAAAGTCAATTATCTAAAGCCAAAATGG 1920
V1      AGAAGGGGACAGTGTCTGTTTCTTCTCAGCGAGAAAGTCAATTATCTAAAGCCAAAATGG 1920
        ************************************************************

V2      TGGCCCAAACCGCCTCCGGTCCTCCAGCTCTGGAGTGGTCTGCCACAAACGAGGAGGATG 1980
V1      TGGCCCAAACCGCCTCCGGTCCTCCAGCTCTGGAGTGGTCTGCCACAAACGAGGAGGATG 1980
        ************************************************************
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V2      ACCTATCTGTGGAGGCTGAAATCGCTCACCAGATTGCTGAAAGCTTTTCCAAGAAGTATA 2040
V1      ACCTATCTGTGGAGGCTGAAATCGCTCACCAGATTGCTGAAAGCTTTTCCAAGAAGTATA 2040
        ************************************************************

V2      AATTTCCCTCTCGATCATCAGGGATATTCTTGTATAATTTTGAGCAGTTGAAGATGAACC 2100
V1      AATTTCCCTCTCGATCATCAGGGATATTCTTGTATAATTTTGAGCAGTTGAAGATGAACC 2100
        ************************************************************

V2      TGGATGACATCGTTAAGGAGTCAAAAAATGTGCCGAGCGTAACCCGCTTAGCCCACGATG 2160
V1      TGGATGACATCGTTAAAGAGTCAAAAAATGTGCCGAGCGTAACCCGCTTAGCCCACGATG 2160
        *************** ************************************************

V2      GATCCAAACTTCCTCTGAGGTGTGTGTTGGGGTGGGTCGCTCTAGCCAACTCCAAAAAGT 2220
V1      GATCCAAACTCCCTCTGAGGTGTGTGTTGGGGTGGGTCGCTCTAGCCAACTCCAAAAAGT 2220
        ********** ************************************************

V2      TCCAGCTGTTAGTTGAGCCTGACAAGCTAAATAAGATAATGCAAGACGACCTGAATCGTT 2280
V1      TCCAGCTGTTAGTTGAGCCTGACAAGCTAAATAAGATAATGCAAGACGACCTGAATCGTT 2280
        ************************************************************

                                                  <-P
V2      ATGTGTTCTGTTGACCACACCCTCAGGCTCAGTCCCACCA TGCAATCAAAACCAACCTGT 2340
V1      ATGTGTTCTGTTGACCACACCCCAGGCTCAGTCCCACCA TGCAATCAAAACCAACCTGT 2340
        ******************** ************************************

                          M->
V2      TCCAAATCCAATGTGAAAAAAA CAGGCAACACCACTGATAAGATGAACTTCCTACGCAAG 2400
V1      TCCAAATCCAATGTGAAAAAAACAGGCAACACCACTGATAAGATGAACTTCCTACGCAAG 2400
        ************************************************************

V2      ATAGTAAAGAACTGTAGAGATGAGGACACTCAGAAGCCCTCTCTCTTGTCGGCCCCTCCA 2460
V1      ATAGTAAAGAACTGTAGAGATGAGGACACTCAGAAGCCCTCTCTCTTGTCGGCCCCTCCA 2460
        ************************************************************

V2      GATGATGATGACTTGTGGTTACCCCCTCCGGAATATGTTCCGCTGAAGGAGCTCACAGGC 2520
V1      GATGATGATGACTTGTGGTTACCCCCTCCGGAATATGTTCCGCTGAAGGAGCTCACAGGC 2520
        ************************************************************

V2      AAGAAAAACATGAGGAACTTCTGTGTAAATGGAGAGGTCAAAGTGTGTAGTCCAAACGGC 2580
V1      AAGAAAAACATGAGGAACTTCTGTGTAAATGGAGAGGTCAAAGTGTGTAGTCCAAACGGC 2580
        ************************************************************

V2      TATTCATTTAGAATCCTGCGGCACATTTTGAAGTCATTCGACGAGATCTATTCTGGAAAT 2640
V1      TATTCATTTAGAATCCTGCGGCACATTTTGAAGTCATTCGACGAGATCTATTCTGGAAAT 2640
        ************************************************************

V2      CAAAGAATGATCGGGTTGGTTAAAGTTGTTGTTGGACTTGCACTGTCAGGGGCTCCAGTC 2700
V1      CAAAGAATGATCGGGTTGGTTAAAGTTGTTGTTGGACTTGCACTGTCAGGGGCTCCAGTC 2700
        ************************************************************

V2      CCGGAGGGCATGAACTGGGTATACAAATTGAGGAGAACTCTTATCTTCCAGTGGGCTGAC 2760
V1      CCGGAGGGCATGAACTGGGTATACAAATTGAGGAGAACTCTTATCTTCCAGTGGGCTGAC 2760
        ************************************************************

V2      TCTAGGGGCCCTCTAGAAGGGGAGGAGTTAGAATACTCTCAGGAGATTACATGGGACGAC 2820
V1      TCTAGGGGCCCTCTAGAAGGGGAGGAGTTAGAATACTCTCAGGAGATTACATGGGACGAC 2820
        ************************************************************

V2      GATGCCGAATTTGTCGGATTAGAAATACGAGTGAGTGCAAGGCAATGCCATATACAGGGC 2880
V1      GATGCCGAATTTGTCGGATTAGAAATACGAGTGAGTGCAAGGCAATGCCATATACAGGGC 2880
        ************************************************************

V2      AGGATTTGGTGTATTAACATGAACTCTAGGGCATGTCAACTATGGTCTGACATGTCTCTT 2940
V1      AGGATTTGGTGTATTAACATGAACTCTAGGGCATGTCAACTATGGTCTGACATGTCTCTT 2940
        ************************************************************

                                                          <-M
V2      CAAACACAAAGGTCTGAGGAGGACAAAGACTCTTCAGTGCTTCTGGAATAGTCAATTTAC 3000
V1      CAAACACAAAGGTCTGAGGAGGACAAAGACTCTTCAGTGCTTCTGGAATAGTCAATTTAC 3000
        ************************************************************
```

```
V2      ATCCTACAAATTCCTCAATTGTTTACCTCTGGAGGAGAGAGCACATGGACTTAACTCCAA 3060
V1      ATCCTACAAATTCCTCAATTGTTTACCTCTGGAGGAGAGAGCACATGGACTTAACTCCAA 3060
        ************************************************************

V2      CCTTCGGGAGCAATAGAACAAAAACATGTTATGGTGCCGTTGAATTGCTGCATTTTATCA 3120
V1      CCTTCGGGAGCAATAGAACAAAAACATGTTATGGTGCCGTTGAATTGCTGCATTTTATCA 3120
        ************************************************************
                                                              G->
V2      GAGTCAAATCAATTACCTTTGCATTTTTGAGCCTCTCGGGTGTGAAAAAAACAATT AACA 3180
V1      GAGTCAAATCAATTACCTTTGCATTTTTGAGCCTCTCGGGTGTGAAAAAAACAATT AACA 3180
        ************************************************************

V2      TCCCTCAAAAGACCTAAGGAAGATGATCCCTCACGCTCTTCTGTCTGTGCCTCTTCTAAT 3240
V1      TCCCTCAAAAGACCTAAGGAAGATGATCCCTCACGCTCTTCTGTCTGTGCCTCTTCTAAT 3240
        ************************************************************

V2      TTCCTCGTTGTGTTTCGGGAAATTCCCCATCTACACGATACCAGAGAAGCTCGGCCCTTG 3300
V1      TTCCTCGTTGTGTTTCGGGAAATTCCCCATCTACACGATACCAGACAAGCTCGGCCCTTG 3300
        ******************************************** **************

V2      GAGTCCCATCGACATACATCACCTCAGCTGTCCTAACAATTTGGTTGTGGAGGATGAGGG 3360
V1      GAGTCCCATCGACATACATCACCTCAGCTGTCCTAACAATTTGGTTGTGGAGGATGAGGG 3360
        ************************************************************

V2      ATGCAACAGTCTGTCAGGGTTTTCTTACATGGAACTGAAGGTGGGATACATCTCTGCCAT 3420
V1      ATGCAACAGTCTGTCAGGGTTTTCTTACATGGAACTGAAGGTGGGATACATCTCTGCCAT 3420
        ************************************************************

V2      AAAAGTTAATGGGTTCACTTGTACTGGTGTTGTGACAGAAGCTGAGACCTACACCAACTT 3480
V1      AAAAGTTAATGGGTTCACTTGTACTGGTGTTGTGACAGAAGCTGAGACCTACACCAACTT 3480
        ************************************************************

V2      TGTTGGTTACGTCACCACCACATTCAAGAGAAAACACTTCCGCCCTATGCCGGATGCATG 3540
V1      TGTTGGTTACGTCACCACCACATTCAAGAGAAAACACTTCCGCCCTATGCCGGATGCATG 3540
        ************************************************************

V2      CAGAGCCGCATACAACTGGAAGACGGCCGGCGATCCTAGATATGAAGAGTCCCTTCACAA 3600
V1      CAGAGCCGCATACAACTGGAAGACGGCCGGCGATCCTAGATATGAAGAGTCCCTTCACAA 3600
        ************************************************************

V2      TCCTTATCCTGATTACCATTGGCTAAGGACTGTAAAAACCACCAAAGAATCCCTTATTAT 3660
V1      TCCTTATCCTGATTACCATTGGCTAAGGACTGTAAAAACCACCAAAGAATCCCTTATTAT 3660
        ************************************************************

V2      CATATCGCCAAGTGTGGCTGATTTGGACCCCTACGACAAATCTCTTCATTCAAGAATCTT 3720
V1      CATATCGCCAAGTGTGGCTGATTTGGACCCCTACGACAAATCTCTTCATTCAAGAATCTT 3720
        ************************************************************

V2      CCCTGGTGGGAAATGCTTGGGAATAACAATCTCTTCCACCTACTGTTCAACTAACCATGA 3780
V1      CCCTGGTGGGAAATGCTTGGGAATAACAATCTCTTCCACCTACTGTTCAACTAACCATGA 3780
        ************************************************************

V2      TTATACTATCTGGATGCCCGAAGAAGCAAGACTCGGGACATCTTGTGACATTTTTACCAA 3840
V1      TTATACTATCTGGATGCCCGAAGAAGCAAGACTCGGGACATCTTGTGACATTTTTACCAA 3840
        ************************************************************

V2      CAGCAAAGGGAAGAGAGCATCCAAGGGGGGGTAGGACTTGTGGATTCGTGGATGAACGGGG 3900
V1      CAGCAAAGGGAAGAGAGCATCCAAGGGGGGGTAGGACTTGTGGATTCGTGGATGAACGGGG 3900
        ************************************************************

V2      CTTGTATAAGTCTCTAAAAGGGGCATGTAAACTTAAGCTGTGCGGAGTTCCTGGACTTAG 3960
V1      CTTGTATAAGTCTCTAAAAGGGGCATGTAAACTTAAGCTGTGCGGAGTTCCTGGACTTAG 3960
        ************************************************************

V2      ACTTATGGATGGAACGTGGGTCGCTATTCAGACACCAAATGAGACCAAATGGTGCTCTCC 4020
V1      ACTTATGGATGGAACGTGGGTCGCTATTCAGACACCAAATGAGACCAAATGGTGCTCTCC 4020
        ************************************************************

V2      TGATCAGCTGGTGAATGTACATGACTTTCGCTCAGATGAGATAGAACATCTCGTCGTGGA 4080
V1      TGATCAGCTGGTGAATGTACATGACTTTCGCTCAGATGAGATAGAACATCTCGTCGTGGA 4080
        ************************************************************
```

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V2      GGAGTTGATCAGGAAAAGGGAAGAATGTCTAGATGCACTGGAGTCCATCATGACTACAAA 4140
V1      GGAGTTGATCAGGAAAAGGGAAGAATGTCTAGATGCACTGGAGTCCATCATGACTACAAA 4140
        ************************************************************

V2      ATCAGTAAGCTTCAGGCGTCTCAGCAACTTGAGAAAACTTGTTCCTGGGTTTGGAAAGGC 4200
V1      ATCAGTAAGCTTCAGGCGTCTCAGCAACTTGAGGAAACTTGTTCCTGGGTTTGGAAAGGC 4200
        ******************************** ************************

V2      ATATACTATATTCAACAAAACCTTAATGGAGGCTGATGCTCACTACAAGTCAGTTCGGAC 4260
V1      ATATACTATATTCAACAAAACCTTAATGGAGGCTGATGCTCACTACAAGTCAGTTCGGAC 4260
        ************************************************************

V2      TTGGAATGAAATCATCCCCTCAAGAGGGTGCCTGAAAGTCGGAGGGAGGTGTCATCCTCA 4320
V1      TTGGAATGAAATCATCCCCTCAAGAGGGTGCCTGAAAGTCGGAGGGAGGTGTCATCCTCA 4320
        ************************************************************

V2      TGTGAATGGAGTGTTTTTCAATGGTATAATTTTGGGTCCGGATGGGCATATCCTGATTCC 4380
V1      TGTGAATGGAGTGTTTTTCAATGGTATAATTTTGGGTCCGGATGGGCATATCCTGATTCC 4380
        ************************************************************

V2      GGAGATGCAATCATCCCTCCTCCAACAGCATATGGAGCTGTTAGAATCCTCTGTAATCCC 4440
V1      GGAGATGCAATCATCCCTCCTCCAACAGCATATGGAGCTGTTAGAATCCTCTGTAATCCC 4440
        ************************************************************

V2      CTTAATACATCCCCTGGCAGACCCATCAACAGTCTTCAAGGACGGTGATGAGGCGGAGGA 4500
V1      CTTAATACATCCTCTGGCAGACCCATCAACAGTCTTCAAGGACGGTGATGAGGCGGAGGA 4500
        ************ ********************************************

V2      CTTTGTTGAGGTTCACCTTCCGGACGTTCACCAACAGGTCTCAGGGGTTGACCTCGGTCT 4560
V1      CTTTGTTGAGGTTCACCTTCCGGACGTTCACCAACAGGTCTCAGGGGTTGACCTCGGTCT 4560
        ************************************************************

V2      CCCAGACTGGGGGAAGTATGTGCTGATGAGTGCAGGTGCTCTAGCGACCGTGATGCTGAC 4620
V1      CCCAGACTGGGGGAAGTATGTGCTGATGAGTGCAGGTGCTCTAGCAACCGTGATGCTGAC 4620
        ******************************************** **************

V2      AATATTCTTGATAACCTGTTGCAGAAGGGTCAAGAGAACAGAATCAGTACAACAAGGTCT 4680
V1      AATATTCTTGATAACCTGTTGCAGAAGGGTCAAGAGTGCAGAATCAGTACAACAAGGTCT 4680
        ********************************** ***********************

V2      TGGTGAGTCAGGAAGGAAAGTGTCGGTAGCTCCCCAAAACGGAAAAGTCATGTCCTCATG 4740
V1      TGGTGAGTCAGGAAGGAAAGTGTCGGTAGCTCCCCAAAACGGAAAAGTCATGTCCTCATG 4740
        ************************************************************

V2      GGAATATTACAAGAGTGGAGGCGGGACCAGGCTGTGAGAGCAGGTCATCTCCTCCGTACC 4800
V1      GGAATATTACAAGAGTGGAGGCGGGACCAGGCTGTGAGAGCAGGTCATCTCCTCCGTACC 4800
        ************************************************************

V2      TTGCGTGTGGATGATCATCTCTCCTCTAGATCTGGGGAGATCTCTTGTTTTGACAGTCCT 4860
V1      TTGCGTGTGGATGATCATCTCTCCTCTAGATCTGGGGAGATCTCTTGTTTTGACAGTCCT 4860
        ************************************************************

V2      CTATGGACTTCGTGCGACTAGGTAAATTTAAGAGTCAAGAAACTTTCATTAATCATCTCA 4920
V1      CTATGGACTTCGTGCGACAAGGTAAATTTAAGAGTCAAGAAACTTTCATTAATCATCTCA 4920
        ****************** ****************************************

V2      ACTGATCAGACACAGTCATGTAGATTTTGATGATGTATGATTTCTTCTGACAGTATCAGT 4980
V1      ACTGATCAGACACAGTCATGTAGATTTTGATGATGTATGATTTCTTCTGACAGTATCAGT 4980
        ************************************************************

V2      GACTAATGGTGCTCTCATTTCCTAAGGACTGATACCAAAGGCTTTGGACAATCCAACTGA 5040
V1      GACTAATGGTGCTCTCATTTCCTAAGGACTGATACCAAAGGCTTTGGACAATCCAACTGA 5040
        ************************************************************

V2      TATATCGGCT---------------------------------------------------- 5050
V1      TATATCGGAT---------------------------------------------------- 5050
        ******** *
```

Table 2.5 Amino acid comparison between *Efv1* (V1) and *Efv2* (V2). The sequence begins at the N gene and continues through the G gene. Differences in deduced amino acid sequence are highlighted.

```
      |->N protein
V2    AAMQFFEGTCPDDWTSYGILIARKGDKITPNSLVDIKRTNVEGNWALTGGMELTRDPTVP 120
V1    AAMQFFEGTCPDDWTSYGILIARKGDKITPNSLVDIKRTNVEGNWALTGGMELTRDPTVP 120
      ************************************************************

V2    EHASLVGLLLSLYRLSKISGQNTGNYKTNIADRIEQIFETAPFVKIVEHHTLMTTHKMCA 180
V1    EHASLVGLLLSLYRLSKISGQNTGNYKTNIADRIEQIFETAPFVKIVEHHTLMTTHKMCA 180
      ************************************************************

V2    NWSTIPNFRFLAGTYDMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQTNLTARE 240
V1    NWSTIPNFRFLAGTYDMFFSRIEHLYSAIRVGTVVTAYEDCSGLVSFTGFIKQTNLTARE 240
      ************************************************************

V2    AILYFFHKNFEEEIRRMFEPGQETAVPHSYFIHFRSLGLSGKSPYSSNAVGHVFNLIHFV 300
V1    AILYLFHKNFEEEIRRMFEPGQETAVPHSYFIHFRSLGLSGKSPYSSNAVGHVFNLIHFV 300
      *****:******************************************************

V2    GCYMGQVRSLNATVIATCAPHEMSVLGGYLGEEFFGKGTFERRFFRDEKELQEYEAAEST 360
V1    GCYMGQVRSLNATVIATCAPHEMSVLGGYLGEEFFGKGTFERRFFRDEKELQEYEAAEST 360
      ************************************************************

V2    KTDVALADDGTVNSDDEDYFSGETRSPEAVYTRIMINGGRLKRSHIRRYVSVSSNHQARP 420
V1    KTDVALADDGTVNSDDEDYFSGETRSPEAVYTRIMINGGRLKRSHIRRYVSVSSNHQARP 420
      ************************************************************
                    |->M protein
V2    NSFAEFLNKTYSNDSMSKIFVNPSAIRAGLANLEMAEETVDLIAKNIEDNQAHLQGEPIE 480
V1    NSFAEFLNKTYSNDSMSKIFVNPSAIRAGLANLEMAEETVDLIAKNIEDNQAHLQGEPIE 480
      ************************************************************

V2    VDSLPEDMKRLQLEDEKPSGLGEMAKSGESKCQEDFQMDEGEDPALLFQSYLDNVGVHMV 540
V1    VDSLPEDMKRLQLEDEKPSGLGEMAKSGESKCQEDFQMDEGEDPALLFQSYLDNVGVHMV 540
      ************************************************************

V2    RQMRSGERFLKIWSHTVEEIISYVMVNFPSLPRKSSEDKATQTVNRELKKGTVSVSSQRE 600
V1    RQMRSGERFLKIWSHTVEEIISYVMVNFPSLPRKSSEDKATQTVNRELKKGTVSVSSQRE 600
      ************************************************************

V2    SQLSKAKMVAQTASGPPALEWSATNEEDDLSVEAEIAHQIAESFSKKYKFPSRSSGIFLY 660
V1    SQLSKAKMVAQTASGPPALEWSATNEEDDLSVEAEIAHQIAESFSKKYKFPSRSSGIFLY 660
      ************************************************************

V2    NFEQLKMNLDDIVKESKNVPSVTRLAHDGSKLPLRCVLGWVALANSKKFQLLVEPDKLNK 720
V1    NFEQLKMNLDDIVKESKNVPSVTRLAHDGSKLPLRCVLGWVALANSKKFQLLVEPDKLNK 720
      ************************************************************
                  |->P protein
V2    IMQDDLNRYVFCMNFLRKIVKNCRDEDTQKPSLLSAPPDDDDLWLPPPEYVPLKELTGKK 780
V1    IMQDDLNRYVFCMNFLRKIVKNCRDEDTQKPSLLSAPPDDDDLWLPPPEYVPLKELTGKK 780
      ************************************************************

V2    NMRNFCVNGEVKVCSPNGYSFRILRHILKSFDEIYSGNQRMIGLVKVVVGLALSGAPVPE 840
V1    NMRNFCVNGEVKVCSPNGYSFRILRHILKSFDEIYSGNQRMIGLVKVVVGLALSGAPVPE 840
      ************************************************************

V2    GMNWVYKLRRTLIFQWADSRGPLEGEELEYSQEITWDDDAEFVGLEIRVSARQCHIQGRI 900
V1    GMNWVYKLRRTLIFQWADSRGPLEGEELEYSQEITWDDDAEFVGLEIRVSARQCHIQGRI 900
      ************************************************************
                                |-> G protein
V2    WCINMNSRACQLWSDMSLQTQRSEEDKDSSVLLEMIPHALLSVPLLISSLCFGKFPIYTI 960
V1    WCINMNSRACQLWSDMSLQTQRSEEDKDSSVLLEMIPHALLSVPLLISSLCFGKFPIYTI 960
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V2    PEKLGPWSPIDIHHLSCPNNLVVEDEGCNSLSGFSYMELKVGYISAIKVNGFTCTGVVTE 1020
V1    PDKLGPWSPIDIHHLSCPNNLVVEDEGCNSLSGFSYMELKVGYISAIKVNGFTCTGVVTE 1020
      *:************************************************************


V2    AETYTNFVGYVTTTFKRKHFRPMPDACRAAYNWKTAGDPRYEESLHNPYPDYHWLRTVKT 1080
V1    AETYTNFVGYVTTTFKRKHFRPMPDACRAAYNWKTAGDPRYEESLHNPYPDYHWLRTVKT 1080
      ************************************************************

V2    TKESLIIISPSVADLDPYDKSLHSRIFPGGKCLGITISSTYCSTNHDYTIWMPEEARLGT 1140
V1    TKESLIIISPSVADLDPYDKSLHSRIFPGGKCLGITISSTYCSTNHDYTIWMPEEARLGT 1140
      ************************************************************

V2    SCDIFTNSKGKRASKGGRTCGFVDERGLYKSLKGACKLKLCGVPGLRLMDGTWVAIQTPN 1200
V1    SCDIFTNSKGKRASKGGRTCGFVDERGLYKSLKGACKLKLCGVPGLRLMDGTWVAIQTPN 1200
      ************************************************************

V2    ETKWCSPDQLVNVHDFRSDEIEHLVVEELIRKREECLDALESIMTTKSVSFRRLSNLRKL 1260
V1    ETKWCSPDQLVNVHDFRSDEIEHLVVEELIRKREECLDALESIMTTKSVSFRRLSNLRKL 1260
      ************************************************************

V2    VPGFGKAYTIFNKTLMEADAHYKSVRTWNEIIPSRGCLKVGGRCHPHVNGVFFNGIILGP 1320
V1    VPGFGKAYTIFNKTLMEADAHYKSVRTWNEIIPSRGCLKVGGRCHPHVNGVFFNGIILGP 1320
      ************************************************************

V2    DGHILIPEMQSSLLQQHMELLESSVIPLIHPLADPSTVFKDGDEAEDFVEVHLPDVHQQV 1380
V1    DGHILIPEMQSSLLQQHMELLESSVIPLIHPLADPSTVFKDGDEAEDFVEVHLPDVHQQV 1380
      ************************************************************

V2    SGVDLGLPDWGKYVLMSAGALATVMLTIFLITCCRRVKRTESVQQGLGESGRKVSVAPQN 1440
V1    SGVDLGLPDWGKYVLMSAGALATVMLTIFLITCCRRVKSAESVQQGLGESGRKVSVAPQN 1440
      ************************************ :********************

V2    GKVMSSWEYYKSGGGTRL 1458
V1    GKVMSSWEYYKSGGGTRL 1458
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RESULTS

Bats were held in quarantine within a biosafety level 3 facility in groups of 4-5 for at least 6 months. All bats entering the captive colony were bled from the uropatagial vein, weighed, and an oral swab and a wing biopsy were taken. Blood was tested for the presence of anti-rabies viral neutralizing antibodies (VNAs) (Table 2.6).

Bats inoculated with *Efv1* virus

Five of the six bats that received 10^4 $TCID_{50}$ (group 1) demonstrated an increase in anti-rabies viral neutralizing antibodies within two months of the first inoculation. All bats receiving 10^3 $TCID_{50}$ (group 3) seroconverted within the first month of inoculation. Five of the six bats that received 10^2 $TCID_{50}$ (group 5) seroconverted within six months after the first inoculation. All bats in groups 1 and 3 survived the duration of the study and most maintained circulating anti-rabies viral neutralizing antibodies (VNAs) for at least two months. Following the six month challenge, *Efu* 6 in group 5, developed clinical signs compatible with rabies virus infection three weeks following second rabies inoculation and was euthanized. A brain smear was positive for rabies viral antigen. An oral swab from this bat was positive 13 days following the second challenge (four days prior to clinical signs), but no other oral swab from this bat was positive (Table 2.7, figure 2.1)

Bats inoculated with *Efv2* virus

An increase in circulating anti-rabies VNAs occurred in all bats in group 2 (seropositive bats) following the primary intramuscular challenge. Three of these bats remained seropositive at month six of the study and all bats in group 2 had detectable levels of circulating VNAs following the six-month challenge. All bats exposed to 10^3

$TCID_{50}$ (group 4) became seropositive within one month following the primary intramuscular challenge. Within one month, four of the six bats died of rabies and the remaining two developed clinical signs compatible with rabies virus infection including unusual vocalizations, anorexia, aggression, ataxia, and disorientation. Both were fed beef baby food and given 0.5ml injections of SQ lactated ringer's solution for two days. Bat *Efu* 2 in group 4 had more mild clinical signs than *Efu* 3 in group 4 and began to improve, 12 hours before *Efu* 3, 36 hours following the onset of clinical signs. Virus was isolated from the oral swabs from both bats during clinical illness. Both bats recovered but *Efu* 3 demonstrated neurological sequelae including lack of grooming, copious food consumption, weight gain, irritability, and somnolence. By month 5, VNAs in both bats were undetectable (Table 2.7, Figure 2.2).

Four of the five bats exposed to 10^2 $TCID_{50}$ *Efv2* (group 6) developed anti-rabies antibodies within two weeks. Two bats died of rabies prior to the one month bleed including the one that remained seronegative. All three remaining bats were seropositive. Only one bat was seropositive at the 4-month bleed and by month six, all *Efv2* bats were seronegative (Figure 2.2).

Mice Inoculated with *Efv1* and *Efv2* Viruses

Within one month following the primary intramuscular challenge six mice and developed VNA. One mouse in the *Efv2* 10^3 $TCID_{50}$ group died of rabies 29 days following inoculation. All mice were seronegative within three months of the primary intramuscular challenge. All mice exposed to *Efv1* survived the experiment. Following the six-month challenge with Efv3, three mice demonstrated an increase in circulating anti-rabies antibodies (Figure 2.3)

Table 2.6. Range of rabies VNA titers in bats within each group during the six month quarantine period. All bats were bled the day they entered the captive colony at CSU. The sera was tested for the presence or absence of anti-rabies VNAs, results are reported in International Units (IU A titer of ≤0.0625 indicates antibodies were not present or below the limit of detected of the RFFIT. Primary virus challenge was performed on 5/15/03, two weeks following the last blood sample.

Variant[a]	Virus Dose[b] ($TCID_{50}$)	Group[c]	1 day[d]	2mo[e]	4mo[e]	6mo[e,f]
Efv1	10^4	1	≤0.0625-0.6	≤0.0625	≤0.0625	≤0.0625
Efv2	10^3	2	≤0.0625 –8.4	≤0.0625 -9.8	≤0.0625 –0.7	≤0.0625-3
Efv1	10^3	3	≤0.0625	≤0.0625	≤0.0625	≤0.0625
Efv2	10^3	4	≤0.0625 -1.3	≤0.0625	≤0.0625	≤0.0625
Efv1	10^2	5	≤0.0625	≤0.0625 -0.6	≤0.0625	≤0.0625
Efv2	10^2	6	≤0.0625	≤0.0625	≤0.0625	≤0.0625

[a] After the six month quarantine period, this column lists the variant that each bat in the group was exposed to.
[b]The amount of virus inoculated i.m. at the time of the first challenge, six months after the quarantine period.
[c]All bats were placed into one of six groups in the colony. All bats were placed in their respective group following the results of the serology and remained in the group throughout the duration of the experiment.
[d]The numbers in this column are the serology results from the first bleed and helped us determine which group to place each bat.
[e]These are the serology results 2, 4, and 6 months after the bats entered the colony.
[f]All bats were bled after six months of quarantine. Bats were first challenged two weeks after this bleed.

Table 2.7 Range of antibody titers of bats in experiment 1. Primary challenge inoculation employing 10^2-10^4 *Efv1* or 10^2–10^3 *Efv2* on 5/15/03. A titer of ≤0.0625 indicates antibodies were not present or below the limit of detected of the RFFIT.

[a] A second challenge inoculation was done on 11/29/06 employing 10^4 *Efv3*.

Variant	Dose	Group	Number of bats	Bleeding date						
				5/29/2003	6/15/2003	7/11/2003	9/1/2003	10/2/2003	11/1/2003[a]	12/1/2003[b]
Efv1	10^4	1	6	≤0.0625-7	≤0.0625-1.8	≤0.0625-7	≤0.0625	≤0.0625	≤0.0625	≤0.0625-7
Efv2	10^3	2	5	≤0.0625-37.9	≤0.0625-36.7	≤0.0625-7.7	≤0.0625-0.9	≤0.0625	≤0.0625	≤0.0625-1
Efv1	10^3	3	6	≤0.0625-2.5	≤0.0625-1.7	≤0.0625-36.7	≤0.0625-0.9	0.0625	≤0.0625	≤0.0625-1
Efv2	10^3	4[c]	6	≤0.0625-11.4	≤0.0625-219	7.3-138	1-7.3	≤0.0625	≤0.0625	≤0.0625-20
Efv1	10^2	5	6	≤0.0625-1.6	≤0.0625-1.4	≤0.0625-36.7	0.0625-1	≤0.0625-0.9	≤0.0625	≤0.5-20
Efv2	10^2	6	5	≤0.0625-36.7	1.6-37.9	≤0.0625-8.4	≤0.0625	≤0.0625	≤0.0625	≤0.0625-0.9
Efv2	10^2	6	5	≤0.0625-36.7	1.6-37.9	≤0.0625-8.4	≤0.0625	≤0.0625	≤0.0625	≤0.0625-0.9

[b] All bats were euthanized on 12/1/03 indicating the last serum sample obtained.
[c] Two bats in this group developed signs compatible with clinical rabies virus infection and were not challenged a second time

Oral Swabs

With few exceptions, all the oral swabs were negative for virus isolation. All positive swabs were taken within two weeks of clinical illness. Thirteen days prior to clinical illness and fourteen days prior to euthanasia, rabies was isolated in cell culture from an oral swab from a bat in group 6. Four days prior to clinical signs, rabies virus was isolated from an oral swab from a bat in group 4. Rabies virus was isolated from oral swabs from bats *Efu* 2 and 3 (group 4), seventeen days following inoculation. Both were demonstrating clinical signs of rabies at the time the swabs were collected.

***Efv1* and *Efv2* bats following 6 month challenge**

Following the second intramuscular challenge on 11/29/03, the majority of bats in groups 1, 5, and 6 demonstrated an increase in circulating antibodies. All of the bats in groups 2, 3, and 4 had a similar response (Table 2.7, Figures 2.1, 2.2). Following euthanasia, salivary glands were removed from all rabid bats during necropsy. In four of the six bats, there was no viral antigen present in the salivary glands when examined with the dFAT. A small amount of antigen was present in the salivary glands from a bat exposed to *Efv* 2 10^3 $TCID_{50}$ but virus was not isolated in cell culture. Virus was isolated in cell culture from the salivary gland homogenate of a bat inoculated with 10^2TCID_{50} *Efv* 2 virus.

Oral Swabs

Four days prior to the onset of clinical signs, rabies virus was isolated from an oral swab from an asymptomatic bat that had been exposed to *Efv*1 10^2TCID_{50} during the first challenge. This bat developed clinical signs of rabies virus infection 17 days following the second rabies virus challenge

Figure 2.1 A Bats were inoculated with 10^4 $TCID_{50}$ *Efv 1*. Bats were bled two weeks following inoculation, then monthly. Six months following the first inoculation, all bats were inoculated a second time with 10^4 $TCID_{50}$ *Efv 3*. Bats were bled two weeks following the second inoculation.

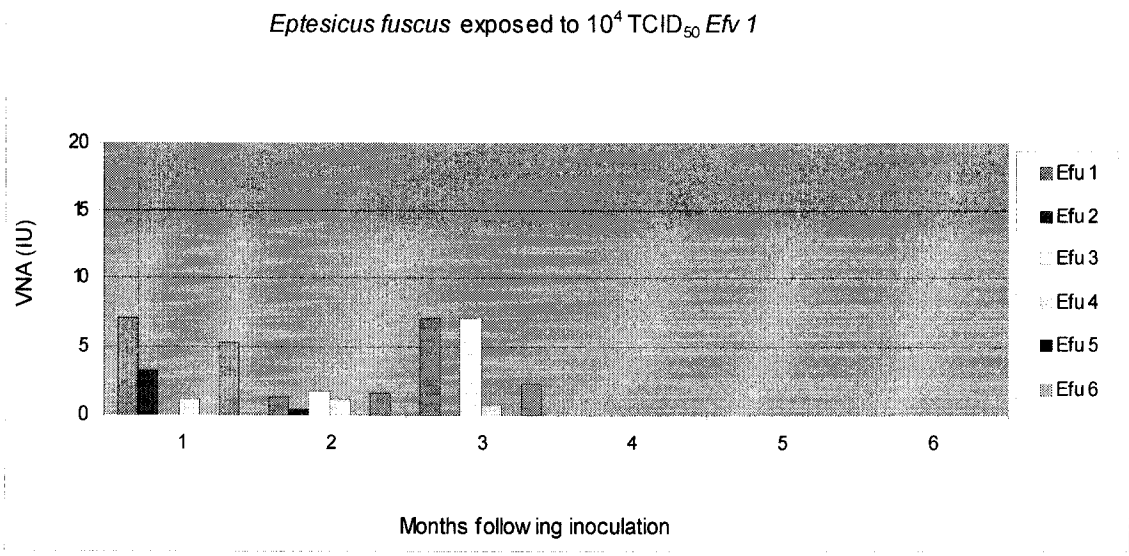


Figure 2.1 B Bats were inoculated with 10^3 $TCID_{50}$ *Efv 1*. Bats were bled two weeks following inoculation, then monthly. Six months following the first inoculation, all bats were inoculated a second time with 10^4 $TCID_{50}$ *Efv 3*. Bats were bled two weeks following the second inoculation.

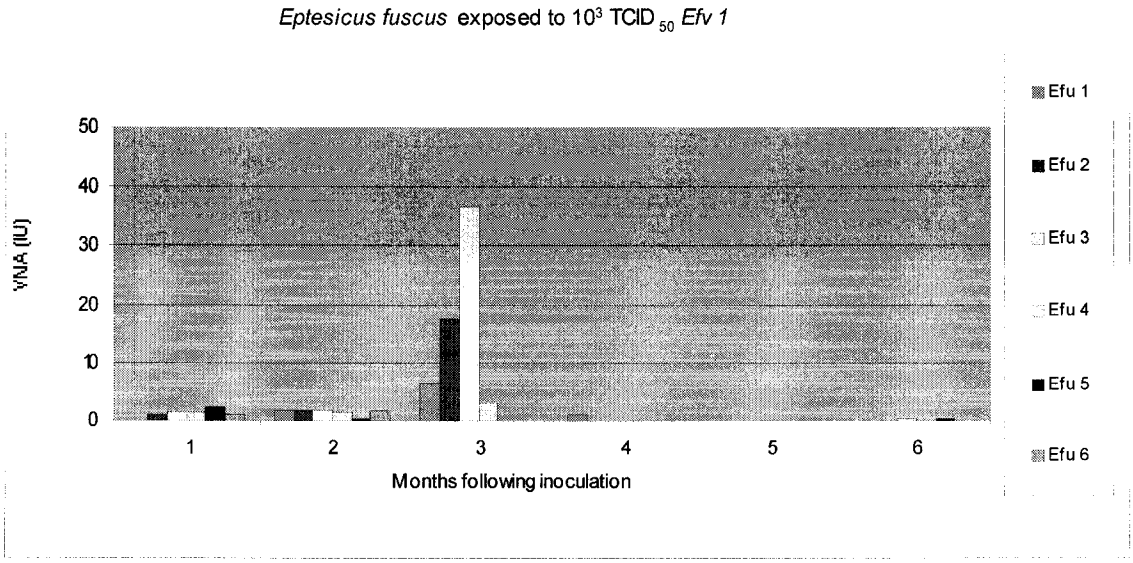


Figure 2.1 C Bats were inoculated with 10^2 $TCID_{50}$ *Efv 1*. Bats were bled two weeks following inoculation, then monthly. Six months following the first inoculation, all bats were inoculated a second time with 10^4 $TCID_{50}$ *Efv 3*. Bats were bled two weeks following the second inoculation.

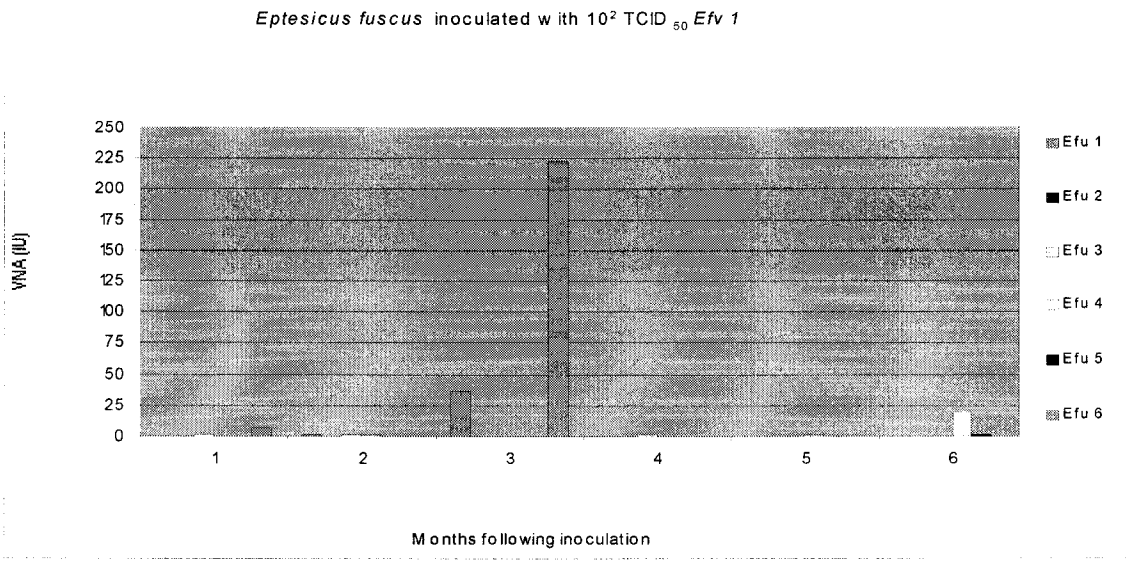


Figure 2.2 A Bats were inoculated with 10^3 $TCID_{50}$ *Efv 2*. Bats were bled two weeks following inoculation, then monthly. Six months following the first inoculation, bats were inoculated a second time with 10^4 $TCID_{50}$ *Efv 3*. Bats were bled two weeks following the second inoculation

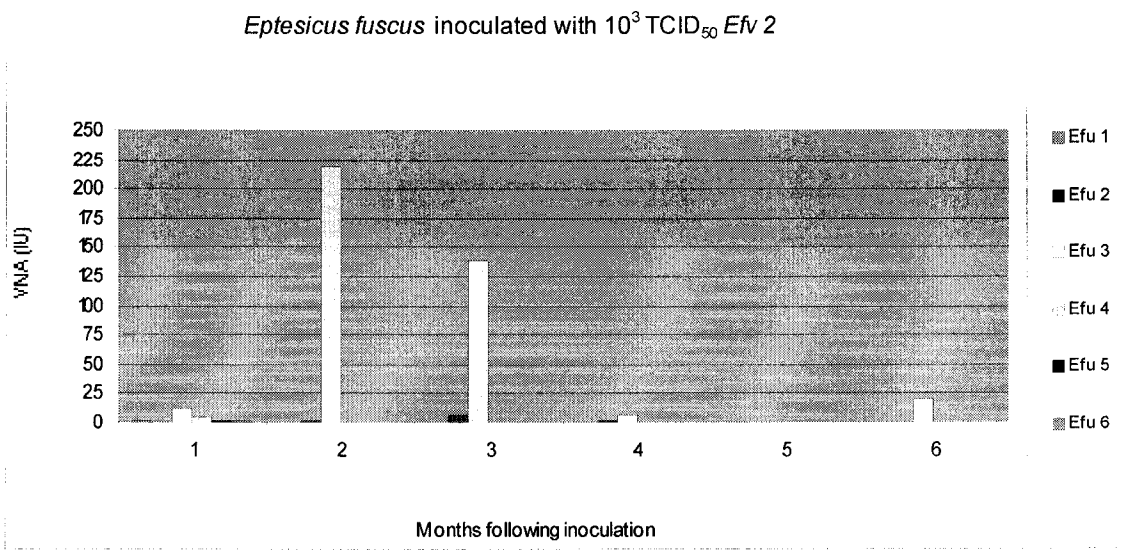


Figure 2.2 B. Bats were inoculated with 10^3 $TCID_{50}$ *Efv 2*. Bats were bled two weeks following inoculation, then monthly. Six months following the first inoculation, bats were inoculated a second time with 10^4 $TCID_{50}$ *Efv 3*. Bats were bled two weeks following the second inoculation.

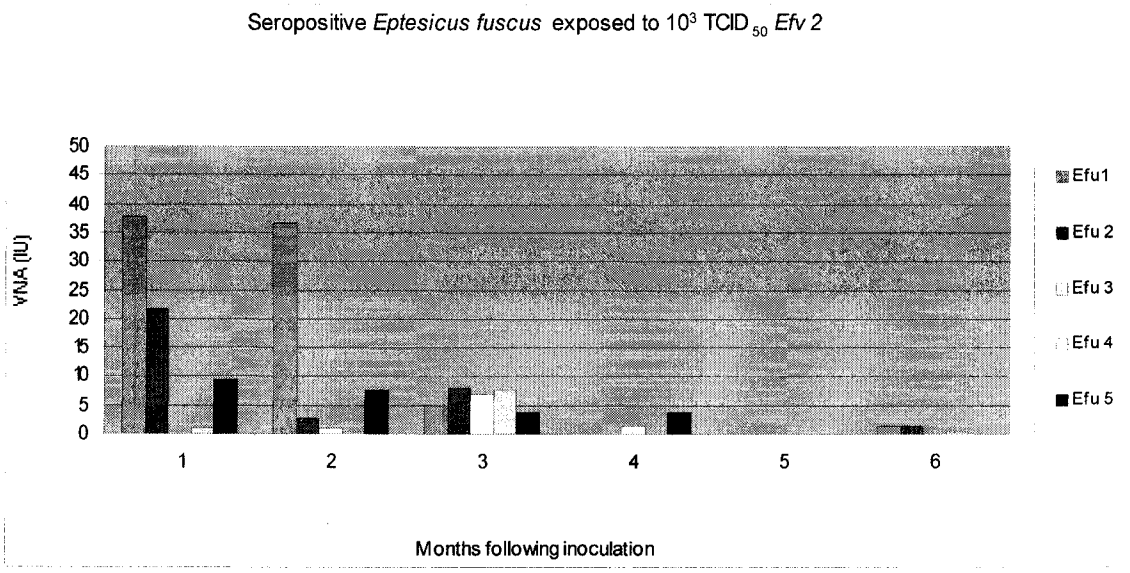


Figure 2.2 C Bats were inoculated with 10^2 $TCID_{50}$ *Efv 2*. Bats were bled two weeks following inoculation, then monthly. Six months following the first inoculation, bats were inoculated a second time with 10^4 $TCID_{50}$ *Efv 3*. Bats were bled two weeks following the second inoculation.

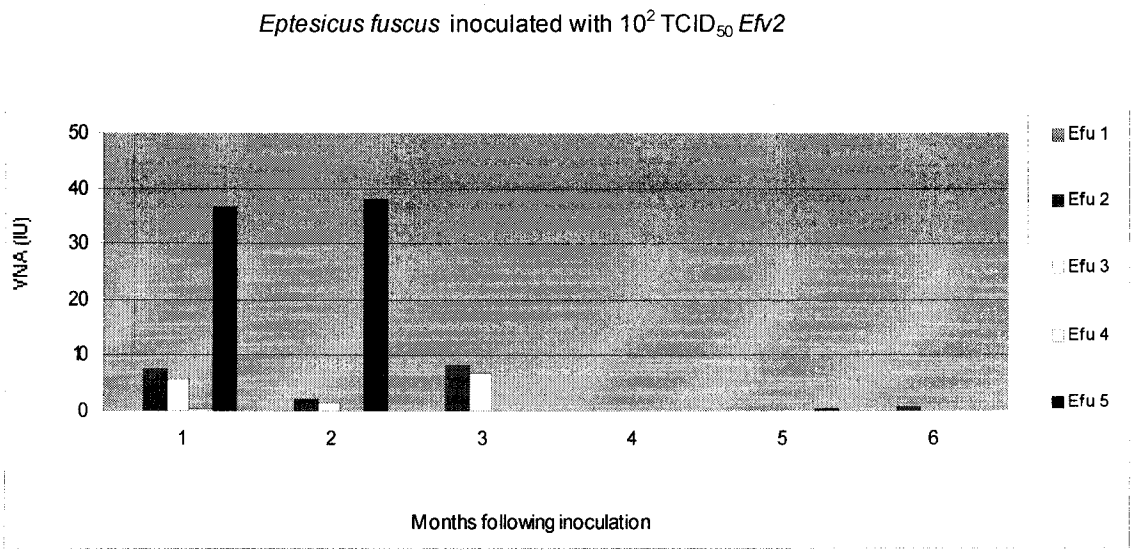


Figure 2.3 A. Mice were inoculated with either 10^4 $TCID_{50}$ *Efv 2*. Mice were bled two weeks following inoculation, then monthly. Six months following the first inoculation, mice were inoculated a second time with 10^4 $TCID_{50}$ *Efv 3*. Mice were bled two weeks following the second inoculation.

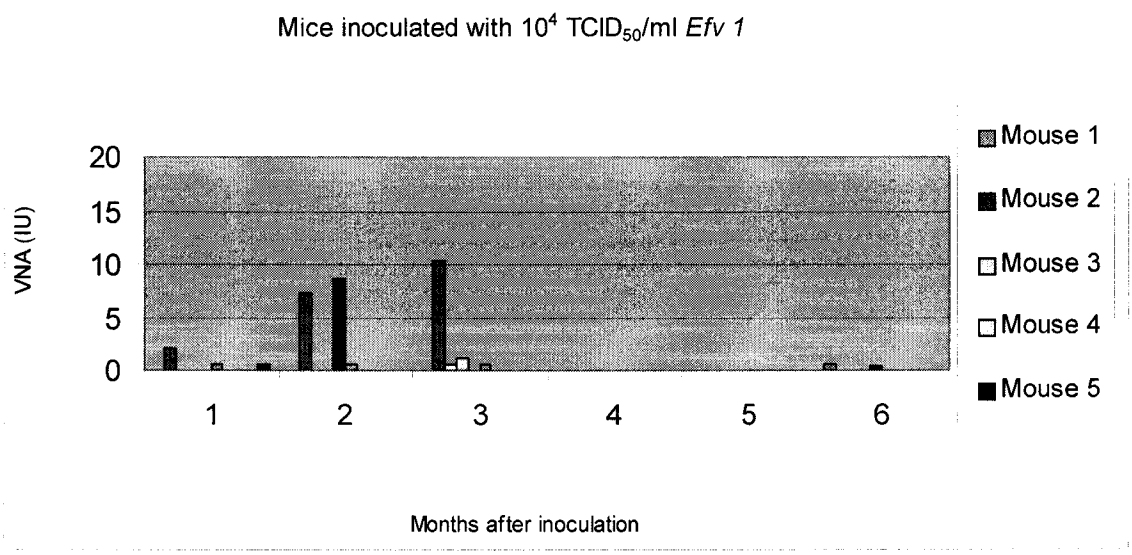


Figure 2.3 B. Mice were inoculated with 10^3 $TCID_{50}$ *Efv 2*. Mice were bled two weeks following inoculation, then monthly. Six months following the first inoculation, mice were inoculated a second time with 10^4 $TCID_{50}$ *Efv 3*. Mice were bled two weeks following the second inoculation.

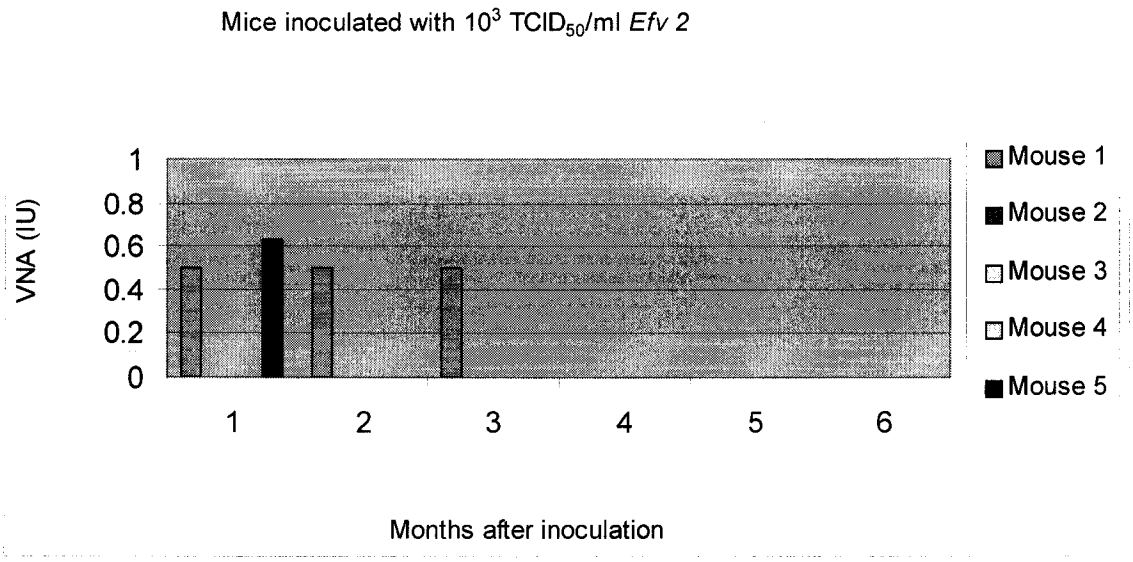
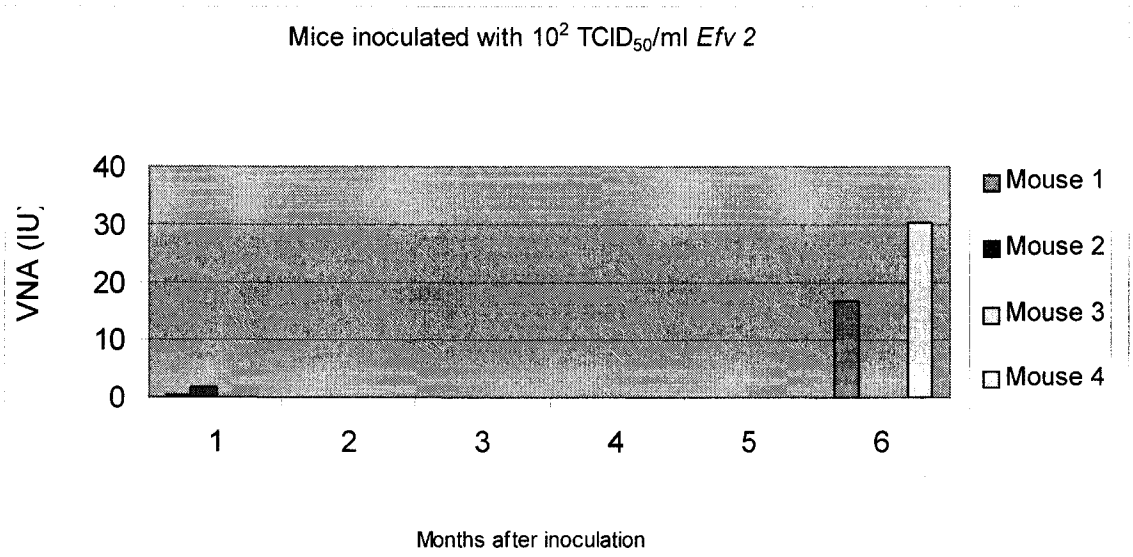


Figure 2.3 C. Mice were inoculated with 10^2 $TCID_{50}$ *Efv 2*. Mice were bled two weeks following inoculation, then monthly. Six months following the first inoculation, mice were inoculated a second time with 10^4 $TCID_{50}$ *Efv 3*. Mice were bled two weeks following the second inoculation.



2.4 EXPERIMENT II

MATERIALS AND METHODS

Viral isolates were obtained and processed as described in Experiment 1. Since the amount of *Efv1* and *Efv2* inoculum remaining following Experiment 1 was inadequate for the second experiment, both isolates were passed three times in mouse neuroblastoma cells to obtain enough virus for the animal inoculations of Experiment 2.

Following three passages in cell culture, both virus isolates, *Efv1* and *Efv2*, were amplified with the primers 1087Deg and 304 as described in Experiment 1 and had identical sequences. Following the passages in neuroblastoma cells, the sequences were further compared employing primers L5 and 304; the two were identical to the sequences determined in Experiment 1.

The challenge virus in Experiment 2 was *Efv* 2. Table 2.1 details the primer sequences employed. The techniques employed in animal capture, quarantine, husbandry, molecular analysis, serology, virus isolation, and antigen detection in the brain and salivary glands were identical to the descriptions in Experiment 1

Bats inoculated with *Efv1* and *Efv2* Viruses

During Experiment 2, all bats were inoculated with 10^4 $TCID_{50}$ of the passaged *Efv1* or *Efv 2* virus in the right deltoid muscle. Following virus inoculation, body weights and oral swabs were collected twice weekly. Bats were checked at least twice a day for changes in behavior. Animals with a weight loss of >2g were placed in an isolation cage and hand fed beef baby food and given subcutaneous lactated ringers saline. Any animal that deteriorated within 24 hours was anesthetized with pentobarbital and terminally bled by cardiac puncture. Serum was separated by centrifugation for 5 minutes and stored at

–80C until processing. All bats were necropsied and a brain smear was made to identify the presence or absence of rabies antigen as described in the rabies compendium (CDC, 2006).

During Experiment 2, bats surviving the primary challenge were challenged a second time, six months after the primary challenge, with 10^4 $TCID_{50}$ *Efv* 2. All inoculated bats were bled two weeks prior to and two weeks after the second inoculation then monthly for the next 5 months. Oral swabs and weighing continued twice weekly.

Six months after the second intramuscular challenge, bats were anesthetized with pentobarbital and were terminally bled though cardiac puncture. Approximately 0.03g portion of the brain was used for dFA, the remainder was stored at -80^0C.

Mice Inoculated with *Efv1* and *Efv2* Viruses

During Experiment 2, ten mice were inoculated intramuscularly: five mice were inoculated with 10^4 $TCID_{50}$ *Efv1*and five were inoculated with 10^4 $TCID_{50}$ *Efv2.* All mice were sedated with ketamine/xylazine combination prior to intramuscular inoculation.

All mice that survived the primary challenge described above were challenged intramuscularly a second time six months later with 10^4 $TCID_{50}$ *Efv2* virus. In both experiments, all mice surviving the second challenge were sedated with an intraperitoneal injection of pentobarbital (80mg/kg body weight) and terminally bled by cardiac puncture. Approximately 0.03g of tissue from the brain was used for dFA, the remainder was stored at -80^0C.

RESULTS

Bats Inoculated with *Efv1* Virus

Following the primary challenge, all bats inoculated with10^4 $TCID_{50}$ *Efv1* developed anti-rabies VNAs. All bats, with the exception of bat #327, maintained VNAs six months post inoculation. At the three month bleeding, bat # 327 was seronegative but at the four month bleed a low level of anti-rabies VNAs was detected. Five months post inoculation bat #327 developed clinical signs compatible with rabies virus infection and was euthanized. Rabies antigen was demonstrated in the brain, yet results of the terminal bleed showed no detectable level of anti-rabies VNAs. It is interesting to note that bat 339, a female, spent her days roosting from the roof of the cage with her wings wrapped around bat 339 a male. Bat 339, housed with bat 327 was attacked the night before bat 327 demonstrated clinical signs of rabies virus infection. The bats were separated, bat 327 was euthanized, and bat 339 was hand fed and given SQ lactated ringer's saline. Bat 339 died two days later of undetermined causes, likely the result of severe emotional distress after being attacked and separated from bat 327. Bat 339 was tested for rabies and found negative (Table 2.8, Figure 2.4).

Bat 083 was housed with bat 328 and developed clinical signs compatible with rabies virus infection 6 months after primary inoculation and was euthanized. The brain smear was positive for rabies antigen. Eight weeks following the death of 083, bat 328 developed clinical signs of rabies virus infection. The brain of bat 328 was positive for rabies antigen and the infecting variant was the *Efv 1* rabies variant. It is unknown if bat 328 contracted rabies though a bite from 083 or had been incubating rabies virus for 8 months. Because the infecting variant was the *Efv 1* variant, one can be sure the infection was not the result of the six month challenge

Bats Inoculated with *Efv2* Virus

All bats inoculated with 10^4TCID$_{50}$ *Efv2* developed anti-rabies VNAs within one month of inoculation. Approximately one month following the primary challenge, bat 065 developed clinical signs compatible with rabies virus infection. Bat 065 was euthanized and was rabid on dFA. Bats 779 and 597 developed clinical signs compatible with rabies virus infection two months following the primary challenge. Both were euthanized and found to be rabid on dFAT. Anti-rabies VNAs continued to be present in the remaining two bats. Anti-rabies VNAs were highest in all bats one month post inoculation and remained high two months post inoculation. The levels of circulating VNAs decreased sharply three months following the primary inoculation (Table 2.8, Figure 2.2).

Table 2.8 Experiment 2 Bats Antibody titers.

The primary challenge inoculation was done on 7/13/04. Five bats were exposed to *Efv1* 10^4 TCID$_{50}$ and five bats were exposed to *Efv2* 10^4 TCID$_{50}$. Bats were bled two weeks following inoculation, then monthly. Numbers represent the range of antibody titers in International Units (IU). A titer of ≤0.0625 indicates antibodies were not present or below the limit of detected of the RFFIT.

Virus	Date of bleeding						
	7/29/04	8/31/04	9/30/04	10/20/04	11/9/04	12/19/04	1/24/04
Efv1 10^4	0.9-3.5	0.8-63.7	≤0.0625-22.8	0.9-3.5	≤0.0625 - 2.4	≤0.0625 - 1.2	≤0.0625 - 1.3
Efv2 10^4	≤0.0625 - 3	13.6-59	≤0.0625 - 28.6	≤0.0625 – 1	0.62-0.8	0.9-3.8	2.3-2.6

Table 2.9. Variant of rabies isolated from the salivary glands of rabid bats. The homogenates were inoculated into cell culture to determine titer. The RNA of the infecting variant was analyzed by RT-PCR and sequence determination.

Infecting Variant	Bat ID	Titer in culture
Efv1	83	10*3.5
Efv1	327	10*3
Efv1	328	Neg
Efv2	65	Neg
Efv2	597	Neg
Efv2	779	Neg

Mice Inoculated with *Efv1* and *Efv2* Viruses

Three of the five mice exposed to *Efv1* developed anti-rabies VNAs within the first two weeks of first inoculation. Within three months, all five mice had demonstrable antibody titers. Table 2.10, Figure 2.5. One mouse that became clinically rabid two weeks following inoculation died prior to the first bleeding date.

Table 2.10 Experiment 2, mouse antibody titers following the primary challenge inoculation performed on 7/13/04. Five mice were inoculated intramuscularly with *Efv1* 10^4 $TCID_{50}$ and five others were inoculated intramuscularly with *Efv2* 10^4 $TCID_{50}$. Mice were bled two weeks following inoculation, then monthly. Numbers represent the range of titers in International Units (IU). A titer of ≤0.0625 indicates antibodies were not present or below the limit of detected of the RFFIT.

Dose	Date of bleeding						
	7/29/04	8/31/04	9/30/04	10/20/04	11/9/04	12/19/04	1/24/05
Efv1 10^4	≤0.0625-0.8	≤0.0625	≤0.0625	≤0.0625-0.7	≤0.0625-1.1	≤0.0625-0.9	≤0.0625 –1
Efv2 10^4	≤0.0625	≤0.0625	≤0.0625	≤0.0625	≤0.0625–51	≤0.0625-4.9	≤0.0625–39

Six Month Challenge of Bats Inoculated with *Efv1* and *Efv2* Viruses

Following the six-month challenge with *Efv2*, one of the five bats in the *Efv1* group and all the remaining bats in the *Efv2* group demonstrated an increase in VNA. One bat in the *Efv1* group that did not demonstrate an increase in VNA died of rabies following the six-month challenge. Surviving bats in the *Efv2* group remained healthy throughout the duration of the study and two were seronegative at the terminal blood collection (Table 2.11, Figure 2.4).

Table 2.11 Experiment 2 bats, antibody titers after six-month challenge.

A second challenge with *Efv2* 10^4 $TCID_{50}$ via intramuscular injection occurred on 1/22/05, six months following the primary challenge. Bats were bled two weeks prior and two weeks following inoculation, then monthly. Numbers represent range of titers in international units. A titer of ≤0.0625 indicates antibodies were not present or below the limit of detected of the RFFIT.

Virus[a]	2/9/04	2/23/05	3/30/05	4/28/05	6/7/05	7/18/05	8/18/05
Efv1	≤0.0625	≤0.0625-2.9	≤0.0625	≤0.0625-6.3	≤0.0625-0.8	≤0.0625-2.8	≤0.0625
Efv2	≤0.0625	≤0.0625-1.8	≤0.0625	1.4-10.2	2.1-10	1.5	≤0.0625

[a]The rabies virus variant bats were inoculated with during the first virus challenge.

Efv1 and *Efv2* Mice Following the Second Challenge

The mouse with the highest titer, > 32IU, four months after the primary challenge with 10^4 $TCID_{50}$ *Efv* 2, developed clinical signs of rabies infection one month following the second challenge. Two weeks prior to clinical signs, this mouse was seronegative. Approximately one month following the second challenge with 10^4 $TCID_{50}$ *Efv* 2, an increase in circulating VNA's was demonstrated in all the mice that were first challenged with 10^4 $TCID_{50}$ *Efv* 2. Titers in mice that were initially challenged with 10^4 $TCID_{50}$ *Efv* 1 were lower following the six-month challenge with 10^4 $TCID_{50}$ *Efv* 2 than mice initially challenged with 10^4 $TCID_{50}$ *Efv* 2 virus (Table 2.12, Figure 2.5).

Table 2.15 Experiment 2; Antibody titers of mice following the second challenge with 10^4 $TCID_{50}$ *Efv* 2 Following primary challenge on 7/13/04 with either 10^4 $TCID_{50}$ *Efv* 1 or 10^4 $TCID_{50}$ *Efv* 2, mice were challenged on 2/11/04 with 10^4 $TCID_{50}$ *Efv* 2. Mice were bled two weeks prior to and following the second challenge, then monthly. A titer of ≤0.0625 indicates antibodies were not present or below the limit of detected of the RFFIT.

Dose[a]	Date of bleeding					
	2/23/05	3/30/05	4/28/05	6/7/05	7/18/05	8/18/05[b]
Efv1 10^4	≤0.0625[c]	≤0.0625 –14	≤0.0625 -0.8	≤0.0625	≤0.0625 -1.3	≤0.0625
Efv2 10^4	≤0.0625 – 1.3	22.9-39	0.8-11.3	≤0.0625	0.7-1.7	≤0.0625

[a] Dose and variant of rabies virus variant mice were inoculated with during the first challenge.
[b] All mice were euthanized and bled on 8/18/05
[c] Numbers represent range of titers in International Units (IU).

Figure 2.4 *Eptesicus fuscus* bats were inoculated with 10^4 $TCID_{50}$ *Efv 1* . Bats were bled two weeks following inoculation, then monthly. Six months following the first inoculation, bats were inoculated a second time with 10^4 $TCID_{50}$ *Efv 2*. All bats were bled two weeks following the second inoculation, then monthly. Twelve months after the first inoculation, all animals were euthanized. Data at 12 months post inoculation is from the terminal bleed.

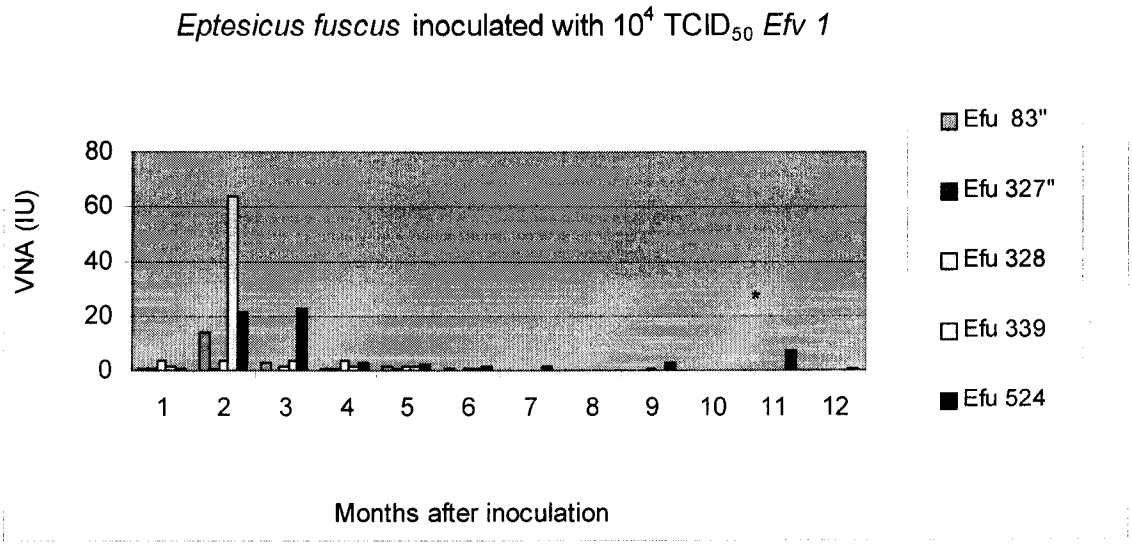


Figure 2.4 *Eptesicus fuscus* bats were inoculated with 10^4 $TCID_{50}$ *Efv 2* . Bats were bled two weeks following inoculation, then monthly. Six months following the first inoculation, bats were inoculated a second time with 10^4 $TCID_{50}$ *Efv 2*. All bats were bled two weeks following the second inoculation, then monthly. Twelve months after the first inoculation, all animals were euthanized. Data at 12 months post inoculation is from the terminal bleed.

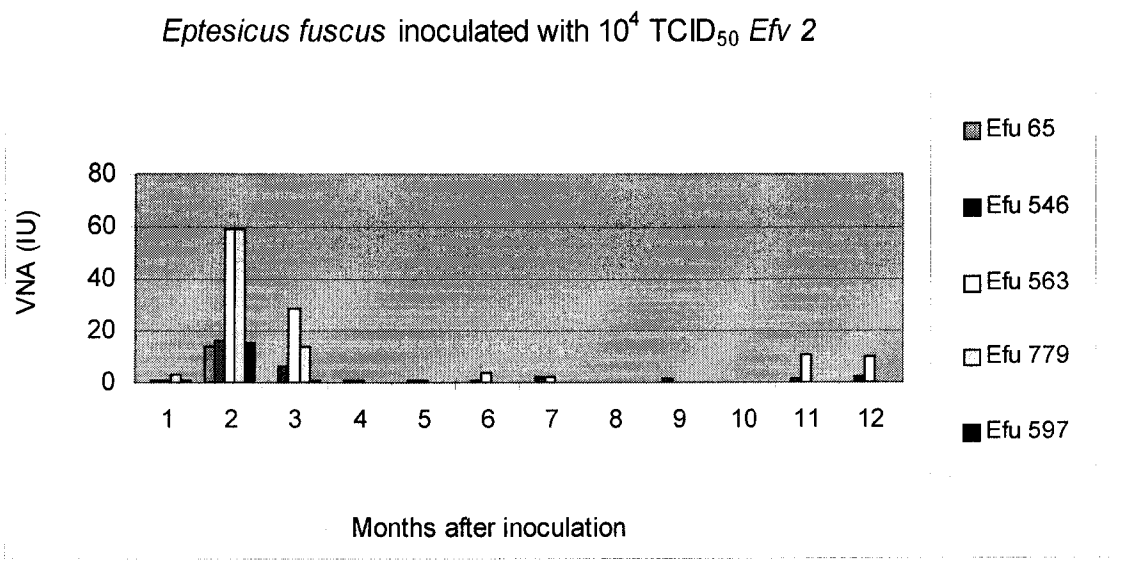


Figure 2.5 Mice were inoculated with 10^4 $TCID_{50}$ *Efv 1* . Mice were bled two weeks following inoculation, then monthly. Six months following the first inoculation, all mice were inoculated a second time with 10^4 $TCID_{50}$ *Efv 2*. Mice were bled two weeks following the second inoculation, then monthly. Twelve months after the first inoculation, all animals were euthanized. Data at 12 months post inoculation is from the terminal bleed.

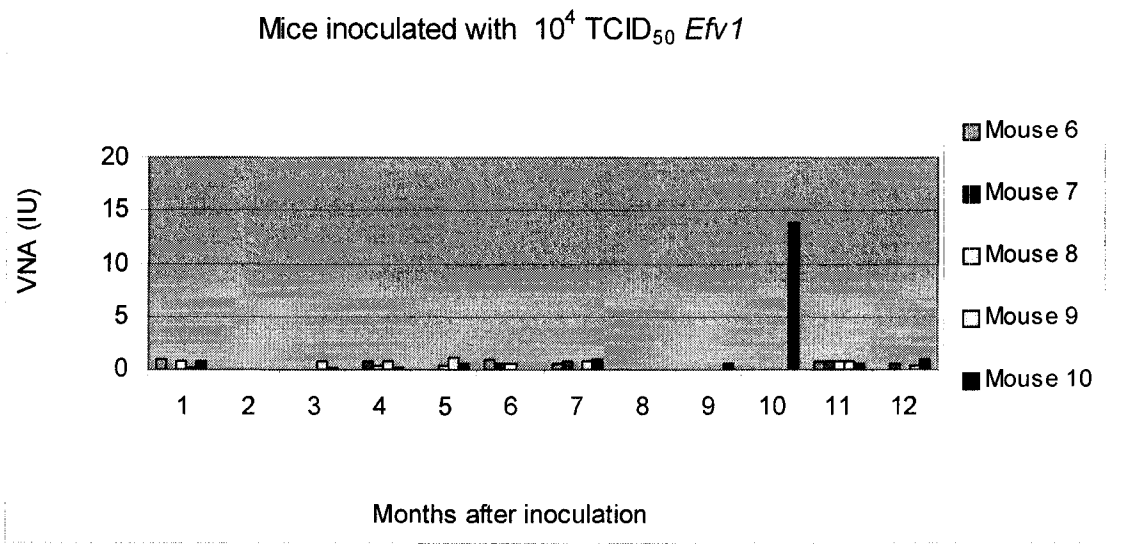
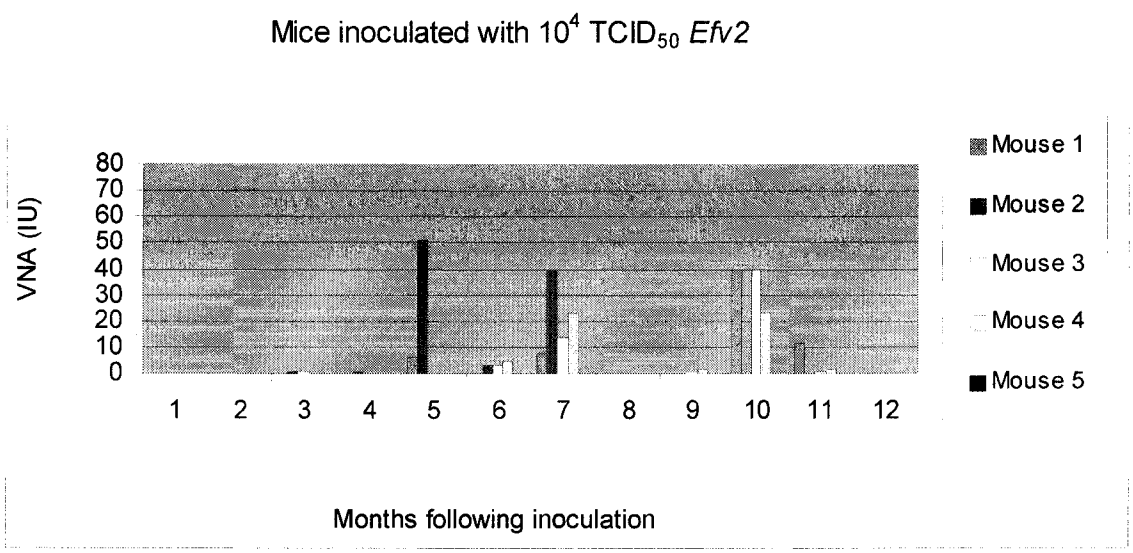


Figure 2.5 Mice were inoculated with 10^4 $TCID_{50}$ *Efv 2*. Mice were bled two weeks following inoculation, then monthly. Six months following the first inoculation, all mice were inoculated a second time with 10^4 $TCID_{50}$ *Efv 2* and is depicted by a descending arrow. Mice were bled two weeks following the second inoculation, then monthly. Twelve months after the first inoculation, all animals were euthanized. Data at 12 months post inoculation is from the terminal bleed.



2.5 DISCUSSION

Sequence differences between RNA of two rabies virus variants isolated from two different *E. fuscus* bats.

We identified several nucleotide differences between the *Efv1* and *Efv2* genomes, most of which did not alter the amino acid sequence. It is notable that there were only four amino acid differences between the proteins encoded by the two genomes, three of which were located in the G protein and one in the N protein. As described previously, the amino acid sequence of the N protein is the most conserved of the viral genome (Wunner, 2002, Bourhy et al., 1993). The residue at site 265 in the N protein is phenylalanine in *Efv2* and leucine in *Efv1*, both of which are neutral and non-polar, although phenylalanine has a larger side chain containing a benzene ring. Potential virulence factors in the N protein have not been as widely studied as the G protein and it has been suggested that the N protein is not as important for viral virulence (Takayama-Ito et al., 2006; Yamada, et al., 2006; Faber et al. 2004; Morimoto et al., 1996). The N protein is involved in regulating RNA transcription, modulating transcription and replication, encapsulation of the genome, and protecting the RNA template (Wunner, 2002; Kissi et al., 1995; Bourhy et al., 1993). The N protein is also immunogenic and may stimulate the immune system to increase the production of viral neutralizing antibodies (Kissi et al., 1995; Lafon, 1992). The N protein is purported to be an super antigen, increasing the VNA response following inoculation and enhancing the activation of peripheral blood lymphocytes (Lafon, 1992). It is not known how a residue change at site 265 may directly impact the virulence of the virus.

The first 19 amino acid residues (-19 to 0) of rabies virus glycoprotein represent the signal peptide providing the membrane insertion signal. This peptide is cleaved from the N terminus of the nascent G molecule in the Golgi apparatus (Wunner, 2002). The ectodomain of the glycoprotein is composed of residues 1-438 that extend from the virus surface to interact with rabies virus cellular receptors and is believed to be involved for viral pathogenesis (Yamada, 2006; Sarmento et al., 2006). The transmembrane portion of the glycoprotein includes residues 439-461 and interacts with the M proteins involved in virus assembly. The remaining residues extend into the cytoplasm of the infected cell (Wunner, 2002).

The first amino acid difference between *Efv1 and Efv2* in the G protein is residue number 9, an aspartic acid in *Efv1* and glutamic acid in *Efv2*. This amino acid change is on the ectodomain of the glycoprotein. Amino acids 466 and 467 are also different; residue 466 is a serine in *Efv1* and an arginine in *Efv*. Serine is neutral and polar, arginine is basic and polar. Residue 467 in *Efv1* is alanine, a neutral non-polar amino acid and threonine, a neutral polar amino acid in 467 of *Efv2*. Although the cytoplasmic domain of the glycoprotein does not interact with cellular receptors, the efficiency of viral budding and viral production is improved by the interaction between G the RNP-M complex (Yamada, 2006; Sarmento, 2006; Wang, 2005). Morimoto et al. (2000) exchanged the cytoplasmic domain of a *L. noctivagans* variant with that of a nonvirulent variant, SN-10G, and found no difference in the production of infectious virus. The cytoplasmic domain of SN-10G is 43% different from the cytoplasmic domain of the wild-type *L. noctivagans* variant (Morimoto et al., 2000) whereas the cytoplasmic domain of *Efv1* is approximately 0.5% different from that of *Efv2* virus. The impact of

these specific regions of variation on the interaction with the cytoplasmic domain of the G protein and the RNP complex-M protein is unknown. Although the variation between *Efv1* and *Efv2* is less drastic than that of *L. noctivagans* and SN-10G, the amino acid changes may be consequential for virulence.

Characterization of the clinical progression of the disease caused by two rabies variants associated with *E. fuscus*

Experiment 1

Two *E. fuscus* virus variants that were identical throughout a 320 nucleotide region of the N gene, but varied in the G protein were employed to study the pattern of rabies virus infection in *E. fuscus* bats and produced very different courses of infection. Clinical signs induced by infection with *Efv2* virus occurred within one month of inoculation, whereas infection with *Efv1* virus required more than four months to produce clinical infection. In the first experiment, eight of the eleven bats exposed to the *Efv2* virus developed clinical signs within 31 days of virus inoculation, and six of these eight were diagnosed as rabid upon death. The two surviving bats developed high levels of anti-rabies VNA and virus was isolated from the saliva during clinical illness. During clinical illness, virus was also isolated from the saliva of two bats exposed to *Efv1*. Of the two bats surviving clinical rabies infection, the animal with more drastic clinical signs developed an antibody titer of >218 IU one month following the primary challenge with 10^3 $TCID_{50}$ *Efv* 2. The second bat was also inoculated i.m. with 10^3 $TCID_{50}$ *Efv* 2 for the initial challenge and developed clinical signs of rabies virus infection. One month following the initial challenge her antibody titer was 1.7 IU but increased to 7.3 the

following month. Her clinical signs were much less drastic; she vocalized abnormally, developed ataxia, anorexia, and became mildly aggressive.

A decrease in antibody titer occurred in the seropositive bats between months two and four of the study. Six months after the second challenge, only three in group 2 had detectable circulating anti-rabies VNA. Bats in group 2 were seropositive prior to the first inoculation and the inoculation may have produced an anamnestic response in these animals. Two bats exposed to *Efv1* never developed a demonstrable immune response. After the second challenge, six-months following the initial challenge, both became seropositive. This is similar to a 1984 study in dogs during which dogs remained healthy and seronegative after a primary inoculation, but after second challenge, the dogs continued to remain healthy but seroconverted (Fekadu and Shaddock, 1984). In the present study, one bat that had naturally occurring antibodies during the quarantine period demonstrated an increase in titer following the first challenge. Two months prior to the six month challenge, the animal was seronegative and lacked an increase in titer following the six-month challenge with *Efv 2* . This bat subsequently developed rabies after a 53 day incubation period. It is possible that circulating VNA moved into the injection site to neutralize infection rendering the level of antibodies below the detection limits of the RFFIT.

Experiment II

The second study in which two groups of bats were exposed to 10^4 $TCID_{50}$ of *Efv1* or *Efv2* produced very different data from Experiment 1. The incubation period in the *Efv2* bats was almost twice as long as the incubation period observed in the first experiment. This may be the result of passing the virus in cell culture, a procedure that

may attenuate the virulence of viruses (Wunner and Clark, 1980, Wiktor et al., 1977). As the virus replicates, the number of infectious virus particles increases along with amount of non-infectious virus particles. These particles are incomplete virions that contain the RNA of the rabies viral genome but are shorter in length than the standard infectious rabies genome (Wunner, 2002). The amount of the *Efv2* virus inoculated was ten-fold higher than it was in the first experiment. As the amount of virus increases, so do the amount of DI particles, and it is possible that the increased dose delivered in the second experiment was associated with a proportional increase in defective interfering (DI) particles (Wunner and Clark, 1980). According to Wiktor et al. (1977), DI particles enhance the antibody response to rabies virus by increasing the ratio of antigenic mass to infectivity, not by inhibiting replication at the molecular level (Wunner and Clark, 1980, Wiktor et al., 1977). It is also possible the number of DI particles were increased during passage in cell culture, increasing the number of DI particles in the inoculum.

In four of the cases where bats succumbed to rabies virus infection, their VNA titers decreased prior to death. This may be the result of circulating VNAs moving out of the blood into the tissue where virus was present. The rise of VNA and subsequent decrease may reflect an initial B cell response followed by an overwhelming production of antigen inducing the movement of VNA out of the circulation. It is not known if, or how often, this may happen in the terminal stages of chiropteran rabies infection.

Data from both experiments demonstrate the ability of bats to develop immunity to rabies virus. In the first experiment, all but one bat developed antibodies following an intramuscular inoculation. Some of the bats that developed VNAs succumbed to the virus, typically following a decrease in circulating VNAs. Bell and coworkers (1983)

and Baer and colleagues (1975) reported the movement of B cells into the tissue following viral replication within the CNS. During both experiments, the decrease in circulating VNAs in bats between sampling times was substantial, decreasing from 5 IU twelve days after inoculation to <1.0 IU fourteen days after inoculation. During the first experiment, six bats died following the primary challenge with rabies virus. Of these six bats, titers in three decreased during the terminal phase of the disease and one remained seronegative.

In the second experiment, three bats and two mice developed clinical rabies infection following the primary challenge of 10^4 $TCID_{50}$ *Efv* 2 rabies virus. Three bats that were initially challenged with 10^4 $TCID_{50}$ *Efv* 1 developed clinical rabies infection. The amount of VNAs in five of the six bats decreased or became undetectable during the terminal stages of the disease. In one bat the titer increased from undetectable to 14 IU. In one mouse, VNAs were undetectable during the terminal stage of the disease and the second mouse never produced VNAs following rabies virus inoculation.

This study demonstrated that the presence and/or quantity of antibodies do not necessarily correlate with survival or mortality. In both experiments, some of the animals with high VNA titers died of rabies virus infection while others did not. Similarly, some of the bats with lower titers died of rabies virus infection, some did not. In the second experiment, the bats with the highest antibody titer two weeks following the first challenge with 10^4 $TCID_{50}$ *Efv* 2 died of rabies virus infection. This phenomenon was not seen in the first experiment. In the second experiment, bat 563 initially had a lower titer than bat 779, on the second bleed the titers were similar. One month post inoculation, the titer of bat 779 dropped from 59 IU to 13 IU and bat 779 subsequently

died. The titer of bat 563 dropped from 59 IU to 28 IU and bat 563 survived. The antibody enhancement theory, (Andral and Blancou, 1981, Prabhakarm and Nathanson, 1981) which suggests the presence of antibodies may accelerate, but not initiate, the clinical course of rabies virus infection, does not help to explain the deaths of these bats as the deaths did not occur earlier in animals with high titers. It is unknown why some bats succumb to rabies virus infection whereas some develop immunity. Constantine (1988) proposed that bats are relatively resistant to rabies virus infection and that those bats that develop clinical rabies are not immunocompetent (reviewed in Niezgoda et al., 2002, and Brass, 1994).

There are several immune components involved in protecting the host including cytokines (Faber et al., 2005; Prehaud et al., 2005; Theerasurakarn and Ubol, 1998) Other host factors such as steroid hormone levels, previous exposure to the agent, and genetic makeup are likely to be involved in the ability of the host to resist clinical rabies virus infection (Brass, 1994; Lodmell, 1993) Thus, the ability of bats to resist clinical infection with rabies virus is likely to be based on much more than what was measured during these experiments. It is clear that exposure to rabies virus does not always manifest itself in clinical infection. Furthermore, significant differences in virulence may be the result of minor changes within the virus. It is plausible that viruses with increased virulence are more likely to produce clinical illness in immunocompetent bats yet these highly virulent viruses are more likely to kill the animal prior to centrifugal movement to the salivary glands. Therefore, it is unlikely these more virulent viruses will be maintained at a high level within a population. Conversely, a less virulent virus is much slower in producing clinical illness, providing the virus time for centrifugal spread to the

salivary glands. It is likely this would be the more commonly circulating virus within the bat populations, resulting in seroconversion in healthy bats or producing clinical infections in bats that were immunosuppressed due to other diseases or environmental stressors.

Few of the virus-exposed mice developed clinical rabies infection. It has been proposed that a variant that does not kill mice will not kill bats, as mice are believed to be more susceptible to rabies virus infections. The present data contradict this hypothesis as 80% mice survived primary challenge with *Efv2* and 100% survived primary challenge with *Efv1* during the first experiment as compared to survival of 45% bats exposed to *Efv2* and 94% bats exposed to *Efv1*. During the second experiment, 60% of the mice exposed to *Efv2* in the primary challenge survived. All the mice exposed to *Efv1* in the primary challenge survived. The survival rate of bats was less than that of the mice; 40% of the bats survived the primary challenge with either *Efv1* or *Efv2*. Findings from Lodmell (1993) demonstrate some level of genetic control over an animal's resistance to rabies virus infection. Rabies variants are better adapted to their own host species and are more likely to spread within that host species population than spill over and adapt to a new host (Hughes et al., 2005; Kobayashi et al., 2004; Hughes et al., 2002; Smith, 2002). Our data support this theory, as mice appeared to be less susceptible to infection than were the host species, *E. fuscus*. By expecting a virus to be non-pathogenic to a bat because it is relatively non-pathogenic to a mouse, rabies viruses highly virulent in their host species may go unnoticed in a rabies study that employs a mouse model. Perhaps future studies should not be based on the pathogenesis of a rabies virus variant in mice as this does not appear to be indicative of the viral variant characteristics in the host species.

Nathanson and Scarano (1990) reported that anti-rabies VNA titers increased throughout infection, reaching the highest levels during the terminal phase of infection. Furthermore it has been proposed that following exposure to rabies virus, surviving animals will develop high antibody titers for months to years. Neither hypothesis was substantiated in experiments 1 or 2. Anti-rabies VNAs were maintained in experimentally exposed animals between three and six months following inoculation. The decrease in anti-rabies VNAs was recently demonstrated in a 2005 study that exposed *Desmodus rotundus*, the common vampire bat, to an intramuscular inoculation of the *D. rotundus* variant. Almeida et al. (2005) reported that 53% of the bats were seropositive thirty days post inoculation, 44% at sixty days post inoculation, and 35% at ninety days post inoculation.

The decrease in titers that occurred during Experiment 1 and the subsequent increase in VNAs following the six-month challenge suggest seronegative bats in the wild may have had previous exposure. Bat pups are born seronegative and may later become seropositive from consumption of antibody-laden milk (Steece and Altenbach, 1989). A study by Steece and Altenbach (1989) found 70% of healthy adult female *T. brasiliensis* to be seropositive, few of which had circulating IgM neutralizing antibodies. Furthermore, less than 1% of the bats tested were rabies antigen positive on dFA (Steece and Altenback, 1989). These numbers remained constant over the duration of their study. Maternal antibodies are typically present in juvenile *T. brasiliensis* from May to September (Steece and Altenback, 1989). Unlike the adult females, the percentage of seropositive *T. brasiliensis* juveniles drops substantially between mid-June and mid-July, during which time the number of dFA positive juveniles is approximately 2% (Steece and

Altenback, 1989). Concurrent to this drop is a slight increase in rabid juvenile bats (Steece and Altenback, 1989). The increase in rabid bats is not proportional to the number of juvenile bats with IgM VNAs. In mid-July, the percentage of juvenile bats with circulating IgM neutralizing antibodies remained low (10%) but markedly increased as the summer progressed (Steece and Altenbach, 1989). Steece and Altenbach (1989) hypothesized that *T. brasiliensis* are exposed to rabies very early in life and that, as the maternal antibodies wane, the juvenile bats become susceptible to rabies virus infection. It is possible that juvenile bats are exposed to rabies virus when maternal antibodies are present as can be seen by the presence of both IgM and maternal antibodies in juvenile bats. The presence of maternal antibodies may be adequate to protect against clinical infection but at the same time low enough to allow the development of active immunity. Data from oral rabies vaccine trials present conflicting evidence on the role of maternal antibodies as hindering or having no effect on the development of active immunity (Mackowiak et al., 1999; Blasco et al., 2001; Hostnik, 2003).

Experiment 1 provided some data on naturally occurring "immunity" in wild caught bats. One bat exposed to 10^3 *Efv 2* and one bat exposed to 10^2 *Efv 2* had low antibody titers during the first two months of quarantine. Both bats were seronegative at least three months prior to the initial experimental inoculation. Both bats subsequently died of rabies within one month of inoculation with *Efv2*, suggesting naturally occurring antibodies may not always protect against infection. Furthermore, the type of exposure that occurred prior to captivity is unknown and may have induced VNAs that were not protective. It could also be the ability of this variant to override any protection VNAs may provide. Conversely, four of the five seropositive bats in group 2 demonstrated an

increase in VNAs two weeks following the first inoculation with *Efv2*. Two bats exposed to 10^4 *Efv 1*, two bats exposed to 10^3 *Efv 1*, and two bats exposed to 10^2 *Efv 1* had low levels of VNAs within the first two months of quarantine. All of these bats survived the initial inoculation and the six month challenge. All bats in the second experiment were seronegative at the beginning of the quarantine period.

The lack of virus in the saliva is not surprising. The amount of virus present in the saliva is likely to be small and may go undetected in cell culture isolation tests. Rabies virus is known to be shed intermittently in the saliva and it is possible swabs were not taken during the shedding period (Fekadu and Shaddock, 1984; Fekadu et al., 1982; Vaughn et al., 1965). Although oral swabs were collected when rabid animals were euthanized, these swabs were negative, possibly due to a lack of centrifugal spread or viral clearance (Hooper, 2005, Orciari et al., 2001, Dietzschold et al., 1993). The paucity of viral shedding in the saliva helps to explain the lack of outbreaks in bat populations and allows the virus to move slowly though bat populations.

The purpose of this study was to characterize the differences between two *E. fuscus* isolates, *Efv1* and *Efv2* with respect to the progression of the disease, dissemination of the virus, and genetic differences between the variants. Although this study was begun with the presumption that viral pathogenesis would be comparable between viruses with similar genomes, this was not the case. Our results demonstrate that a small number of amino acid changes in the protein may be consequential for viral pathogenesis. We also found that VNA produced following inoculation with a live rabies virus are not always detectable six months later, yet the animals that survived the first inoculation are more than 75% more likely of surviving a second challenge.

Furthermore, our study does not support the suggestion that the murine animal model is a reliable model for characterizing rabies virus pathogenesis. We found that rabies virus produced clinical illness in more than 34% of inoculated bats compared to 13% of inoculated mice. Of these, 80% were infected with *Efv 2* where as 100% of the mice that developed rabies were infected with *Efv2*, suggesting mice are not susceptible to all variants of rabies virus that may be involved in laboratory studies or found in nature. These tentative conclusions must be tempered by the small number of animals involved in these experiments.

CHAPTER III

EFFECTS OF AEROSOLIZED RABIES VIRUS ON BATS AND MICE

3.1 INTRODUCTION

Rabies is a neurotropic virus resulting in a neurologic disease that has the ability to affect all mammals. Man is not the primary host of this viral disease, yet it is estimated that worldwide, rabies virus kills in excess of 55,000 humans annually (WHO, 2005). Dogs remain the most epidemiologically significant host of rabies virus, as they have for the past 4000 years of recorded history (Wilkinson, 2002). In the 1950s, bats were recognized as competent vectors of rabies and the presence of rabies in bat populations was observed to pose public health threat (Brass, 1994). The natural biting behavior of hematophagous bats, the close associations with man and commensal bat species, and the recently described theory of cryptogenic rabies exemplify the concerns presented by bat rabies (Gibbons, 2002 and Messenger et al., 2003).

Rabies virus is maintained in host populations and transmitted to other species, including humans, largely through bites by infected animals. Aerosol transmission has been postulated as an alternative route of exposure to rabies virus following a small number of both laboratory incidents/cases and apparently natural occurrences. Aerosol transmission has been implied in four reports of human rabies cases and documented in

experimental work with animals (Winkler et al., 1973, Winkler et al., 1972, Hronovsky and Benda, 1969, Hronovsky and Benda, 1969, Constantine, 1967, and Constantine, 1962). In all instances when aerosol transmission was cited as the route of human infection, more plausible explanations may exist (Gibbons, 2002). In the two cases involving spelunkers working in Frio Cave (Constantine, 1967, and Irons et al., 1957], there are notations of direct contact between the spelunkers and the bats although no documented bites were reported. In the two cases involving laboratory-acquired infections (CDC, 1977 and Winkler et al., 1973) the scientists had other opportunities to be exposed to rabies virus in situations that did not include aerosol productions. Constantine (Constantine, 1967) documented aerosol transmission of rabies virus to experimental animals in Frio Cave. This Texas cave, which is home to over 10 million bats, was the location where two humans were believed to have contracted rabies via aerosol. Constantine was able to demonstrate transmission of rabies virus to animals housed in cages that excluded all but cave atmosphere. Rabies virus was isolated from samples collected via air condensation techniques which were used to monitor the atmosphere in this cave (Winkler, 1968).

Additional work is warranted if we are to understand the role that aerosol exposures play in the epizootiology of rabies in mammals. The objectives of the work described here were to investigate whether rabies virus, especially bat variants, can be transmitted to animals through aerosol exposures in the laboratory, and to characterize the pathogenic and immunologic consequences of such exposures.

Based on our knowledge, we expected the amount of virus to which the animals would be exposed to following the aerosolization process to be greatly reduced. Because

rabies virus is an enveloped virus that is easily destroyed by through desiccation and chemical disinfection, it was unknown if aerosolized rabies virus would retain the ability to infect various lines of cell cultures. If rabies virus were capable of infecting cell culture, the experiment would progress to an animal model involving bats and mice. Although cell culture would demonstrate infectivity in vitro, our animal models would allow us to characterize aerosol exposure in mammal models in which this type of exposure could occur.

3.2 MATERIALS AND METHODS

Cell Culture

Mouse C-1300 neuroblastoma (NA) cells were grown to confluence in T-25 flasks containing 7 ml Eagle's growth medium (GM) consisting of minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 2.0 mM glutamate, 100 IU penicillin G, 50 ug streptomycin, and 2.5 μg amphotericin B/ml. The numbers of cells containing rabies antigen were enumerated by immunofluorescence as described previously (Rudd and Trimarchi, 1989).

Viruses

Four rabies virus variants were used in this experiment: a variant isolated from *Tadarida brasiliensis mexicana* (Mexican free-tailed bat) (*Tbv*); two variants isolated from *Eptesicus fuscus* (big brown bat) (*Efv1* and *Efv2*), and a variant isolated from *Lasionycteris noctivagans* (silver-haired bat) (*Lnv*). Virus variant identity was verified by the utilization of BLAST analysis (www.ncbi.nlm.hih.gov/blast) based on N gene sequence. The Colorado Department of Public Health and Environment provided

carcasses of rabid *E. fuscus* and *L. noctivagans* bats, which were submitted for rabies diagnosis. Rabid *T. brasiliensis* were collected at a Texas field study site where they were found grounded and moribund underneath a roosting colony. Bat collection and handling followed protocols that were in compliance with established procedures of the Colorado State University (CSU) Institutional Animal Care and Use Committee. Bats were dissected; brains and salivary glands were removed. Brain tissue from all bats was examined by the direct fluorescent antibody procedure (DFA) (Trimarchi and Smith, 2002) to identify and verify rabid individuals. Homogenates (10% w/v) were made of salivary glands with a Ten-Broeck tissue grinder, using 1.0 ml of GM as diluent. To achieve consistent virus titers and adequate volumes for the aerosol and inoculation experiments, all virus isolates were passaged three times in NA cells. The resulting viral stocks contained infectivity titers for the *Efv1*, *Efv2* and *Lnv* isolates of 1.0 X 10^5 50% tissue culture infectious doses ($TCID_{50}$)/ml and for *Tbv* isolate 1.0 X 10^6 $TCID_{50}$ /ml. The virus employed in all intramuscular challenges, *Efv2,* was obtained from a rabid *E. fuscus;* in a previously performed experiment, a challenge dose of 10^3 $TCID_{50}$ of this virus produced mortality levels of 67% (4/6) in *E. fuscus* and 20% (1/5) in mice (unpublished data).

Viral Genotyping

RNA extraction and RT-PCR were performed on all inoculae employed in this experiment, as well as on the tissue samples from euthanized experimental animals. For RNA isolation, 50 µl of the viral suspension were added to 1.0 ml TRI-Reagent and processed using 1-bromo-3-chlorpropane (BCP) as per the manufacturer's recommendations (Molecular Research Center, Inc., Cincinnati, OH). The resultant 50µl

volume containing isolated RNA was stored at –20°C for at least 1 h before we performed the reverse transcriptase (RT) procedure. The RT procedure was carried out with the SuperScript First-Strand Synthesis System™ according to manufacturer (Invitrogen, Carlsbad, CA), recommendations, with minor modifications. Random hexamers from the SuperScript First- Strand Synthesis System™ were used in cDNA synthesis. The total volume of the RT reaction was 20 µl. The RT reaction conditions followed the manufacturer's recommendations. The cDNA was stored at –20 ° C until processed further.

Viral N gene cDNA was amplified using a Robocycler (Stratagene, La Jolla, CA). The optimal reaction using the TURBO™ system (Stratagene) contained 5 µl cDNA, 33 µl water, 5 µl 10X ($MgCl_2$) Turbo cloned pfu buffer, 1 µl 50 mM $MgCl_2$,
4 µl dNTP, 1 µl of each primer (20 mM), and 1 µl 500 U pfu TURBO DNA polymerase. To amplify a 320-bp PCR product, the optimal annealing temperature for the PCR primers 21F (5'-ATGTAACACCCCTACAATG-3') and 304R (5'-TTGACGAAGATCTTGCTCAT-3') was 55°C. Two negative controls were run with each PCR, one substituting 5 µl water for cDNA, and a second substituting 5 µl cDNA from the RT negative control. Positive controls were done with 5 µl cDNA from CVS-11 rabies virus. PCR conditions were as follows: 1 cycle of 94°C for 1 min, 40 cycles of 94°C for 1 min, 55°C for 1 min, 2 min at 72°C, followed by one 10-min cycle at 72°C.

Amplicons were electrophoresed on a 1% SeaKem agarose gel (BMA Products, Rockland, ME), and to verify the size of the amplicon,
a 1-kb ladder was electrophoresed next to the negative control. The appropriate band was excised with a sterile razor blade and placed in a labeled sterile microcentrifuge tube.

Electrophoresed cDNA was purified using the Qiagen Quick kit following manufacturer's recommendations (Qiagen, Valencia, CA). In separate microcentrifuge tubes, the purified PCR product and 8 μl of each primer (at 3 mM) were sent to Davis Sequencing (UC Davis, Davis, CA). Initial sequence analysis was done with CHROMAS version 1.45 (School of Health and Science, Griffith University, Brisbane, Australia). Sequences were compared to other rabies sequences from the NCBI-site (www.ncbi.nlm.hih.gov/blast).

Serology

The RFFIT procedure, as described by Smith (1996) and Shanker (2004), was employed to determine the concentration of virus neutralizing antibody(Shankar et al., 2004 and Smith et al., 1996).

Animals

Before laboratory exposure to virus, wild-caught *E. fuscus* and *T. brasiliensis* bats were quarantined for 8 and 6 months, respectively. For identification of individual bats, a passive integrated transponder (PIT) tag was inserted subdermally into *E. fuscus* and an identification number was tattooed onto the wing membrane of each *T .brasiliensis.* Four-to-eight-week old outbred laboratory mice (ICR) were obtained from Taconic Farms (Germantown, NY) and were identified by ear holes. Mealworms were fed ad libitum to *E. fuscus; T. brasiliensis* were fed a mixture of blended mealworms and beef baby food, and the mice were fed laboratory rodent pellets. All animals had continuous access to clean water. Bats were caged in 76 x 61 x 61 cm enclosures lined with fly screen. Each cage housed four bats; species were not co-mingled. Mice were

caged in 76 x 31 x 31 cm enclosures with three mice per cage. The temperature was maintained between 29.4°C and 31.1°C and the humidity at approximately 40%.

Ill bats were given subcutaneous (s.c.) lactated Ringers solution and were hand-fed beef baby food during initial stages of clinical rabies, before the disease was differentiable from other illnesses that are occasionally encountered and successfully treated in captive bats. If clinical signs did not improve within 12 h, the ill bat was euthanized by intraperitoneal injection of pentobarbital (80mg/kg). Mice were euthanized at the first sign of clinical illness using the same technique as described for bats. All animals were necropsied following euthanasia.

Following aerosol exposure to the virus, *E. fuscus* and mice were bled monthly. *T. brasiliensis* were bled bimonthly, due to fragility in captivity. Bats were bled from the uropatagial vein and mice were bled from the tail vein, both by means of needle puncture with collection of blood in hematocrit tubes.

Aerosol Exposure Procedure

Aerosols containing rabies virus were produced using an Inhalation Exposure System (Glas-Col Inc., Terre Haute, IN). The apparatus was located in a BSL - 3 suite and a strict safety protocol was implemented to assure operator safety. A wire mesh basket containing five compartments was used to house the animals during the aerosolization process. Bats and mice were exposed to virus simultaneously, but were kept in separate compartments. *E. fuscus* and *T. brasiliensis* (four of each species) and mice (three) were exposed to one of three bat variants of rabies virus (*Efv1, Tbv,* and *Lnv*) in three separate aerosolization experiments (one virus variant per experiment), in an attempt to determine the immunological and pathological effects of an exposure to

homologous and heterologous variants of rabies virus. For all experiments, a total of 10^6 $TCID_{50}$ of virus in 10 ml of GM was introduced as an aerosol into the chamber, over an 80-min time period. The infection chamber had a volume of 0.1416 m^3. Humidity and temperature were recorded during aerosol exposure; humidity was 45%, and temperature was 27.8 to 28.9° C.

Following exposure to the aerosolized virus, the animals were visually checked three times a day and weights were recorded twice a week. Animals that became ill were euthanized, necropsied, and brain smears were tested by the DFA test for the presence of rabies virus antigen.

Six-Month Challenge

Six months after exposure to aerosolized rabies virus, all surviving animals were challenged by intramuscular (i.m.) inoculation into the right deltoid muscle with 1.0 X 10^3 $TCID_{50}$ of *Efv2* challenge virus. Mice were anesthetized for challenge with 100 µl ketamine/xylazine, and bats were injected without sedation. All animals were bled 2 weeks before and 2 weeks after challenge, then either monthly (*E. fuscus* and the mice) or bimonthly (*T. brasiliensis*). Weights were recorded twice weekly. Euthanized animals were necropsied, and brain smears were tested by DFA for the presence of rabies virus antigen.

To ascertain if animals that became rabid following i.m. challenge were infected with the aerosolized rabies variant or with the challenge variant, sequence analysis of the N gene on viruses isolated from brain was performed, as described above. The sequence of the virus isolated from the euthanized animal was compared to the sequence of the

aerosolized virus to which the animal had been exposed, and to that of the challenge virus.

3.3 RESULTS

Response of bats to aerosolized rabies virus

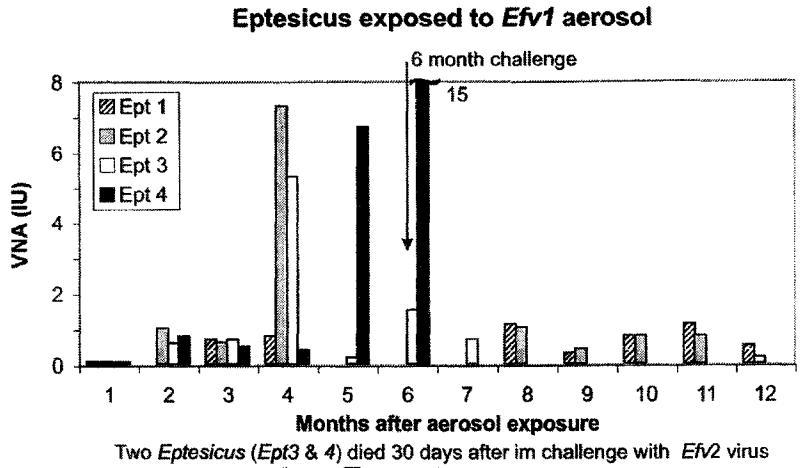
All 24 bats (12 *E. fuscus* and 12 *T. brasiliensis*) survived the 6-month observation period following exposure to aerosolized rabies virus and developed anti-rabies viral neutralizing antibodies within 2 months of aerosol exposure, with titers ranging from 0.6 to 12.3 IU/ml (Table 3.1 and 3.2). The average VNA titer of bats exposed to the *Efv1* aerosol varied, depending on the species of bat exposed; titers in *T. brasiliensis* bats peaked 2 months after aerosol exposure, and titers in *E. fuscus* peaked at 5 months post exposure. Peak titers in all bats exposed to *Tbv* aerosol occurred 2 months following exposure. Among the bats exposed to *Lnv* virus aerosol, *E. fuscus* bats developed peak antibody titers 3 months after exposure, and *T. brasiliensis* bats developed peak titers 4 months after exposure. Anti-rabies viral neutralizing antibodies were undetectable 6 months after aerosol exposure in one *T. brasiliensis* bat exposed to *Lnv*, and in two *E. fuscus* bats exposed to *Efv1*.

Response of mice to aerosolized rabies virus

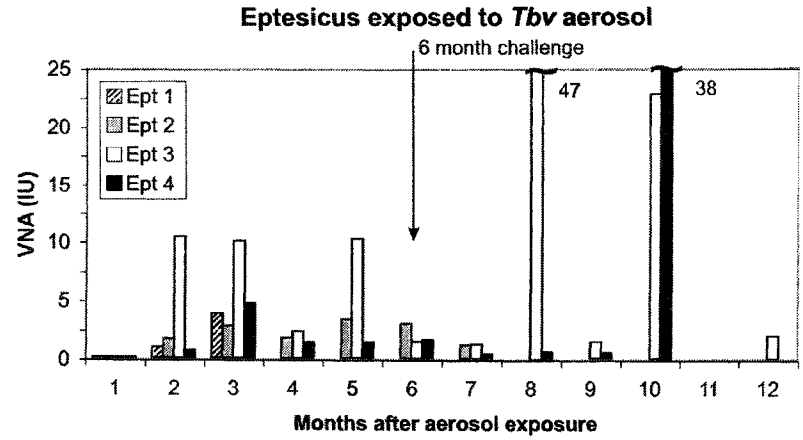
One mouse exposed to *Efv1* and one mouse exposed to *Lnv*, respectively, developed signs of rabies 17 and 8 days after aerosol exposure. Two mice exposed to *Tbv* developed signs of rabies, at 10 and 14 days post aerosol exposure. All four mice were euthanized and found to be rabies-positive by the DFA. The five surviving mice developed anti-

rabies viral neutralizing antibodies (Table 3.3). At approximately 5 months after aerosol exposure the remaining mouse exposed to *Tbv* died of unknown causes.

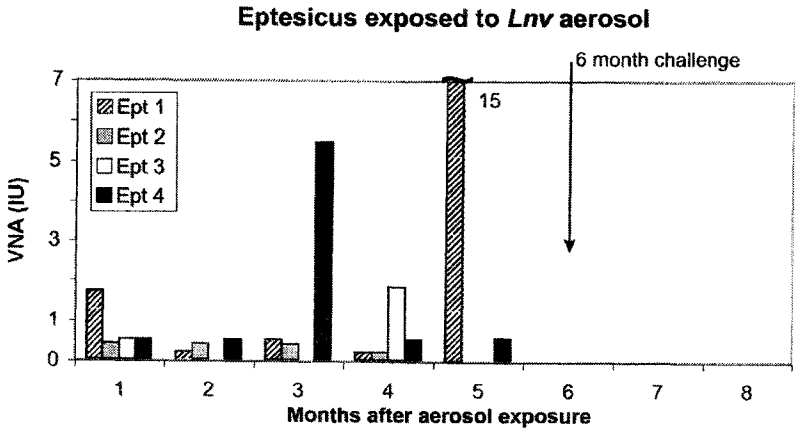
Figure 3.1 Responses of *E. fuscus* following exposure to aerosolized rabies virus.



Two *Eptesicus* (*Ept3* & *4*) died 30 days after im challenge with *Efv2* virus

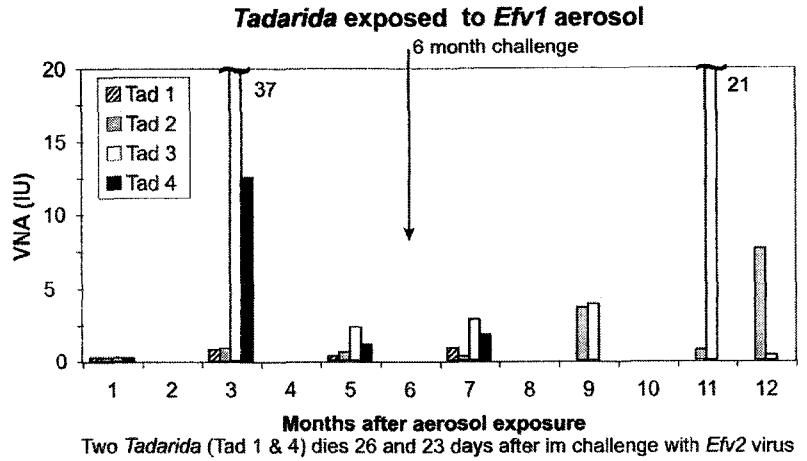


One bat (*Ept1*) died 32 days after im challenge with *Efv2* virus

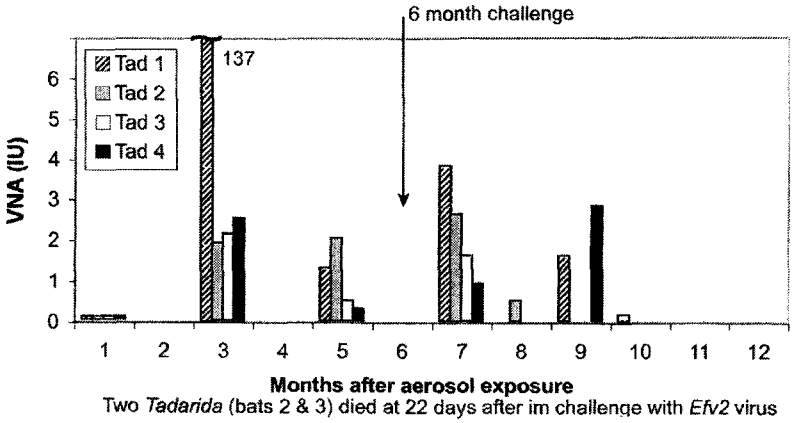


Two *Eptesicus* (*Ept2* & *3*) developed rabies 30 and 31 days after im challenge with *Efv2* virus

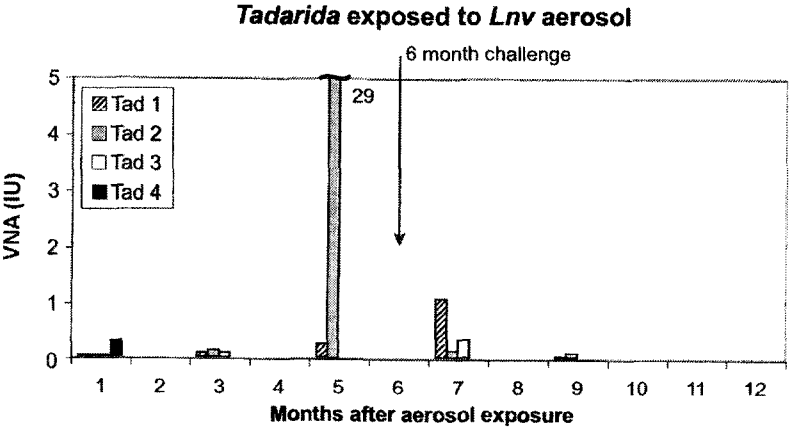
Figure 3.2 Responses of *T. brasiliensis* following exposure to aerosolized rabies virus.



Two *Tadarida* (Tad 1 & 4) dies 26 and 23 days after im challenge with *Efv2* virus

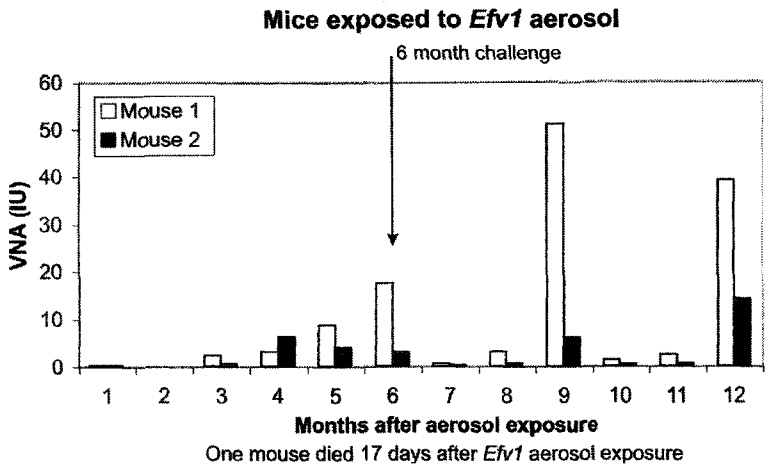


Two *Tadarida* (bats 2 & 3) died at 22 days after im challenge with *Efv2* virus

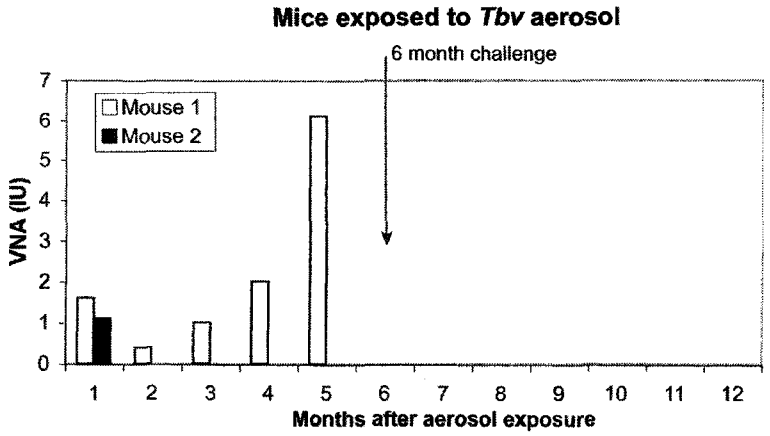


One *Tadarida* (Tad 4) developed rabies 26 days after im challenge with *Efv2* virus

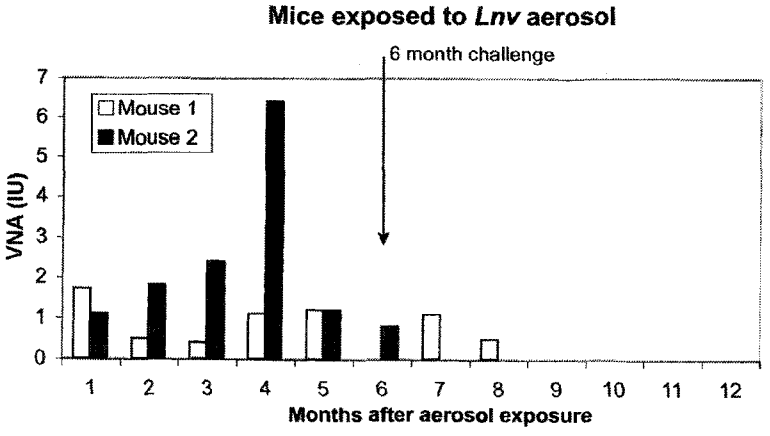
Figure 3.3 Responses of laboratory mice following exposure to aerosolized rabies virus.



One mouse died 17 days after *Efv1* aerosol exposure



Two mice developed rabies 10 and 14 days post aerosol exposure to *Tbv* virus.



One mouse developed rabies 8 days after *Lnv* aerosol exposure. One mouse (#2) developed rabies 16 days following im challenge with *Efv2* virus

Response of bats to intramuscular challenge

Approximately 2-4 weeks following i.m. challenge, three to four bats in each group developed respiratory distress and ataxia. Of the 24 bats given an i.m. challenge, 10 developed rabies (Tables 3.1 and 3.2). Bats with respiratory signs and ataxia were euthanized and all tested positive for rabies virus antigen by DFA. Sequence analysis of the virus detected in brain indicated that it was the challenge virus, rather than the virus originally delivered by aerosol.

The concentration of viral neutralizing antibodies in bats after i.m. challenge varied. Two weeks after i.m. challenge, titers ranged from undetectable to 5.7 IU/ml. An increase in titer was noted in 21 bats 3 weeks after the i.m. challenge; nine of these bats subsequently developed signs of rabies, were euthanized, and were found to be rabies-positive via the DFA test. The bats that survived the i.m. challenge had a titer ranging from 0 to 28.7 IU/ml immediately before i.m. challenge, while the titers of bats that did not survive the i.m. challenge ranged from 0-14.7 IU/ml immediately before i.m. challenge.

Response of mice to intramuscular challenge

With one exception, the five mice that survived for 6 months after aerosol exposure had detectable virus neutralizing antibody before i.m. challenge. Two weeks following the i.m. challenge, the titer of the one mouse initially lacking antibody had increased to 1.1 IU/ml. On day 16 following i.m. challenge, a mouse that had been exposed to *Lnv* aerosol appeared dehydrated and slightly ataxic. Before challenge, the antibody titer of this mouse was 0.8 IU. The mouse was euthanized, and rabies antigen was detected in the brain by the DFA. This was the only mouse to develop signs of rabies following i.m. challenge.

Sequence analysis of the infecting rabies strain indicated that it was consistent with the challenge virus.

3.4 DISCUSSION

In the present study, 24 bats (12 *E. fuscus* and 12 *T. brasiliensis*) and nine outbreed mice were exposed to one of three bat variant rabies viruses was via aerosol. Survivorship and antibody levels were tracked after animals were initially exposed to the aerosolized rabies virus, and then after an i.m. challenge 6 months following aerosol exposure. All bats were sero-negative for rabies neutralizing antibody prior to the experiments, but developed demonstrable neutralizing antibody following aerosol exposure and survived for 6 months until the i.m. challenge. Four of the nine mice died of rabies as a result of the aerosol exposure and the remaining five mice developed demonstrable neutralizing antibody. One mouse died of rabies following the 6-month i.m. challenge.

The bats used in this study were wild-caught, and had possibly been previously exposed, and thus potentially immune to rabies virus. Such priming could account for the immune response to aerosolized rabies virus that was seen in all bats, and for the failure of the aerosol exposure to produce clinical rabies virus infection in any bat. Alternatively, the posited co-evolution between bats and lyssaviruses, induced partially by the host response, could help to explain the low incidence of clinical disease in bats (Messenger et al, 2003, and Selimov et al., 1969).

Previous experiments employing aerosolized rabies virus have examined the lethality and the viscerotropic nature of aerosol exposure in laboratory settings (Hronovsky and Benda, 1969a, Hronovsky and Benda, 1969b, Atanasiu, 1965 and

Niezgoda et al., 2002). Under field conditions, Constantine demonstrated that certain species of animals can develop rabies when exposed to aerosolized virus (Constantine 1967 and Constantine 1962). Susceptibility ranking among animals exposed to rabies (Selimov et al., 1969) can only be verified by laboratory inoculation experiments (Winkler et a l., 1972). The present study, utilizing aerosol inoculation under laboratory conditions, suggests that bats are less susceptible than laboratory mice to three variants of bat rabies virus when administered by aerosol.

The presence of serum rabies neutralizing antibody alone does not define protection to clinical rabies virus infection (Templeton et al., 1978, Wiktor et al., 1978,Wiktor et al., 1977, Wiktor et al., 1972). Antibody levels of 1:20 to 1:39, as measured by the RFFIT (IU data were not provided), sufficed to protect 98 of 98 dogs and 67 of 68 cats when they were challenged i.m. with a 100% lethal dose of rabies virus (Bunn et al., 1984). In our study, the presence of aerosol-induced anti-rabies viral neutralizing antibodies provided little or no protection from an i.m. challenge.

In the present study, the isotype of the VNA induced in bats and mice by aerosol exposure to rabies virus not determined. The immune response resulting from the presentation of antigens to the mucosal immune system is principally of the isotype S-IgA (Childers et al., 2000). S-IgA, induced by antigens presented to mucosal inductive sites of the upper respiratory tract would, by mucosal homing, be directed to mucosal effector sites (Van Ginkel et al., 2000). Serum levels of IgA antibodies do not reflect the mucosal level of IgA, nor are serum IgA and mucosal IgA produced in the same compartments (Burrer et al., 2001). Monoclonal IgA has previously failed to protect when administered i.m. in mice 24 h prior to i.m. challenge with rabies virus; the same

IgA monoclonal antibody was protective against rabies virus *in vitro* (Dietzschold et al, 1992). Nasal immunization has been shown to protect against certain infections when the infectious agent's normal portal of infection is respiratory (Childers et al., 2000). In the majority of cases, rabies virus has a transcutaneous portal of entry. This brings into question the protective effect of aerosol-induced IgA antibody in resistance to a parenteral challenge of rabies virus. Perhaps, in the present study, an aerosol challenge with rabies virus would have resulted in a different outcome.

The prevalence of neutralizing anti-rabies antibody in bat populations has been reported, but the source of the immune-system stimulation that elicits this antibody has not been identified (2005, and O'Shea et al., 2004, Steece and Altenbach, 1989, Trimarchi and Debbie, 1977, and Constantine et al., 1968). Colonial bat species live in very dense clusters, suggesting an obvious scenario for repeated aerosol exposures to rabies. Constant vocalization and frequent echolocation in densely packed colonies are potential sources of aerosol from the oral and nasal mucosa of bats that are infected with rabies virus. Clinically ill rabid bats have been observed to emit incessant, abnormal vocalization (Constantine et al., 1972). Published reports (Murphy, 1977) and unpublished data (Davis, unpublished) demonstrate the presence of infected tissue in the nasal and oral mucosa of colonial bat species. Our study offers evidence of the ability of aerosolized rabies virus to induce production of serum neutralizing antibody to rabies virus in exposed bats and mice. However, what role, if any, is played by aerosolized rabies virus in naturally arising rabies immunity is not understood. The lack of morbidity and mortality in bats following exposure to aerosolized rabies virus suggests that such an aerosol route of exposure does not play a major role in bat-to-bat transmission of rabies,

although it could be responsible for the naturally occurring antibody found in bat populations.

CHAPTER IV

Epidemiology of Rabies in Bats from a Texas Bridge Colony And Throughout Colorado

4.1 INTRODUCTION

The state of Texas is home to the largest bat colonies in the world, some with upwards of 30 million bats. *Tadarida brasiliensis*, which make up these large colonies, are very successful in taking up residence in caves, abandoned houses, and under bridges (Constantine, 1967). At times the number of bats exceeds 300 per square foot (Constantine, 1967). The Congress street bridge in Austin, Texas is the world's largest urban bat colony and home to approximately 1.5 million *T. brasiliensis* (Bat Conservation International (BCI), 2005). This site is famous worldwide and draws approximately 3 million visitors annually and adds over 8 million dollars annually into the Texas economy (BCI, 2000).

Like *T. brasiliensis*, the big brown bat, *Eptesicus fuscus* are highly colonial, albeit on a much smaller scale. A large colony of *E. fuscus* would consist of hundreds of bats rather than hundreds of thousands. Both species lack high roost fidelity and a single bat may inhabit several roosts throughout the summer months. With the continuous movement of bats between roosts, it is likely bats are exposed to a variety of pathogens, including rabies virus. With such an enormous amount of interaction between bats and

the close proximity in which they roost, the low frequency of clinically rabid bats within bat populations is surprising.

It has been estimated that less than 1% of wild bats are infected with rabies virus at any given time (Debbie and Trimarchi, 1977 and Childs, 2001). The numbers from diagnostic laboratories are skewed much higher as these laboratories receive highly biased cases, such as grounded bats or bats flying during the day. Depending on the geographic location, the bat species most commonly submitted to diagnostic laboratories are *E. fuscus* or *T. brasiliensis*. Approximately 17% of *E. fuscus*, and 16% of *T. brasiliensis* submitted to certain diagnostic laboratories in the U.S. are rabies positive (Pape et al., 1999, Rohde et al., 2004). The number of bats reported rabid from field studies is low and large outbreaks have not been reported.

The reasons for the low intraspecies transmission rate of rabies virus remain a mystery. Constantine (1989) hypothesized that animals that develop clinical rabies are more likely to be immunocompromised than animals that do not develop rabies. Alternately, highly virulent viruses may be fatal to the bat prior to centrifugal migration to the salivary glands, thereby preventing the bat from transmitting the virus. (Brass, 1994). It is also possible that juvenile bats may be exposed to rabies virus as maternal antibodies wane yet the remaining maternal antibodies may continue to provide a low but adequate defense against rabies virus infection (Steech and Atenbach, 1989)

The experiments in this chapter were designed to estimate the number of rabid bats in a wild population, the percentage of rabid bats with live virus in their salivary glands, and the presence of anti-rabies viral neutralizing antibodies in rabid bats during the

terminal stages of disease. Although rabies in bats is a public health issue in the United States, large-scale bat die offs are not typically associated with rabies virus infection.

4.2 METHODS AND MATERIALS

Immunostaining for detection of rabies virus antigen

To detect rabies antigen in the brain, slides were prepared using approximately 0.03 g of tissue. Approximately 0.05 g of macerated salivary gland tissue was employed in creating slides to detect rabies antigen in the salivary glands. Slides were dried and fixed in 100% acetone at -20 C overnight. Further processing of slides for the direct fluorescent antibody (dFA) testing followed the rabies compendium protocol (CDC, 2006) employing the Chemicon or Centocor anti-rabies conjugate. Slides were viewed on a Nikon fluorescent microscope at 150X and 450X.

Serum Neutralization

The bats in this study were euthanized as a result of illness, injury, or being found grounded and unable to fly. Bats were first anesthetized with pentobarbitol then blood was collected via cardiosentesis with a sterile 26-gauge needle. Blood was centrifuged for 5 minutes; serum was separated and placed on dry ice until further processing. Virus neutralizing antibodies (VNA) were measured by the rapid fluorescent focus inhibition test (RFFIT) (Shankar et al., 2004, Smith et al., 1996). To inactivate complement, serum was heated at 56^0C for 40 minutes. Fivefold dilutions were mixed with a constant dose of challenge virus standard-11 (CVS-11) rabies virus in the Lab-Tek Chamber Slide ™ system (Nalge, Nunc Rochester, NY,USA). CVS-11 was obtained from the New York State Department of Health Rabies Laboratory. Infection of mouse neuroblastomas

(Neuroblastomas C-1300, compliments of New York State Department of Health) was demonstrated by the use of Chemicon anti-rabies conjugate. The CVS-11 virus was diluted as recommended by Smith et al. (1996) and Trimarchi et al. (1996). The U.S. Standard Rabies Immunoglobulin (R-3), a human derived rabies immunoglobulin (Bethesda Maryland, Food and Drug Administration, Office of Biologics Research and Review) was employed as the positive control in each RFFIT. Antibody titers are reported in International Units (IU) unless otherwise specified. A titer of $\geq$ 0.5 IU is considered acceptable for individuals working with rabies virus (World Health Organization, 2005, Briggs, 2002).

Because a limited amount of blood can be collected from a bat, the volume of serum per sample assayed ranged from 3 µl to 50 µl. Approximately 90% of the sample volumes were between 20-30 µl. The RFFIT detected anti- rabies antibodies in serum samples containing a volume of 5µl or greater and with a limit of sensitivity of 0.0625 IU or greater.

Virus isolation and titration

Virus was isolated from the salivary glands as described in Rudd and Trimarchi (1989, 1987). The salivary glands were removed and homogenates (10%w/V) were made with a Ten-broeck tissue grinder using 1.0 ml of GM (minimal essential medium supplemented with 10% fetal bovine serum, 2.0 mM glutamate, 100 IU penicillin,G, 50µg streptomycin, and 2.5µg/ml amphotericin B) as a diluent. Salivary gland homogenates were frozen at –80 ^{0}C until further processing could take place. In 96-well plates, viral titration was done in duplicate employing neuroblastoma cells (NA-1300) as described in Trimarchi et al., 1996. Briefly, 50 µl of test inoculum was added to 50µl

neuroblastoma cells and 150 µl GM. Test inoculum was diluted 1:5 in each individual well. The plates were incubated in 5% CO_2 for 72 hours. After 72 hours the plates were removed and the fluid was aspirated from the wells. The wells were washed twice in phosphate buffered saline (pH 7.6) for 5 min, air dried, and fixed in 75% acetone at -20C^0 for at least 1hour. Further processing of slides for the direct fluorescent antibody (dFA) testing followed the rabies compendium protocol (CDC, 2006) employing the Chemicon (Chemicon International, Temecula, CA) or Centocor (Centocor Inc. Horsham, PA) anti-rabies conjugate. Following application of the anti-rabies conjugate, the plates were placed in a 5% CO_2 incubator for 1 hour. The conjugate was decanted and the wells were washed twice as described above and allowed to air dry. One or two drops of mountant media (Rudd et al., 2006) were placed in each well and the cells were viewed with a Nikon fluorescent microscope. Titers were calculated using the Reed and Munch equation.

RNA was isolated by adding 100 µl of the viral suspension to 1ml Tri-Reagent and processed using 1-bromo-3-chlorpropane (BCP) as per the manufacturer's recommendations (Molecular Research Center, Inc., Cincinnati, OH). Fifty µl of the RNA extract was stored at –20 ^{0}C for at least one hour prior to the reverse transcriptase (RT) procedure.

Bats

Bats were obtained for from three sources.

Colorado State Department of Health and Prevention: Rabid bats submitted to the Colorado State Department of Health and Prevention (CDHPE) during the years 2000-2004 were provided to CSU to determine the presence or absence of rabies virus antigen in salivary

gland tissue. The bats were speciated originally by CDHPE personnel and a second time by Dr. Tom O'Shea (United States Geological Service, Fort Collins, CO). Each bat was necropsied with sterile scissors. Approximately 0.1g of brain tissue was removed. Both mandibular salivary glands from all bats were removed. All tissues were immunologically stained to detect rabies virus antigen as described above. The amount of antigen present in the brain and salivary glands was determined employing a scale of 0 to +5, with 0 indicating a negative specimen, and +5 indicating the presence of viral antigen in all observable cells.

Captive colonies of *E. fuscus and T. brasiliensis*: From 2001 to 2005, four colonies of *E. fuscus* were established at Colorado State University. The *E. fuscus* were removed from buildings or caught in mist nets over drinking sites. Between 2002 and 2003 two colonies of wild caught *T. brasiliensis* were established from bats captured at a bridge in Austin, Texas. During quarantine, bats were monitored at least twice a day. Any bat that died or was euthanized while in captivity was necropsied and a brain smear was done to identify the rabies status of the animal.

Tadarida brasiliensis from Round Rock, Austin, Texas: Grounded bats were collected twice a day for eight days from the base of a bridge outside of Austin, Texas, in late September 2004. Bats were sedated immediately following collection from the ground, and following blood collection, the bats were euthanized and placed on dry ice. Blood was placed into sterile 1.7ml collection tubes. Serum was separated by centrifugation for 5 minutes and stored at on dry ice. All samples were brought to CSU for further processing. The carcasses were thawed, a brain smear prepared, and salivary glands were removed and processed for detection of rabies virus antigen and infectious virus. Sera were assayed for neutralizing antibody employing the tissue culture serum neutralization (Trimarchi et al. 1996).

4.3 RESULTS

Bats Received from the Colorado State Department of Health and Prevention

A total of 67 rabid bats, representing five species, were provided from the CDHPE during 2000-2004. Rabies virus antigen was detected in the brain of all bats, and in the salivary glands of 51% .(Table 4.1). Rabies virus antigen was present in the salivary glands of 100% (1/1) of the *Myotis* spp., 50% of the *Lasiurus cinerus* (7/14), 50% of the *E. fuscus* (23/46), 100% of the *L. noctivagans* (2/2), and 25% of the *Nyctonomops macrotis* (1/4). Bats were received in varying states of decomposition ranging from recently euthanized to mummified. Virus isolation from the salivary glands was attempted for all bats, and virus was isolated from 34% of the salivary gland suspensions (Table 1).

Table 1 Summary of viral antigen detection in brain and salivary glands.

Characteristic	Amount of Viral Antigen in Brain				
	5+	4+	3+	2+	1+
Average amount of viral antigen in salivary glands	+1.6	+1.8	+1.4	+1.0	0
Percentage of bats with virus antigen in salivary glands	8%	54%	14%	24%	0%
Percentage of bats with infectious virus in salivary glands	8%	41%	30%	21%	0%

Captive Colonies of *E. fuscus* and *T. brasiliensis*

Four colonies of captive *E. fuscus* bats were brought maintained at CSU between 2001 and 2004. The 2001 colony consisted of 35 bats, two of which developed clinical rabies while in captivity. The first bat developed signs consistent with rabies virus infection approximately four weeks following introduction into the captive colony. This

bat was euthanized and found to be rabid by the direct fluorescent test (dFAT) (Shanker et al., 2004). Sixteen days later a second bat developed clinical signs compatible with rabies virus infection, was euthanized, and found to be rabies positive by the dFAT (Shanker et al., 2004). Rabies virus was isolated from the salivary glands of both rabid bats.

During 2002 a second colony of 89 *E. fuscus* was maintained at CSU to study West Nile virus in chiropteria. One developed rabies 4.5 months following introduction into the captive colony, 10 days following inoculation with West Nile virus (Davis, et al. 2005). The salivary glands from this bat were not available for virus isolation.

A third colony of 12 *E. fuscus* bats was maintained at CSU during 2003. None of these bats developed clinical rabies.

A fourth colony was composed of 62 *E. fuscus* bats was maintained at CSU in 2004. Two bats (4%) developed clinical signs compatible with rabies virus infection. The first bat became aggressive and ataxic more than 4 months following captivity, and the second bat developed clinical signs of rabies more than 6 months following admission into the captive colony, 58 days after the first bat. Rabies virus was detected by immunofluorescence in the brains of both of these animals, but virus was not isolated from salivary gland suspension from either.

From the 199 *E. fuscus* brought into the captive colonies during 2001-2004, five (2.5%) developed rabies during the six-month quarantine period. Of the five bats that developed rabies while in captivity, at least three bats were naturally infected in the wild whereas two may have been exposed while in captivity. While in captivity, the three bats developed clinical signs prior to experimental rabies inoculation and had no exposure to

rabies virus (Table 2). The two bats that subsequently developed rabies while in captivity were clinically healthy when their cage mates developed clinical illness. In some instances, fighting was observed between the ill and healthy bats. It is possible that the incubation period in these two bats was longer than in their three cage-mates following exposure in the wild. Conversely, it is possible these two bats were infected by their rabid cage mates while in captivity.

Table 4.2. Development of rabies and detection of rabies virus infection in captive colonies of *E. fuscus*.

	Year				
	2001	2002	2003	2004	Total
Number of bats	35	89	12	63	199
Number of bats developing rabies	2	1	0	2	5
(% of total)	(6%)	(1%)	(0%)	(4%)	(2.5%)
Number of bats from which rabies virus was	2	ND	ND	0	2
isolated from salivary glands (% of rabid)	(100%)			(0%)	(40%)

In 2002 and 2003, 160 *T. brasiliensis* were collected in Austin, TX and maintained in a captive colony at CSU. At the time of capture, one bat (0.6%) was easily removed by reaching up into a bridge crevasse, was unresponsive, showed mild paralysis, and was euthanized 12 hours later. The bat was positive for rabies antigen by the direct fluorescent antibody testing of brain. During the two-month quarantine period, 70% of the remaining *T. brasiliensis* died or were euthanized following the onset of severe respiratory symptoms. None of these bats were found to be rabid. The remaining 30% remained in captivity without development of clinical signs of rabies.

Tadarida brasiliensis from Round Rock, Austin, Texas

A total of 81 downed bats were collected from under the bridge outside of Austin, TX. Of these animals, 86% were diagnosed as rabid by the direct fluorescent antibody testing on brain tissue. Rabies virus antigen was demonstrated in the salivary glands of 58% of these rabid bats. Of these rabid bats, rabies virus was isolated in cell culture from 30% of the salivary glands, five of which did not have detectable antigen in the salivary glands. Thirty-six rabid bats (42%) had serum anti-rabies viral neutralizing antibodies with titers ranging from 0.5 IU to >17.6 IU. The number of bats collected each day and the percentage that were rabid is listed in Table 3.

Live virus was not isolated from the salivary glands of *T. brasiliensis* in which the amount of brain antigen was +1 or +5. The amount of antigen in the brain of all bats from which virus was isolated from the salivary glands was +4.

Table 3. Rabies virus detection in downed bats collected under the bridge in Austin, TX.

Date collected	Bats collected	Number of bats positive brains	Percent SG FAT positive	Percent SG Virus isolation positive
9/11/2004	12	11	83%	10%
9/12/2004	9	8	88%	25%
9/13/2004	5	5	100%	20%
9/14/2004	14	13	68%	38%
9/15/2004	26	21	81%	28%
9/16/2004	12	9	75%	44%
9/17/2004	3	3	100%	0%
Total or mean	**81**	**70**	**58%**	**30%**

4.4 DISCUSSION

The paucity of rabies outbreaks in bat populations has not been widely studied but several hypotheses have been proposed. Evidence of adaptation between rabies virus and bats over the last eleven thousand years has been described by Hughes et al. (2005) and Tordo et al. (2001), employing the use of molecular clocks. The co-evolution in bat populations is characterized by low levels of rabies virus circulating within bat populations, a low percentage of rabid bats with infectious virus in salivary gland tissue, no large die-offs due to rabies virus infection, several bat specific variants, a low percentage of rabies virus positive bats with circulating anti-rabies VNAs, and a long history of naturally acquired anti-rabies neutralizing antibody in healthy bats (Smith, 2002, Brass, 1994, Steech and Atenbach, 1989).

Conversely, the more recent spillover of bat rabies into the raccoon population is characterized by a high incidence of rabies in the wild raccoon population, a high percentage of rabid raccoons with infectious virus in salivary gland tissue, the continuous spread into naïve areas, the maintenance of one raccoon rabies variant, and the recent identification of animals with naturally acquired anti-rabies VNAs in $\geq$20% of apparently healthy raccoons tested (Bigler, et al., 1973 and Niezgoda et al., 2001).

Canine rabies was described more than four thousand years ago and continues to kill more than 40,000 humans annually (WHO, 2005, Childs, 2002). Canine and chiropterian rabies virus variants are similar in many regards: there are several variants of the viruses, typically of geographical origin; both have become endemic in several areas throughout the world; and naturally occurring antibodies can be found throughout certain

canine and chiropterian populations ranging from 20% to 80% (Mebatsion et al., 1992, Wosu and Anyanwu, 1990, and Ogunkoya et al., 1990). Unlike chiropterian rabies, outbreaks of canine rabies continue to occur in many areas of the world. Although the canine rabies virus has continued to evolve with the dog population, it does not appear to be as well adapted to the host population as chiropterian rabies viruses.

Outbreaks of bovine rabies resulting from rabid vampire bats have been described for decades, outbreaks of rabies in vampire bat populations have not (Baer, 1999). Similar to insectivorous bats, the incidence of rabies in vampire bats is less than 3% and extensive surveys have found the incidence rate varied from 0.46% to 0.75%. Furthermore, in 56 wild caught vampire bats, 36% had circulating anti-rabies viral neutralizing antibodies and the brains for all 56 were negative when tested with the direct fluorescent antibody test (Baer, 1999).

Occurrences of spillover events are not unheard of but when they do occur, they typically result in a small number of cases, and the variant is not sustained in the new host. In a recent spillover event, an *E. fuscus* bat rabies variant began to circulate in a small skunk population in Arizona. Hughes and Rupprecht (2003) experimentally inoculated Arizona skunks with either an Arizona skunk rabies variant or the *E. fuscus* variant circulating in the skunks. All animals developed clinical rabies infection and at euthanasia, they found 25% of the skunks inoculated with the skunk rabies variant developed neutralizing antibodies, as compared to 100% of the skunks experimentally infected with the *E. fuscus* variant (Hughes and Rupprecht, 2003). This suggests that a variant recently introduced into a population is more immunogenic than the host adapted variant.

Adaptation to rabies virus infection within the *T. brasiliensis* population may be demonstrated by the low morbidity and mortality rates, low percentage of bats with detectable amounts of infectious virus in their salivary glands and high percentage of wild bats with naturally acquired anti-rabies VNAs. Steech and Altenbach (1989) reported that nearly 70% of the 750 wild caught adult *T. brasiliensis* had circulating anti-rabies IgG. IgM was circulating in approximately 2% of the wild caught bats. Less than one percent of the adult wild caught *T. brasiliensis* were rabies positive by immunofluorescence on brain tissue. Juvenile *T. brasiliensis* were 3.5 times as likely to be rabies positive by the immunofluorescence test than adults (2%), and 10% of the 600 juvenile *T. brasiliensis* had circulating IgM antibodies. One hypothesis for the paucity of rabies within bat populations is early exposure to rabies virus and the development of immunity. Although the prevalence of anti-rabies neutralizing antibody in healthy *T. brasiliensis* was not studied here, the number of rabid bats in a large colony was estimated and was similar to that found in other studies, which helps to explain the low prevalence of rabies in bat populations.

In natural exposures, the protection conferred is likely to be dependent on the variant to which the animal was exposed, the host species, health of the host, location of exposure, and type of exposure. In one study it was found that 50-80% of unvaccinated humans contracted rabies following multiple severe head bites, 15-40% following bites to the finger, hand or arm, and 3-10% following bites to the leg (Childs, 2001). Indeed, not all exposures are immunologically equal. The exposure of an animal unintentionally bitten during grooming by a rabid bat that has not yet developed clinical illness is very different than that of a bat that is attacked by a clinically rabid animal. Furthermore, an

animal exposed to aerosolized rabies virus in a roost during commensal grooming or sneezing episodes may result in a different immunological response than an animal that is bitten by a rabid animal.

The prevalence of seropositive rabid bats in our study suggests the *T. brasiliensis* rabies variant has adapted somewhat to the host, thus decreasing the hosts' immunological response to clinical infection. Conversely, the route of inoculation may impact the immunological response to rabies virus. In the current study, only 46% of the rabid *T. brasiliensis* bats were seropositive. All of these bats were in the terminal stages of clinical rabies virus infection at which time the presence of anti-rabies VNA may be expected demonstrating the hosts' attempt at clearing the virus. Due to the neurotropic nature of rabies virus, it is possible the virus was never presented to the immune system to allow an immunological response to develop. Shanker et al. (1991) reported that replication in muscle is not necessary for CNS infection and rabies virus can directly infect neurons. The lack of antibodies to rabies virus in *T. brasiliensis* may have resulted from the virus being sequestered in neurons and never encountering the immune system.

It is unknown what level of immunity is conferred in naturally exposed animals with circulating anti-rabies antibodies. In the studies reported in Chapter II, a majority of animals inoculated with a primary intramuscular challenge developed anti-rabies antibodies and survived a second intramuscular challenge six months later. This experimental evidence provides support for the theory that anti-rabies antibodies in exposed animals may be protective. Some animals with a titer of >0.5 IU succumbed to the first or second IM challenge. As reported in chapter III, all of the animals exposed to aerosolized rabies virus seroconverted but were not protected against subsequent

intramuscular challenge with rabies virus. The two different routes of inoculation are likely to be responsible for the differences in the immune response.

Previous studies examining the prevalence of rabies within wild bat populations have suggested that 0.5% to 2.5% of all wild bats are infected with rabies at any given time, yet more than 10% of bats submitted to public health laboratories are rabies positive (Pape et al., 1999). The abnormal behavior of rabid bats such as daytime flight, becoming grounded, inappropriate roosting in human dwellings, and possible aggression all result in an increased interaction between bats and humans. Unlike healthy bats, which typically avoid interaction with humans, it is the bats that come into contact with humans that are submitted to public health laboratories, consequently explaining the discrepancy between rabies in wild bat populations and rabies in bats submitted to public health laboratories. In our studies, 0.6% and 1.6% of wild caught and apparently healthy *T. brasiliensis* and *E. fuscus,* respectively were rabies virus positive via immunofluorescence. Conversely, 86% of grounded *T. brasiliensis* tested positive for rabies virus.

Large-scale bat die offs are rare and prior to laboratory testing, were often believed to be the result of a rabies outbreak (Constantine, 1978). In all cases, laboratory results demonstrated a lack of rabies involvement and typically lead to the diagnosis of pesticide poisoning, other bat-related diseases, or weather. Rabies virus infection in bats is maintained in low levels within bat populations as demonstrated by this and previous studies. The low percentage of rabies virus infection in healthy, wild caught bats and the presence of antibodies in these bats help to explain the paucity of rabies in bat populations.

Infectious rabies virus was isolated from salivary glands of 30% of rabid *T. brasiliensis*. If 0.6% and 1.6% of wild caught *T. brasiliensis* and *E. fuscus* were incubating rabies, using the data that rabies virus can be isolated from the salivary glands of 30% of rabid *T. brasiliensis* and 40% of *E. fuscus*, one can approximate that 0.18% of the healthy wild-caught rabid *T. brasiliensis* and 0.64% of the healthy wild-caught rabid *E. fuscus* would be capable of transmitting rabies. Because the frequency of rabies virus shedding from healthy bats incubating rabies is unknown, these numbers may not adequately reflect the ability of a healthy bat to transmit rabies virus. The discrepancy between infectious rabies virus in the salivary glands of rabid bats and the presence of viral antigen may be the result of viral clearance by the immune system. Rabies antigen is produced by viral protein complexes and does not represent infectious virions. The presence of infectious rabies virus in bat saliva at any time during infection is variable and has been reported to be intermittent. Fifty-five percent of rabid bats described in chapter II and 10% of the rabid bats described in chapter III had infectious virus in their saliva within 14 days of death. Therefore, our results suggest less than 50% of rabid bats are secreting infectious rabies virus prior to death.

The amount of rabies virus antigen in the brain did not always reflect the amount of antigen in the salivary glands (Table 4.1). It was expected that the amount of antigen in the brain would correlate with infectious virus in the salivary glands. There may be several explanations for the lack of infectious virus in the salivary glands in animals where the brains were rated +5 including infection by a more virulent variant resulting in death prior to centrifugal spread. Currently, there no evidence supporting the hypothesis

that immunosuppressed animals will succumb to the infection prior to centrifugal spread to the salivary glands.

It is estimated that during the summer months 2,000,000 *T. brasiliensis* reside every day at the bridge sampled in this study. An average of 12 bats, or 0.0006% of the estimated population, dies from the bridge population die of rabies each day. If this is extrapolated for 12 bats/day, approximately 0.2% of the bats at the bridge will develop rabies at some point during the year. The time of year during which these bats were collected is when rabies virus is circulating in the colony at its highest level (Steech and Atenbach, 1989), and therefore this estimate is likely higher than what may be occurring on average. This estimate does not account for the bats that become grounded after leaving the bridge roost.

Rabies virus is maintained within bat populations by less than 2% of the population. It has been proposed that the low level of rabies within bat populations can be attributed to a lack of infectious virus in the salivary glands and saliva. Therefore, the majority of rabid bats are likely to be dead end hosts for the virus.

CHAPTER V

SYNOPSIS AND CONCLUSIONS

Rabies virus is a disease of mammals and with a fatality rate of >99%. Rabies is currently one of the most deadly viruses known to exist. Rabies virus can be found throughout the world, with the exception of a few island nations and states. Worldwide, canine rabies variants are the most common variants of rabies, killing in excess of 50,000 people a year, at a rate of one human every 15 minutes, typically children less than 14 years of age. In the US, canine rabies cases began decreasing as a result of vaccination efforts several decades ago. Rabies in insectivorous bats was reported in the US during the 1950's and has since become a major public health concern.

In the last 20 years, cryptic rabies cases in humans are commonly associated with bat rabies variants. Although the individual may not recall a history of a bite, in every case defined as cryptic, there has been some association with bats whether it is a history of a bat in the house or an unnoticed bite after handling a bat. Most of these cryptic rabies cases were the result of infection by one of three bat variants, *L. noctivagans*, *P. subflavus*, or *T. brasiliensis*. With the exception of *T. brasiliensis*, it is rare for these tree dwelling bat species to come into contact with humans. It has been postulated that these variants are more infectious and virulent than other rabies viruses and this has been a focus of rabies research for the past 17 years. Research data have demonstrated that the *L. noctivagans* variant is unique in its ability to replicate at lower temperatures and in

several cell types when compared with a carnivore variant. The differences between bat variants have not been studied and leave a void in our knowledge of bat rabies.

To better understand differences between bat rabies variants, two *E. fuscus* rabies variants where experimentally inoculated into bats and mice. The data from this experiment demonstrated that infrequent changes in the rabies genome may have profound effects on infectivity and virulence. Comparison of the variants in bats demonstrated that the *Efv2* variant resulted in clinical infection much more quickly than *Efv1*. Mice appeared to be less susceptible to clinical infection by either variant. Furthermore, the gold standard for typing, the rabies N gene, was ineffective in identifying changes in the genome. Larger segments of the N gene and inclusion of other rabies genes was necessary to identify changes in the genome that resulted in amino acid differences in the proteins. Mutations in the proteins may be the result of selective pressure, rendering a variant more capable of adapting to the host and improving its probability of centrifugal spread to the salivary glands.

The presence of anti-rabies VNA in bats has been reported for decades, yet it is unknown if their presence was the result of exposure following the bite of a rabid bat or how protective these antibodies were. The data presented in this study depict the development of anti-rabies VNAs in the majority of bats experimentally inoculated with rabies virus. In bats that survived the primary challenge, an increase in anti-rabies VNA was typically demonstrated in the bats following the second challenge. Some of the bats that did develop VNA's succumbed to rabies virus infection.

Following exposure to aerosolized rabies virus, anti-rabies virus antibody was detected in the serum of the majority of bats. When the bats were challenged by

intramuscular inoculation six months later, the number of bats that survived was the same as the control group. The presence of VNAs appears to decrease but not abrogate the susceptibility of a bat to rabies infection upon challenge. The route of exposure by which an animal develops VNAs may also play a role in the susceptibility following a subsequent challenge

Approximately 0.5%-2.5% of all apparently healthy wild bats are believed to be infected with rabies virus. This estimate is supported by the number of healthy bats that were brought into captivity and subsequently developed rabies, and by the field work performed in Texas. The low prevalence of rabies virus infection in bats may be the result of a previous exposure to a less virulent virus, non-infectious dose of virus, and/ or the immune status of the animal. In the majority of rabid bats, rabies virus was not isolated from the salivary glands. This may help explain the lack of rabies outbreaks in bats. Furthermore, this may allow the virus to maintain itself indefinitely within a population

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