Physiological Correlates of Bat Rabies Pathogenesis and Epizootiology

by

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Abstract

Rabies virus (RABV) is one model pathogen to consider for studying the dynamics of emerging infectious diseases under both laboratory and field conditions. The evolutionary history of RABV is characterized by regularly documented spillover infections and a series of notable host shifts. RABV is not a single, genetically homogeneous and unvarying virus, but a virus that has undergone genetic changes in adapting to local hosts and local habitats, such that each variant occupies a unique ecologic niche. The opportunity for re-emergence of rabies in mesocarnivores and the likelihood of sustained transmission of RABV in different species is influenced in part by host and variant-specific factors. Understanding pathogen maintenance and transmission within the natural reservoir may facilitate early detection strategies and targeted interventions prior to human spillover. As such, the general focus of this dissertation is to integrate a contemporary molecular, comparative, and ecological approach to complement our existing knowledge on bat rabies epizootiology and pathogenesis. The multidisciplinary approach of Chapter 2 consists of examining the physiologic response and phylogenetic relationships among bat RABV variants circulating the United States, including those implicated in recent and historical spillover infections in humans and mesocarnivores. To gain insight into viral pathogenesis and identify clinical features among individuals associated with experimental RABV infection, I along with the help of many collaborators evaluated the use of infrared thermography to detect thermographic changes associated with experimental RABV infection in captive big brown bats. These data suggest that infrared thermography has utility for

discriminating rabid bats in natural field settings. In addition, focusing upon RABV circulating in the United States between 2008 - 2013 confirmed spillover events of bat RABV among carnivores and identified cross-species transmission events caused by four lineages of RABV associated with insectivorous bats. Rabies in bats is considered enzootic throughout the New World, but few comparative data are available for most countries in the region. The remedy for this lack of knowledge, Chapter 3 extends information on the geographic distribution of RABV circulation among bats to assess the broader public and veterinary health risks associated with bats in Guatemala. The detection of RABV neutralizing antibodies demonstrated viral circulation among multiple bat species in Guatemala. These data indicate that the proportion of seropositive bats varies significantly across trophic guilds, suggestive of complex intraspecific compartmentalization of RABV perpetuation. Chapter 4 seeks to address potential host shifts by examining differences in susceptibility, pathogenesis, and neurovirulence of major US RABV variants associated with bats and carnivores. The results of this study determined that the incubation period is influenced significantly by variant. These data also support the concept that spillover infections of biologically relevant North American RABV can be transient or dead ends, but dependent upon the isolate, dose, and route, could result in a sustained transmission when epizootiological conditions are ideal. Taken together, this dissertation suggests that the balance between pathogenesis and epizootiology is unique to the RABV variant and particular host.

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List of Abbreviations

ANOVA	analysis of variance
BEAST	bayesian evolutionary analysis sampling trees
BLAST	basic local alignment search tool
CDC	Centers for Disease Control and Prevention
CI	confidence intervals
CNS	central nervous system
COY	coyote
CVS	challenge virus standard
DFA	direct fluorescent antibody
EF	*Eptesicus fuscus*
FFU	fluorescent focus-forming unit
FITC	fluorescein isothiocyanate
G	glycoprotein
GTR	generalized time reversible substitution model
i.c.	intracerebral
i.m.	intramuscular
IHC	immunohistochemistry
IQR	interquartile range

IRT	infrared thermography
IU	international unit
LNRV	*Lasionycteris noctivagans*
mAb	monoclonal antibody
MEM	Dulbecco's modified essential medium
MICLD	mouse intracerebral lethal dose
MNA	mouse neuroblastoma cell culture
mRNA	messenger RNA
N	nucleoprotein
NCSK	north central skunk
NYC	New York City
p.i.	post inoculation
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RABV	*Rabies virus*
RAC	raccoon
RFFIT	rapid fluorescent focus inhibition test
RIG	rabies immune globulin
RT-PCR	reverse transcription polymerase chain reaction
rVNA	rabies virus neutralizing antibody
SCSK	south central skunk
SMSG	submandibular salivary gland

Chapter 1

General Introduction

Emergence of novel high-consequence pathogens appears unpredictable. To date no pathogen has been predicted to emerge before human morbidity and mortality occurred [1]. Origins of novel pathogens are correlated significantly in part with ecological and socio-economic factors. As such, these parameters provide a basis for identifying potential epidemiologically relevant ‘hot-spots’ where novel pathogens are predicted to emerge, providing appropriate targets for enhanced surveillance [2]. Indeed, a focus upon early detection and rapid screening of relevant wildlife populations seems a logical strategy to predict spillover and potential host shifts during disease emergence events. However, the challenge of such an approach is the determination of a measureable signature reflective of relevant pathobiological events within the host populations of interest, which is rarely pathognomonic of infections with specific agents [3]. Variation in individual host physiology, particularly in relation to immunocompetence, influences basic susceptibility and response to infection. Such attention to host attributes often receives less research focus than those facets (e.g. virulence factors) related to the pathogen. Improved introspection of host responses is required to understand the ‘species barrier' limitations associated with host range of a pathogen, and may provide novel surveillance opportunities by allowing focal targeting of those populations at risk and most likely to harbor conditions supportive of the next emerging zoonosis [4]. An approach that would validate such techniques requires that efforts be directed toward likely reservoirs and vectors at the human-animal interfaces, especially where epidemiological modeling suggests an increased risk of such transmission [5].

Bats and Rabies

Rabies is the oldest known zoonosis associated with bats and given both their veterinary and public health significance, the transmission and spillover dynamics associated with bat *Rabies virus* (RABV) offer numerous laboratory and field research opportunities to better understand parameters related to disease emergence, pathogen transmission and perpetuation in host populations and communities. Since 1953 (but likely longer before), RABV has been reported to occur in insectivorous bats in North America [6]. Increased public health awareness and laboratory -based surveillance have identified RABV infection in the majority of bat species throughout the United States and Canada [7]. Numerous New World bat species harbor specific RABV variants. However, relatively few comparative experimental studies have been conducted with RABV variants associated with bats [8-10].

Rabies is an acute, progressive encephalitis resulting from infection with any of the neurotropic viruses in the genus *Lyssavirus*. RABV is just one of several lyssaviruses that cause rabies, but is the major etiologic agent of the disease. Considering its global burden, distribution and public health significance, rabies is an ideal model to better understand disease emergence. All lyssaviruses possess an RNA genome that encodes five structural proteins, the nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and RNA polymerase (L) [11]. Estimates based on coalescent analysis of viral genes demonstrate that the ages of contemporary RABV lineages are close to a thousand years old [12]. While all mammals are considered susceptible to rabies, bats are believed to serve as the ultimate historical source of carnivore infections, based upon epidemiological, ecological, and phylogenetic inferences [13-16].

Rabies is considered the most important viral disease associated with bats from a global perspective, and RABV is the most significant lyssavirus from an epidemiological perspective [16]. The development and application of appropriate research tools provide the opportunity for comparisons of virus-host interactions during natural and experimental infections in bats and other mammals, and may identify those parameters needed to enhance strategies to combat host shifts associated with RABV. Such multi-disciplinary studies require improvement of basic knowledge in bat ecology, physiology, immunology, virology, and genomics, among others. These insights are useful not only for investigation of other emerging bat zoonoses, but also clearly demonstrate that rabies serves as a unique model for exploration of novel areas and modern concepts in science as a whole.

The pathogenesis of RABV infection: Correlates of virus and host factors

Rabies occurs by introduction of infectious RABV virions into a susceptible host's tissue. Routinely, this event occurs as a result of direct bite contact from an infected animal. In a typical case, following intramuscular exposure, RABV virions progress to the neuromuscular spindles as well as to the synaptic cleft at the neuromuscular junctions of the innervating neuron [17]. The glycoprotein spikes on the surface of virions are critical for RABV pathogenicity, as these structures are responsible for specific binding to cellular receptors. Efficient RABV entry into neurons is likely possible through neuron-specific receptors, such as the p75 neurotrophin receptor (p75NTR), neural cell adhesion molecule (NCAM), or nicotinic acetylcholine receptor (NAChR) [18,19]. After receptor binding, viral ribonucleoprotein (RNP) is released into the cytoplasm of the host cell. This requires a pH-dependent fusion of viral and endosomal membranes that occurs following endocytosis. Thereafter, RABV is transported from the

periphery towards the central nervous system (CNS) by retrograde axonal transport via sensory and/or motor pathways averaging 50–100 mm per day [20].

Considering transit from a portal of entry to the CNS, RABVs must overcome the numerous hurdles of host physiology, as replication occurs in the presence of potent host defense mechanisms –including the type I interferon (INF) system, which has powerful antiviral activity and important adjuvant functions in adaptive immunity. Preservation of the cellular functions involved in virus transport may be one reason for the development of self-limiting mechanisms that down-regulate RABV gene transcription. Successful manifestation of RABV infection in the CNS and subsequent transmission to other organisms depends on the integrity of long distance transport mechanisms. A number of RABV protein domains have been implicated in viral virulence [21]. Known for its pronounced INF sensitivity, RABV is suggested to encode mechanisms that prevent IFN expression. For example, the amino acids Phe 273 and Tyr 394 on the N protein of a pathogenic RABV have been shown to play a role in inhibition of the INF system and other cytokines important in the immune response [22]. Inhibition of the INF system prevents expression of anti-viral proteins at the site of infection, allowing the virus to be transported to the CNS. The activity of the RABV P protein may play a critical role in this mechanism [23]. Local viral replication in non-neuronal tissue is suggested to result in variable incubation periods. However, if local viral replication does occur at the exposure site, it would likely be effective in triggering an immune response. Sub-maximal viral gene expression is suggested to delay apoptotic or other counteracting host responses, thereby allowing effective virus dissemination. Additionally, RABV appears to have evolved mechanisms to regulate gene expression from maximal levels to optimal levels allowing for sufficient virus replication and host cell preservation simultaneously [24]. However, considering its simplicity and the

fundamental molecular structure of this negative sense, single-stranded RNA virus, the basic capacity for RABV lineages to generate genetic variation is restricted by the low-fidelity, RNA-dependent RNA polymerases, leaving little genomic blueprint for adaptation of immune evasion genes.

The pathogenic capacity of an infectious RABV virion can be defined by virulence factors associated with the ability to invade and colonize its natural reservoir host and produce transmission – achieved by limited viral replication in the periphery. The regulation of expression levels of antigenic viral proteins is perhaps one of the most notable virulence factors concerning RABV pathogenesis –as minimal replication evades initial detection by the host immune system –thereby conserving neuronal architecture [25]. Transmission of the virus relies on dissemination from the CNS to a completely new micro-environment: the salivary glands of the infected host, and selection pressure may operate on quasispecies viral diversity generated from replication in the periphery to the CNS and to the portals of exit, to impact the ensemble of viral populations that are ultimately transmitted to the next host. Considering that viral transmission occurs before the onset of obvious illness, the virus can exploit effectively a variety of socio-biological mechanisms inherent in mammalian taxa for effective direct host-to-host contact – without the necessity of a secondary advantage – the peculiar mechanisms of behavioral change and biting tendencies during the encephalitic phase.

Host physiological correlates are biomarkers used as an indicator of a biologic state. It is a characteristic that is objectively measured and evaluated as an indicator of normal biologic or pathogenic processes [26]. More specifically, a "biomarker" is a substance or trait that indicates a change in expression or state of a protein that correlates with the risk or progression of a disease, or with the susceptibility of the disease to a given treatment. Once a proposed biomarker has

been validated, it can be used to infer disease risk, presence of disease in an individual, or to tailor treatments for the disease in an individual [27]. A biomarker can also be used to indicate exposure to a virus or vaccine [28,29]. For many viruses, antibodies serve as a biomarker of past virus exposure and correlates of immunity. However, for RABV, the presence of rVNA is clearly not a perfect correlate of immunity/protection in bats, since exposed individuals may develop antibodies without being protected necessarily against disease upon subsequent infection [30]. However, rVNA are routinely used to indicate evidence of RABV circulation in a population or species. Beyond detection of rVNA, few immunological or physiological biomarkers to predict outcome to rabies infection in bats have been validated. In many cases, prediction of pathogen virulence or transmissibility from molecular or phylogenetic data alone is still not feasible, although promising work has been done with *in vitro* techniques to characterize virulence [31-33]. Certain genetic signatures in the G protein may also predict the spillover potential of bat RABV into carnivores [34]. This predictive value will further increase if the molecular data are correlated with epidemiological and clinical data, as our understanding of host requisites for transmission and barriers to cross species transmission improves [2].

The correlation between body temperature and various diseases has been known for centuries. Because of the advent of new technologies, skin temperature measurement obtained via infrared thermography has been used on humans and animals as a non-invasive diagnostic method for measuring physiological or pathological changes in skin temperature resulting from administration of pharmaceutical compounds, surgical procedures, changes in vascularity or blood flow, and both systemic (fever) and local (inflammatory) responses to disease conditions [35-37]. One prior study has indicated thermal profile changes of rabies infected raccoons [38].

Characteristics of RABV epizootiology: Paradigms for Cross Species Spillover Events

A pathogen's basic reproduction ratio (R_0) is a representative measure of fitness. The variant with the highest R_0 outcompetes all others. However, pathogen fitness depends on the average survival time of the hosts, thus, high virulence is not expected to pay off [39]. Outbreaks outside of a major reservoir, characteristic of many multi-host pathogens, can be of variable and unpredictable timing and magnitude. The epidemiological and ecological processes that determine whether a pathogen occurs in a spillover host as sporadic infections, minor outbreaks, or major epidemics remain poorly understood, but are of critical importance in the context of EIDs. The success of RNA viruses in crossing species barriers is enhanced by their high mutation rates (generated by error-prone RNA polymerases and large within-host population sizes), which provide genetic and phenotypic variability that enable ongoing transmission into new hosts [40]. Many bat-associated viruses display broad cellular tropism, and share high genome conservation within the Order: *Chiroptera*, suggesting strong selective constraints [41]. As such, pathogens are therefore expected to evolve towards intermediate levels of virulence that optimizes host exploitation.

Bats of many species have been shown to be involved in the sylvatic RABV cycle (bat-to-bat or bat-to-other mammal-to-other mammal, etc.) and in the dead-end road represented by any mammal which dies before transmitting the virus [42]. The epizootiology of RABV appears simple (infected mammal-to uninfected mammal) but in reality is complex. In one respect, RABV can be transmitted directly from bats (virus-infected bat to uninfected human, equid, bovid, canid, raccoon, skunk, or any other mammal) but the terrestrial mammal that becomes infected may serve as a temporary (until it dies) source of virus which can then infect other

mammals, thus establishing a cycle, not involving bats [43]. The converse, while possible, appears limited on the basis of epidemiological or ecological grounds.

Currently, there are at least two explanations for the evolutionary history of lyssaviruses: that fitness differences among lineages have enabled some to out- compete others, resulting in a selective purging of lineages, or that intermediate lineages have died out because of stochastic processes alone [44]. Although it is possible that the fixation of advantageous mutations that enable RABV to adapt to new host species has occurred but cannot be detected by current methods, suggests that purifying, rather than positive selection, dominates evolutionary dynamics [45]. Therefore, the most parsimonious explanation for current patterns is that random processes have had a more profound effect on RABV evolutionary history than previously thought. Hence, the dual processes of random genetic drift and geographical isolation alone are likely to be sufficient to explain the evolutionary history of carnivore RABV variants [46]. In support of this idea, it is known that terrestrial clades RABV isolates cluster more by geographical origin than by host species, such that the virus forms ‘‘epizootic’’ compartments in which closely related viruses infect a variety of species [47]. Even terrestrial variants of bat origin exhibit similar geographic clustering [47,48].

Viral phylogeny in general correlates with the phylogeny of bats, and it appears that successful spillover infections and host shifts occur more frequently among related species [49]. Estimates of RABV prevalence in North American bats have varied between species and across years within species [50]. In Latin America, vampire bats are undeniably the most important reservoir and vector of rabies to humans and livestock, but the diversity of independent foci associated with insectivorous bats and spillover to other mammals has recently been gaining wider recognition and appreciation [51-53].

Disease emergence requires, in addition to the presence of an infectious agent, an effective bridge from the reservoir host to a susceptible spillover host. Such bridges may be caused by changes to the agent, the host, or the environment. Increasing human development and associated land-use changes, in combination with wildlife populations under stress, with altered foraging and behavioral patterns; facilitate interactions in closer proximity to humans and livestock [54]. As more information has been generated about the underlying drivers or causes of disease emergence, there is an expectation that it might be possible to predict or forecast the emergence of pathogens, but until now there has been little success for predicting how, where or when a novel pathogen might arise, or the spillover events which might precede emergence. Nevertheless, there has been an unprecedented search for emerging pathogens in the past decade, especially at the human– wildlife interface. These are supported by the development of new technologies for detection and identification such as high throughput sequencing technologies, and by the initiation of pathogen discovery programs [55]. Broad pathogen testing across multiple species can facilitate testable models that address several long-standing questions in virology, such as why in the same host does one virus induce severe pathology, whereas a closely related variant induces little or no disease, and how does the same virus induce widely different transmission dynamics in different hosts?

Importance and Relevance of Dissertation Research: An interdisciplinary approach for understanding viral pathogenesis and epizootiology

As critical keystone species and global disease reservoirs, this research investigates the characteristics of RABV variants associated with bats, comparing their relationships with each other, and evaluates their medical, epizootiologic or evolutionary significance associated with

pathobiological parameters. This dissertation explores the critical interplay between population ecology, genetics and host physiology of bat-associated RABV variants within a One Health theoretical framework. Specifically, the goal of this multidisciplinary study design was to focus on specific virus-host interactions that form the basis of a complex causal chain leading from initial infection, to the onset of clinical signs of disease, leading to death or host survival. I also examine the mechanisms through which viral components may redirect or disrupt host functions, and relate how these interactions progress to various clinical syndromes, influencing subsequent RABV transmission and perpetuation.

The sporadic nature of RABV infections in wildlife and lack of routine surveillance hampers an understanding of host factors associated with viral pathogenesis, limiting the opportunities to initiate appropriate medical and veterinary countermeasures to prevent or respond to apparent host shifts. Because of this limitation, animal models are utilized to gain partial insight into the pathogenesis of disease with the overall goal of identifying relevant targets for such intervention [56]. The Big Brown Bat (*Eptesicus fuscus*) is a highly synanthropic rabies reservoir in the United States, and is the most frequently submitted bat species for rabies diagnostic testing [57]. Furthermore, this species can survive well in captive conditions, and is frequently used as an experimental animal model [10,30,58]. To gain insight into thermographic variation during viral pathogenesis, in Chapter 2, I determine the normal infrared thermographic profile in the captive big brown bat (*Eptesicus fuscus*); test the hypothesis that a decrease in facial surface temperature, as determined by the measurement of temperature using infrared thermography, precedes the visible onset of clinical signs of RABV CNS infection (determined by measurement of pre- and post-inoculation facial temperatures) among experimentally infected individuals, and evaluate whether variation in individual thermal profiles has any utility in early

detection of clinical infection in rabid big brown bats. In addition, focusing upon RABV circulating in the United States between 2008 and 2011, I confirm spillover events of bat RABV among carnivores and identify cross-species transmission events caused by four lineages of RABV associated with insectivorous bats.

The most effective strategy to tackle the threat of RABV emergence is to focus epidemiologically upon regions where wildlife biodiversity is high, and where anthropogenic factors (e.g. human development) that may exacerbate disease emergence are operative. As such, multiple areas in the Neotropics provide both a rich biodiversity of mammalian species, as well as burgeoning human and domestic animal populations, often in conflict with wildlife at the agro-ecosystem interface. In particular, vampire bats are found only in the New World. Based upon their unique hematophagous lifestyle, these highly adaptive bats are responsible for significant morbidity and mortality among humans and cattle in a broad region from Mexico to Argentina. With this in mind, recognizing the utility of experimentation performed both in the laboratory and in the field, in Chapter 3, I explore serological indicators of RABV exposure among a diverse Neotropical bat community, describing basic patterns of immunological response to RABV among vampires and other multiple species of bats in Guatemala, to determine if seroprevalence (as a proxy of RABV infection pressure) is associated significantly with either species trophic guild or sex, and explore whether these results would support the role of bats as a substantive reservoir for RABV in Guatemala. Additionally, sequence data from RABV virus isolates obtained as part of this study were examined to provide information on the genetic diversity and origins of vampire bat rabies in Guatemala in relation to circulation elsewhere in Latin America.

The intent of any disease model is to provide insight into the pathogenesis of a pathogen in susceptible host populations for the purposes of designing and testing potential medical or veterinary management interventions. To achieve this, an ideal *in vivo* disease model should reproduce all clinical syndromes as closely as possible in an immunocompetent animal subject following a realistic challenge infection administered via an appropriate exposure route with a wild-type etiological agent of the disease [56]. In Chapter 4, I further explore the concept of RABV host shifts by focusing upon an important terrestrial reservoir infection model, the red fox (*Vulpes vulpes*), to test for variation in susceptibility and pathogenesis between RABV variants (e.g. bat versus carnivore) following inoculation with a standardized dose of a distinct RABV variant. I investigated the following hypotheses: 1) the RABV variant used for inoculation has an effect on incubation period, with the bat variant positively correlated with incubation period length; 2) the pattern of viral dissemination within the CNS is associated with the particular glycoprotein gene sequence of the infecting RABV variant, and that highest antigen distribution is associated with the carnivore variant; 3) foxes infected with a bat RABV variant display a neutralizing antibody response (rVNA) inversely correlated with the degree of viral excretion in the salivary glands.

Comprehensively, this dissertation provides insights towards a more complete understanding of RABV pathogenesis and epizootiology, as well as support for novel strategies in reducing the global burden of human mortality associated with bat rabies.

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Chapter 2

Multidisciplinary approach to epizootiology and pathogenesis of bat rabies viruses in the United States

Abstract

Zoonotic disease surveillance is typically initiated after an animal pathogen has caused disease in humans. Early detection of potentially high-risk pathogens within animal hosts may facilitate medical interventions to cope with an emerging disease. To effectively spillover to a novel host, a pathogen may undergo genetic changes resulting in varying transmission potential in the new host and potentially to humans. *Rabies virus* (RABV) is one model pathogen to consider for studying the dynamics of emerging infectious diseases under both laboratory and field conditions. The evolutionary history of RABV is characterized by regularly documented spillover infections and a series of notable host shifts. Within this context, enhanced field surveillance to improve detection of spillover infections will require validated techniques to non-invasively differentiate infected from non-infected individuals. In this study we evaluated the use of infrared thermography to detect thermal changes associated with experimental RABV infection in big brown bats (*Eptesicus fuscus*) in a captive colony. Our results indicated that 62% of rabid bats had detectable facial temperature decreases (-4.6°C, SD ±2.5), compared to pre-inoculation baseline values. These data suggest potential utility for discriminating rabid bats in natural field settings. In addition, focusing upon RABV circulating in the United States between 2008 - 2011, we confirmed spillover events of bat RABV among carnivores and identified cross-species transmission events caused by four lineages of RABV associated with insectivorous bats. Additionally, our analysis of RABV glycoprotein sequences identified substitutions in antigenic

sites that may affect neutralizing activity associated with monoclonal antibodies proposed for use in human post-exposure prophylaxis. This study provides a glimpse into RABV pathobiology and spillover dynamics among and between bats and a variety of mesocarnivores.

Introduction

Rabies is an acute progressive viral encephalitis (Family *Rhabdoviridae*, genus *Lyssavirus*), and remains one of the oldest and most thoroughly studied zoonoses associated with bats. Fundamentally, bats are believed to drive the natural history and evolution of lyssaviruses (Rupprecht *et al.*, 2011). Among more than a dozen lyssaviruses associated with bats, *Rabies virus* (RABV) is the type species and most important member of the genus from a public health perspective. Concerning recent host shifts into mesocarnivores, the raccoon and south-central skunk RABVs are more closely related to bat RABV lineages than other carnivore lineages (Badrane & Tordo, 2001; Nadin-Davis & Real, 2011). How RABV disseminates into novel hosts, and why spillover events result typically in dead-end rather than sustained infections is unclear. Host taxonomy and social behavior are suggested to play a role in RABV perpetuation, due to the frequency of interaction among con-specifics (Hughes *et al.*, 2005). The RABV host range is also constrained by species barriers (Streicker *et al.*, 2010).

Bats are unique among other mammals regarding thermoregulation, as many are heterothermic. For bats, metabolic activity and body temperature operates within a range of values on a daily basis, fluctuating regularly from a basal resting state to an active state of flight; both of these states can persist for several hours during torpor or daily foraging and even extend to several days or weeks as part of short and long-distance seasonal migrations, or when bats are in hibernation. Whether or not this widely variable metabolic activity has an influence on viral

pathogenesis has not been addressed. A mathematical model parameterized from a longitudinal study of RABV in a Colorado population of big brown bats suggested a critical influence of hibernation in modulating seasonal infection dynamics (George *et al.*, 2011). This model suggests that life history patterns of many temperate-zone bats, coupled with sufficiently long incubation periods, facilitate RABV maintenance. Seasonal variability in bat mortality rates, and specifically low mortality during hibernation, promotes long-term host population viability. Within susceptible populations, sufficiently long incubation periods may allow enough infected individuals to enter hibernation and survive until the following year, and possibly avoid an epizootic fadeout of infection. Further, studies on the influence of environmental temperature on the pathogenesis of experimental RABV infection in Mexican free-tailed bats (*Tadarida brasiliensis)* and little brown bats (*Myotis lucifugus*) provided evidence that minimal viral replication occurs in the host during experimentally induced hibernation, thus raising numerous questions concerning the interactions of environmental temperature on immune function in hibernating bats (Sulkin *et al.*, 1960). Similarly, whether daily (e.g., colonial roosting or refuging) and/or seasonal (e.g., torpor) behavioral adaptations for dealing with temperature and energetic stress in temperate and tropical latitudes also impact intrinsic host susceptibility and ultimately pathogen evolution requires experimental evaluation (Streicker *et al.,* 2012).

Despite the relatively high mutation rates of RNA virus genomes, including RABV, strong purifying selection is the primary force shaping the evolution of lyssaviruses (Rupprecht *et al.*, 2011). All mammals are susceptible to RABV, likely due to evolutionarily conserved host cell receptors (Holmes *et al.,* 2002). However, which host cell molecules interact with RABV glycoproteins (G) to facilitate entry into host cells *in vivo*, particularly with respect to viral dissemination into extraneural tissues, is less clear. The RABV G is the only exterior antigen,

and is responsible for inducing specific RABV virus-neutralizing antibodies (Wunner, 2007). Understanding of antigenic variability of RABV G associated with different reservoir hosts is of particular importance for development of novel biologics for rabies prophylaxis, such as virus-neutralizing monoclonal antibodies (MAb) that are capable of binding to specific linear or conformational epitopes on the viral G (Marissen *et al*., 2005; Bakker *et al.*, 2005; Sloan *et al*., 2007; Wang *et al*., 2011).

Novel schemes for preventing and controlling zoonotic diseases in wildlife can only be developed by integrating studies on host-pathogen ecologies (Hayman *et al.,* In Press). Within this context, enhanced surveillance under field conditions will require validated techniques to non-invasively differentiate infected from non-infected individuals. The multidisciplinary approach of this study consisted of examining infrared thermography and phylogenetic relationships among bat RABV variants that circulate in the U.S., including those implicated in recent and historical spillover infections in humans and mesocarnivores. To gain insight into viral pathogenesis and identify clinical features among individuals, we evaluated the use of infrared thermography to detect thermographic changes associated with experimental RABV infection in captive big brown bats, as evaluated previously in raccoons (*Procyon lotor*) (Dunbar & MacCarthy, 2006). The prevalence of RABV is low in natural populations of bats with ~0-0.5% infection rates reported (Kuzmin & Rupprecht, 2007). Our approach has potential to identify infected individuals and may improve the efficiency of active field surveillance efforts. Here, we utilize *E. fuscus* as an experimental animal model due to its broad geographic distribution, local abundance, and complex genetic structure (Agosta, 2002; Turmelle *et al.*, 2011). Additionally, big brown bats are the most frequently submitted bat species for rabies diagnosis in the U.S., suggesting substantial human exposure (Blanton *et al.*, 2011).

Laboratory-based surveillance is an essential tool for the detection and identification of novel lyssaviruses and RABV diversity (Freuling *et al*., 2011; Marston *et al.*, 2012). Historically, both spillover events and host shifts have been revealed by public health surveillance systems (Flanagan *et al.*, 2011). In the U.S., RABV has been detected in all extant families of bats (Constantine, 2009). Bat RABV lineage diversity is partitioned into clades, which are associated with individual bat species or genera (Smith *et al.*, 1995; Streicker *et al*., 2010). Most of these clades are monophyletic and appear to be dominated by intraspecific exchanges, suggesting that con-specifics maintain circulation of a RABV variant across areas that correspond with the host species geographic distribution, such as the big brown bat, *Eptesicus fuscus,* in North America or various *Lasiurus sp.,* throughout the Americas (Sheeler-Gordon & Smith, 2001; Shankar *et al.*, 2005; Nadin-Davis *et al.*, 2010). Further, molecular and epidemiologic studies have identified RABV variants associated with insectivorous bats in the majority of human rabies cases in the U.S. following the control and elimination of enzootic canine RABV (Velasco-Villa *et al.*, 2008b; Petersen & Rupprecht, 2011).

Materials and Methods

Experimental animals

Twenty-seven big brown bats were collected from colonies in local buildings in GA, U.S. Capture, handling, and experimental procedures were performed in compliance with requirements of CDC Institutional Animal Care and Use Committee. Collection of animals occurred under GA Department of Natural Resources permit #29-WSF-05-14. Bats were marked individually with metal forearm bands and quarantined for approximately one month Bats were

held in groups of three to six animals in stainless steel cages 813 X 305 X 254 mm. All cages were placed into an ABSL-2 facility at 24-27ºC and 30-50% humidity.

Experimental RABV infection

The viral inoculum used in this study was a bat-associated RABV obtained from the salivary glands of a naturally infected gray fox (*Urocyon cinereoargenteus*) from Arizona during 2009 (CDC A09-2400). We chose this RABV because it demonstrated peripheral mortality in this experimental model. On Day 0, 24 bats were inoculated intramuscularly with 25 µl of viral suspension into both left and right masseter muscles (total volume=50 µl). The virus dose was $10^{2.5}$ 50% mouse intracerebral lethal doses ($MICLD_{50}$) per ml, as determined by titration in mice (Dean *et al*., 1996). Three control bats were not inoculated, but co-housed with inoculated bats. Bats were either euthanized post-inoculation (pi) at planned time points (Days 7, 14, and 21) or following clinical manifestation of rabies (i.e. paresis, paralysis, ataxia, atypical aggressive or reclusive behavior).

Infrared Thermography

Thermograms were collected between 0900 and 1530 hr on the day before inoculation and on days 7, 14, 15, 18, 19 and 21 pi. Each bat was restrained by hand at a distance of 0.6-0.7m from a P640 thermal camera (FLIR Systems, North Billerica, MA, USA). Object parameters for the thermogram were either set when the thermogram was captured or corrected during processing. Parameters consisted of emissivity set at 0.96 and reflected temperature, atmospheric temperature and humidity set to the ambient conditions. For processing the thermograms, Reporter 8.5 (FLIR Systems, North Billerica, MA, USA) software was used to acquire the mean

facial temperature of individual bats. We evaluated the threshold of temperature for "normal" bats assessing variations within the temperatures recorded from the day before inoculation (Day -1 pi). The difference between each temperature recorded pi and the pre-inoculation temperature was calculated for individual bats. Due to a slight skew to the data and limited sample sizes on certain days, we used a non-parametric evaluation for significance of the temperature changes. We compared the individual values to the interquartile range (IQR) from the difference between the 75th percentile and 25th percentile of the Day -1 temperatures among individual bats. Temperature was then classified as positive (difference > +IQR), steady (-IQR < difference < +IQR), or negative (difference < -IQR) for each day of thermogram collection. We considered the change significant if the difference was outside the -IQR to +IQR range.

RABV diagnosis

Necropsy was performed on all bats. Brain impressions were fixed in acetone at -20°C, and RABV antigens were detected by the direct fluorescent antibody test (dFA), using fluorescein isothiocyanate (FITC)-labeled monoclonal antibody (mAb) conjugate (Fujirebio Diagnostics, Inc., Malvern, PA, USA), as described (Dean *et al.*, 1996).

RNA extraction, sequencing and phylogenetic reconstruction

Brain specimens from carnivores and bats of various species were collected across the continental US between 1991 and 2011 (Table 2). Total RNA was extracted from brain tissues using TRIzol ™ according to manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Reverse transcription-polymerase chain reaction (RT-PCR) was performed as described (Kuzmin *et al.*, 2003). The G gene was amplified as two overlapping fragments using primer combinations

umf2/994b and 760f/308b (Table 3). The RT-PCR products were purified with Wizard ® PCR Preps DNA Purification System (Promega, Madison, WI, USA), according to the manufacturer's recommendations and sequenced in forward and reverse directions using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit, version 1.1 on an ABI3770 automatic sequencer (Applied Biosystems, Carlsbad, CA, USA). Editing and alignments of sequences were performed using BioEdit (Hall, 1999) and Clustal X (Larkin *et al.*, 2007). Sequences were truncated to the coding region of the G gene (1572 nucleotides), and analyzed by Bayesian methods, implemented in BEAST, version 1.3 (Drummond & Rambaut, 2007). The optimal evolutionary model was chosen with MEGA, version 5.01 (Tamura *et al.*, 2011). The general time-reversible model incorporating both invariant sites and a gamma distribution (GTR+I+G) was the best model for the dataset. Two simultaneous analyses, each with four Markov chains, were run for 10,000,000 generations and sampled every 1,000 generations. The final inference of the tree was summarized from both runs with the initial 10% of samples discarded as burn-in. The remaining trees were used to build a 50% majority rule consensus reconstruction. Posterior probability values were used to assess support at each node. The trees were visualized using FigTree program, version 1.3.1 (Rambaut, 2009).

Results

Results of RABV challenge

Five infected bats per day were euthanized on days 7, 14, 19 and 21 pi, and two per day on Days 15 and 18 pi. With rare exception, bats displayed clinical signs of rabies on the day of euthanasia (Table 1). Among 24 inoculated bats, 21 were diagnosed as rabid by detection of RABV in the brain by dFA, and three were dFA negative. The three dFA negative bats included two animals

euthanized on Day 7 pi and one bat euthanized on Day 21 pi (Table 1). Control bats were euthanized on Day 38 pi. During this time control bats did not display clinical signs of rabies, and RABV antigens were not detected in brain tissue by dFA.

Thermography

Pre-inoculation, the mean facial temperature of bats was 30.9°C and the median was 31.0°C. The difference between the minimum and maximum facial temperature recorded was 3.7°C and the IQR was 1.5°C. Bats euthanized on Day 14, 15, 18, and 19 pi had median temperatures < 30°C. Additionally, on each of these days, ≥50% of the euthanized bats had a facial temperature decrease (individual facial temperature difference < -1.5°C) and none of the bats had facial temperature increases (Table 1). Bats euthanized on Day 7 and 21 pi had a median facial temperature ≥30°C on each day, ≤40% of individuals presented decreased facial temperatures (Table 1). Two euthanized bats presented with increased facial temperatures on Day 7 pi. The median facial temperatures for the three control bats ranged from 31.8°C on Day 7 pi to 30.3°C on Day 19 pi. The facial temperature difference at each sampled time for the control bats remained within ±1.5°C.

At the time of euthanasia, 13 bats (54%) had a decreased facial temperature. The median decrease was 3.4°C (3.5 IQR) and the mean was 4.6°C (±2.5 SD). Seven of the bats had facial temperature decreases of 3.4°C beyond pre-inoculation baseline. Ataxia and/or paralysis occurred in six of the seven bats. One rabid bat with a facial temperature decrease of 6.5°C was euthanized on Day 14 pi and did not display observable clinical signs of rabies. One additional bat displayed ataxia and/or paralysis, and this individual’s temperature decreased by 2.6 °C.

Phylogenetic analysis

In total, 13 major lineages were identified from the phylogenetic analysis of G gene sequences (Figure 1; Genbank accession JQ685912, JQ685914, JQ685924, JQ685928, JQ685952, JQ685965, JQ685973, JQ685998–JQ68610, JQ686012, JQ686013, JX871837–JX871881). These were associated with: *Tadarida brasiliensis* (Tb), *Parastrellus hesperus* (Ph), *Myotis sp.* (My), *Lasiurus intermedius* (Li), *Lasiurus borealis* (Lb), *Lasiurus cinereus* (Lc), *Lasionycteris noctivagans* (Ln), *Perimyotis subflavus* (Ps), *Antrozous pallidus* (Ap), and *Eptesicus fuscus* (Ef). Variants associated with *E. fuscus* further segregated into four lineages: Ef-w1 and Ef-w2 with predominantly western distribution, and Ef-e1 and Ef-e2 with predominantly eastern and central distribution. Moreover, viruses associated with the genus *Myotis* represent substantial diversity among bat RABV lineages. We identified seven putative cross-species transmission events among bats, caused by viruses from four RABV lineages (Lb, Lc, Ap, Ps). Similarly, spillover infections with bat RABV (Ps, Ef-e1, My) were documented in carnivores (Table 2).

In the binding site for MAb CR57 (AA 229-231 [Marrisen *et al.*, 2005]), the substitution L/P_{231} was detected in the majority of bat RABV variants, except viruses from the lineage Ef-w2 and in one of the six available viruses from lineage Ef-e1 (both associated with big brown bats [*Eptesicus fuscus*], and in both available viruses associated with pallid bats (*Antrozous pallidus*; lineage Ap), which retained $L_{231.}$ In addition, RABV associated with Mexican free-tailed bats (*Tadarida brasiliensis*; lineage Tb) had $S_{231.}$

Numerous and variable substitutions were detected in the binding site for MAb CR4098 within antigenic site III (AA 330-338; [Bakker *et al.*, 2005]). For example, substitution $V/I/F_{332}$ was documented in several RABV variants, associated with big brown bats (Ef-w1, Ef-w2, Ef-

e2). Substitution N/D_{336} was detected in the majority of viruses from lineage Ef-e2. However, no sequences contained substitutions in the binding sites for MAbs CR57 and CR4098 simultaneously. Moreover, the presence of combination D_{336}-K_{346} was detected in the majority of viruses from lineage Ef-e2 within the binding site of MAb RAB1 (Wang *et al*., 2011) and HuMAb 17C7 (Sloan *et al*., 2007).

Discussion

These preliminary findings contribute to our understanding of the epizootiology and pathogenesis of RABV in bats. As with other etiological agents associated with bats, a greater appreciation is needed at the individual host-pathogen level, predicated with the diversity of outcomes from the variables of variant, dose, route, and host population. Bats differ from other mammals in that an individual's body temperature when at rest during any season typically approximates their environment (Hock, 1951). The metabolic rate reflects this change, so that metabolism is influenced by body temperature. The experimental infection data suggest a link between thermal changes detectable peri-mortem in big brown bats and clinical display of rabies. Bats displaying multiple signs of rabies, including those associated with an acute progressive encephalopathy (i.e. ataxia, paralysis), had substantially reduced facial temperatures at euthanasia or humane experimental endpoints (Figure 2). Some bats had detectable temperature changes without acute clinical signs. These individuals may have been euthanized before manifestation of illness, warranting further investigation into infrared thermography's ability to enhance earlier detection of rabid animals. Alternatively, individual temperature variations may have been greater than the variation captured from the pre-inoculation baseline thermograms. While some individual positive temperature changes were observed, we were unable to

determine causation from our data. Future research is needed to address the precise timing of changes in facial and body temperature in relation to RABV pathogenesis.

One recent model highlighted the slowing effects of hibernation on metabolic and viral activity maintained in infected individuals until arousal, when susceptible individuals from the annual birth pulse became infected (George *et al.,* 2011). Previous research on the effects of high ambient temperature on various stages of experimental RABV infection in laboratory mice found that exposure to high ambient temperature late in the incubation period delayed clinical manifestation, decreased mortality, and increased frequency of an abortive infection, but exposure to high ambient temperature after clinical manifestation did not affect the course of disease (Hock, 1951, Bell & Moore, 1974). As torpid hibernators, bats can adjust to a wide range of ambient temperatures and reduce their body temperature by 1–2 °C above the ambient temperature, thus drastically decreasing their metabolic rates. The adaptive value of this state is to conserve energy, although hibernating animals arouse periodically and their body temperature metabolic rate returns to normal for a period of usually < 24 hours (Pengelley & Fisher, 1963; Geiser *et al.*, 1990). Torpor induces leukopenia, and during this period lower levels of lymphocyte proliferation and antibody production can be observed, which is hypothesized to be beneficial in terms of energy conservation (Bouma *et al.*, 2010). An appropriate immune response operates most efficiently within narrow temperature confines, constrained by the unique properties of an individual's physiology. However, recent studies have suggested that the immune system may be at rest, or depressed, in torpid or hibernating mammals (Bouma *et al.,* 2010). Support of this concept has been suggested recently as a main factor allowing colonization of *Geomyces destructans*, the fungal agent associated with White Nose Syndrome in North America, which reproduces optimally during winter periods when many temperate bats

undergo torpor (Lorch *et al.,* 2011). The impact of variation in host metabolism resulting from short- or long-distance migration and torpor or hibernation behaviors deserves greater introspection with regard to perpetuation of infectious diseases in bat populations.

The association between geography and the rate of RABV evolution reflects differences in the seasonality of RABV transmission throughout diverse ecotypes (Streicker *et al*., 2012). As such, epizootiologic factors associated with RABV in bats include regional population differences and the effects of roosting habits, on both the proportion of rabid bats and numbers of bats submitted for diagnosis (Patyk *et al*., 2012). Additionally, roosting ecology influences estimates of RABV prevalence, with significantly higher prevalence among passive surveillance submissions of non-synanthropic bat species compared to synanthropic bat species submitted for rabies diagnosis in the U.S., a trend not evident in active surveillance reports (Klug *et al.*, 2011). Patterns of RABV circulation in bats also appear to be different from those described for carnivores (Blanton *et al*., 2011). For example, in Texas, rabid skunk cases peak during March and April (Oertli *et al*. 2009). Host species susceptibility needs to be balanced with pathogenicity of the virus, which is directly associated with host population density, mobility, sex and age composition, frequency of social contacts, and patterns of behavior (Sterner & Smith, 2006). As with any successful virus, these variables should ensure that rabid animals transmit virus to at least one other susceptible individual (i.e., $R_0 \geq 1$). Individual bats in large colonies may experience multiple exposures to RABV throughout their lifetime (Constantine *et al.*, 1972). Repeated sub-lethal exposures may provide a natural booster, confer significant immunological memory, and reduced susceptibility to RABV (Turmelle *et al*., 2010). However, infectious virus and/or antigens are not always present in the salivary glands of rabid bats (Davis *et al.*, 2012). In addition, the immunogenicity of RABV variants likely differs across host reservoir species. The

precise amount of virus introduced by bite or non-bite exposures necessary for a productive infection and lethal disease among insectivorous bats remains unknown.

In this report, phylogenetic analysis of bat RABVs based on their G gene sequences revealed similar phylogenetic groupings as reported previously utilizing other viral genes (Smith *et al*., 1995; Velasco-Villa *et al.,* 2006; Streicker *et al*., 2010; Nadin-Davies and Real, 2011). This is expected, given that major forces of RABV evolution include point mutations, lack of proofreading mechanisms, and strong constraints applied by purifying selection. We also confirm that tri-colored bats (*Perimyotis subflavus*) maintain circulation of a similar but distinct RABV lineage from that associated with the silver-haired bat (*L. noctivagans*), as was suggested from N-gene sequences (Franka *et al*., 2006). Initially, these two viruses were considered one variant, based on the reactivity patterns with anti-nucleocapsid MAbs and partial N-gene sequences (Smith, 1988; Morimoto *et al*., 1996; Messenger *et al.*, 2003). We also documented spillover of selected RABVs among bats and also to carnivores. One human rabies case in 2010 was the result of infection with a Ps RABV variant. Furthermore, viruses from My, Ef-w1, and Ef-w2 were associated with outbreaks in mesocarnivores in Arizona and Oregon during 2008-2010, consistent with similar findings documented during 2001-2005 in Arizona (Leslie *et al.,* 2006). Although substantial diversity was demonstrated for RABVs associated with *Myotis* bats, phenotypic identification of species, and particularly degraded specimens potentially received by state health laboratories, were beyond the scope of this study, and likely require robust morphological and genetic assessment for further resolution of specific RABV associations in this genus (Stadelmann *et al.*, 2007).

Our analysis of binding sites for putative RABV-neutralizing MAbs identified several G gene substitutions. Previous experiments with escape mutants demonstrated that substitution

$L/P/S_{231}$ did not preclude virus neutralization by MAb CR57 (Marissen *et al.*, 2005). However, substitution N/D_{336} precluded virus neutralization by MAb CR4098 in the escape mutant studies (Bakker *et al.*, 2005). No sequences contained substitutions in the both binding sites for MAbs CR57 and CR4098 simultaneously, which supports the use of a MAb cocktail as a replacement of conventional polyclonal rabies immune globulin for human PEP (Goudsmit *et al.*, 2006). In contrast, the proposed use of a single MAb for human PEP seems inappropriate. For example, the substitution N/D_{336}, and the combination D_{336}-K_{346}, was detected in our study in several RABV lineages associated with big brown bats, thus precluding the use of HuMAb 17C7 (Sloan *et al*., 2007) and MAb RAB1 (Wang *et al*., 2011) for human prophylaxis without combination with other MAbs.

The implications of spillover infections and host shifts for public and veterinary health are numerous. Human exposures to rabid carnivores may occur in localities believed to be "free" of rabies, and therefore not attract the necessary medical care including appropriate post-exposure prophylaxis. A preventable example of such an exposure resulted in a human rabies case in the U.S. acquired from a bite of a rabid canine (potentially a fox) in Oaxaca, Mexico, which was subsequently characterized as a bat RABV associated with *T. brasiliensis* (Velasco-Villa *et al.*, 2008a). Early detection of diseased animals within a population may improve surveillance opportunities, reduce economic loss, and minimize the loss of exposed humans, domestic species, and wildlife (Dunbar *et al.*, 2009). Further research is needed to determine the utility of thermography for predicting RABV infection under experimental and field conditions. Such applications may be limited to providing an additional record in determining a humane endpoint during experimental infections, or may have potential use in pathogen discovery and outbreak response by enhanced surveillance at roosts. The use of thermography will depend on

when notable temperature changes occur in relation to disease progression. Thermography is harmless for subjects, and can be performed without anesthesia. This technique has shown promise for detecting fever and inflammation, and is also useful for postsurgical, orthopedic and neurological monitoring. However, diseases with non-specific clinical features (i.e. fever) may produce similar thermal patterns, thus precluding its utility for providing a specific diagnosis, as opposed to generalized illness. With further studies and the advancement of this technology, thermography used in a clinical setting may become a standard tool in both human and veterinary medicine for improved surveillance, prevention and control.

Figure 1. Phylogenetic tree of bat RABV glycoprotein gene sequences rooted at the midpoint, and truncated to the coding region (1572 nucleotides), posterior probability values shown for key nodes

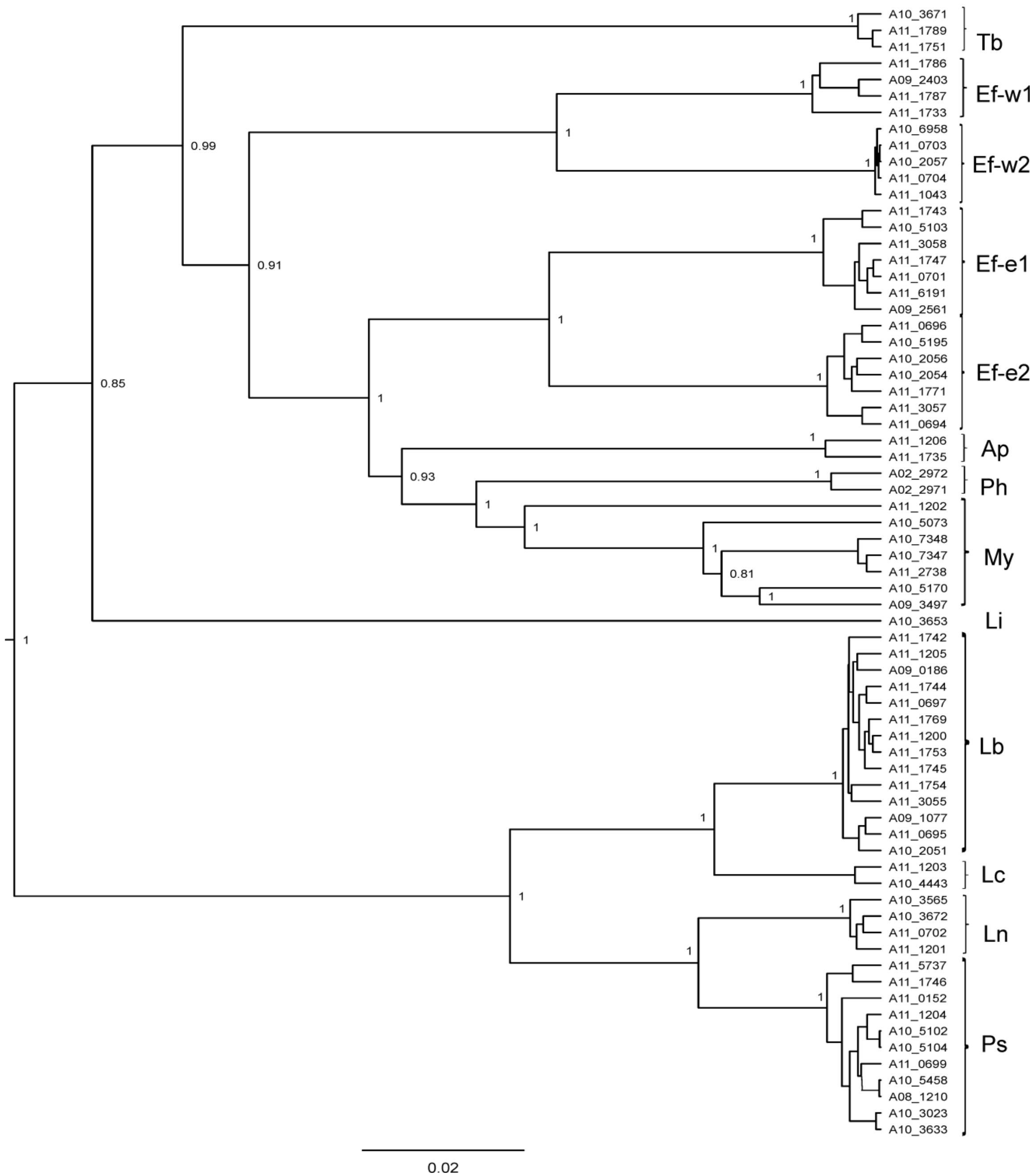

Figure 2. Individual facial thermograms shown in grayscale (top) and color (bottom) for bat 191 observed at (a) day –1 (pre-inoculation) = 37.8°C; (b) day 7 post-inoculation (pi)= 37.9°C; (c) day 14 pi= 34.3°C and (d) day 19 pi= 24.0°C (note: temperature scale modified)

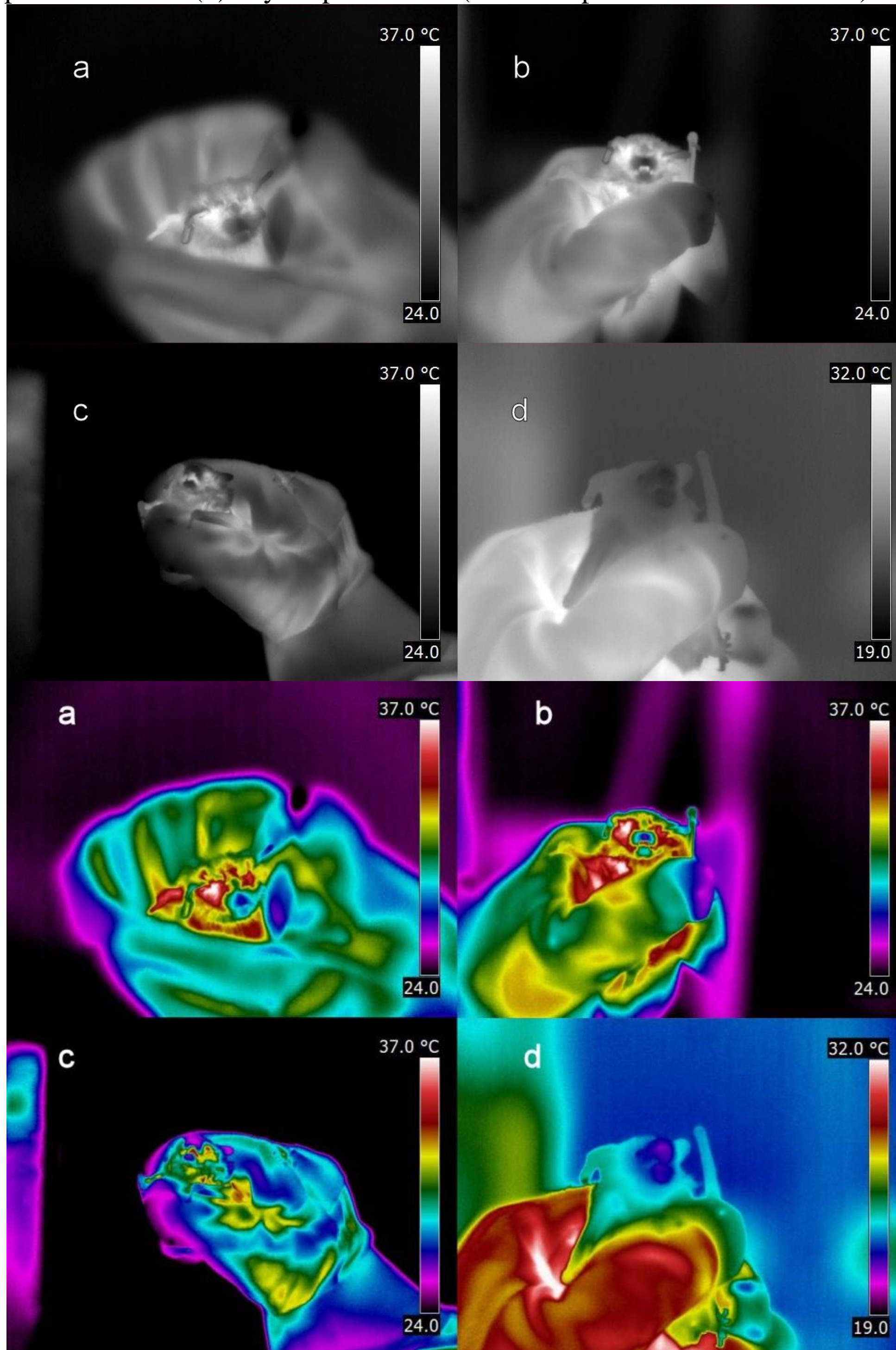

Table 1. Facial temperature of individual bats before inoculation of rabies virus and at time of euthanasia along with observed clinical signs

Animal ID	Day Euthanized	Facial Temperature °C			Clinical Signs	dFA Diagnosis
Inoculated		Pre-Inoculation	Day Euthanized	Change		
179	7	29.3	32.3	3	WNL	+
180	7	30.1	32.3	2.2	WNL	+
181	7	32.2	30	-2.2	WNL	+
182	7	30.5	30.9	0.4	WNL	-
184	7	29.6	30.1	0.5	WNL	-
190	14	30.1	30.6	0.5	WNL	+
197	14	31.6	29.7	-1.9	WNL	+
198	14	31	30.7	-0.3	WNL	+
199	14	32	25.5	-6.5	WNL	+
376	14	30.9	27.9	-3	WNL	+
192	15	29.8	28.6	-1.2	A/L,V,T	+
362	15	31.4	24.6	-6.8	P,T,X	+
178	18	32	28.9	-3.1	A/L,V,T,A	+
188	18	31.8	28.8	-3	A,V	+
189	19	32.2	23.4	-8.8	A/L,V,N,P,T,X	+
191	19	31.9	22.7	-9.2	L,V,N,P,X	+
193	19	30.9	24.9	-6	L,V,P,T,X	+
195	19	30.2	29.6	-0.6	A,V,N,T,D	+
372	19	28.45	25.9	-2.55	L,V,S,T,X	+
177	21	30.6	30	-0.6	A,V,T	+
194	21	31.7	27.8	-3.9	L,V,N,S,T,P	+
196	21	31.3	27.9	-3.4	A/L, V,N,S,P,T,X	+
370	21	30.3	30.7	0.4	WNL	-
375	21	31.3	30.7	-0.6	A,V	+
Uninoculated controls						
364	38	30.8	31.3*	-0.5	WNL	-
379	38	31.5	31.0*	0.5	WNL	-
400	38	31.4	30.3*	1.1	WNL	-

*Temperature from last day of thermogram collection, Day 21 pi

Abbreviation Key for Signs

A	Increased aggression	D	Dysphasia	X	Ataxia
P	Paralysis/Paresis	T	Tremors	L	Lethargy
V	Odd Vocalization	S	Seizure	N	Nystagmus
A/L	Minor Aggression	WNL	Within Normal Limits		

Table 2. RABV specimens referred in this study

CDC ID	Specimen Origin	Lineage	State	Year of isolation
A02-2971	*Parastrellus hesperus*	Ph	CA	2002
A02-2972	*Parastrellus hesperus*	Ph	AZ	2002
A08-1210	*Lasionycteris noctivagans*	Ps	FL	2008
A09-0186	*Lasiurus borealis*	Lb	NC	2009
A09-1077	*Lasiurus borealis*	Lb	NC	2010
A09-2403	*Urocyon cinereoargenteus*	Ef-w1	AZ	2009
A09-2561	*Eptesicus fuscus*	Ef-e1	IA	2010
A09-3497	*Myotis sp.*	My	AZ	2009
A10-2051	*Lasiurus borealis*	Lb	NC	2010
A10-2054	*Eptesicus fuscus*	Ef-e2	NC	2010
A10-2056	*Eptesicus fuscus*	Ef-e2	NC	2010
A10-2057	*Eptesicus fuscus*	Ef-w2	NC	2010
A10-3023	*Eptesicus fuscus*	Ps	AL	2009
A10-3565	*Lasionycteris noctivagans*	Ln	AL	2010
A10-3633	*Perimyotis subflavus*	Ps	NC	2009
A10-3653	*Lasiurus intermedius*	Li	FL	2008
A10-3671	*Tadarida brasiliensis*	Tb	FL	2009
A10-3672	*Lasionycteris noctivagans*	Ln	AL	2009
A10-4443	*Eptesicus fuscus*	Lc	VA	2010
A10-5073	*Myotis sp.*	My	AZ	2009
A10-5102	*Perimyotis subflavus*	Ps	NC	2010
A10-5103	*Eptesicus fuscus*	Ef-e1	NC	2010
A10-5104	*Perimyotis subflavus*	Ps	NC	2010
A10-5170	*Myotis sp.*	My	ID	2010
A10-5195	*Eptesicus fuscus*	Ef-e2	VA	2010
A10-5458	*Perimyotis subflavus*	Ps	NC	2010
A10-6958	*Urocyon cinereoargenteus*	Ef-w2	OR	2010
A10-7347	*Urocyon cinereoargenteus*	My	OR	2010
A10-7348	*Urocyon cinereoargenteus*	My	OR	2010
A11-0152	*Homo sapiens*	Ps	WI	2011
A11-0694	*Eptesicus fuscus*	Ef-e2	TN	2011
A11-0695	*Lasiurus borealis*	Lb	TN	2011
A11-0696	*Eptesicus fuscus*	Ef-e2	TN	2011
A11-0697	*Lasionycteris noctivagans*	Ln	TN	2011
A11-0699	*Perimyotis subflavus*	Ps	TN	2011
A11-0701	*Eptesicus fuscus*	Ef-e1	TN	2011
A11-0702	*Lasionycteris noctivagans*	Ln	IL	2011
A11-0703	*Urocyon cinereoargenteus*	Ef-w2	OR	2011

A11-0704	*Urocyon cinereoargenteus*	Ef-w2	OR	2011
A11-1043	*Canis latrans*	Ef-w2	OR	2011
A11-1200	*Lasiurus borealis*	Lb	NC	2010
A11-1201	*Lasionycteris noctivagans*	Ln	MI	2009
A11-1202	*Myotis thysanodes*	My	CA	1991
A11-1203	*Antrozous pallidus*	Lc	CA	1994
A11-1204	*Perimyotis subflavus*	Ps	NC	2010
A11-1205	*Lasiurus borealis*	Lb	NC	2010
A11-1206	*Antrozous pallidus*	Ap	NC	1993
A11-1733	*Eptesicus fuscus*	Ef-w1	AZ	2011
A11-1735	*Parastrellus hesperus*	Ap	AZ	2011
A11-1742	*Lasiurus borealis*	Lb	GA	2011
A11-1743	*Eptesicus fuscus*	Ef-e1	GA	2011
A11-1744	*Lasiurus borealis*	Lb	GA	2011
A11-1745	*Lasiurus borealis*	Lb	GA	2011
A11-1746	*Lasiurus borealis*	Ps	GA	2011
A11-1747	*Eptesicus fuscus*	Ef-e1	GA	2011
A11-1751	*Tadarida brasiliensis*	Tb	GA	2011
A11-1753	*Lasiurus borealis*	Lb	GA	2011
A11-1754	*Lasiurus borealis*	Lb	GA	2011
A11-1769	*Lasiurus borealis*	Lb	NC	2011
A11-1771	*Eptesicus fuscus*	Ef-e2	NC	2011
A11-1786	*Eptesicus fuscus*	Ef-w1	AZ	2011
A11-1787	*Eptesicus fuscus*	Ef-w1	AZ	2011
A11-1789	*Tadarida brasiliensis*	Tb	GA	2011
A11-2738	*Urocyon cinereoargenteus*	My	OR	2011
A11-3055	*Eptesicus fuscus*	Lb	MO	2011
A11-3057	*Eptesicus fuscus*	Ef-e2	MO	2011
A11-3058	*Eptesicus fuscus*	Ef-e1	MO	2011
A09-2400	*Urocyon cinereoargenteus*	Ef-w1	AZ	2009
A11-5737	*Vulpes vulpes*	Ps	VA	2011
A11-6191	*Vulpes vulpes*	Ef-e1	WV	2011

Table 3. Primers used in this study

Primer	Sense	Viral locus	Sequence (5' – 3')
Umf2	+	M - G	ATGGTGCCRTTRAATYGCTGCATTT
g994b	-	G	TTGAATATRGTRTATGCCTT
g760	+	G	CTDATGGAYGGAACNTGGGT
308deg	-	L - G	ACCTCTCCDGGATCGAKCT

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Chapter 3

Bat rabies in Guatemala

Abstract

Rabies in bats is considered enzootic throughout the New World, but few comparative data are available for most countries in the region. As part of a larger pathogen detection program, enhanced bat rabies surveillance was conducted in Guatemala, between 2009 and 2011. A total of 672 bats of 31 species were sampled and tested for rabies. The prevalence of rabies virus (RABV) detection among all collected bats was low (0.3%). Viral antigens were detected in the brain from two common vampire bats (*Desmodus rotundus*). From these, RABV was isolated from oral swabs, and viral RNA was detected in all of the tissues examined by hemi-nested RT-PCR, except for the liver of one bat. Sequencing of the nucleoprotein gene showed that both viruses were 100% identical, whereas sequencing of the glycoprotein gene revealed one non-synonymous substitution ($302_{T,S}$). The two vampire bat RABV isolates in this study were phylogenetically related to viruses associated with vampire bats in the eastern states of Mexico and El Salvador. Additionally, 7% of sera collected from 398 bats demonstrated RABV neutralizing antibody. The proportion of seropositive bats varied significantly across trophic guilds, suggestive of complex intraspecific compartmentalization of RABV perpetuation. In this study we extend information on the geographic distribution of rabies virus (RABV) circulation among bats to assess the broader public and veterinary health risks associated with bats in Guatemala. The detection of RABV neutralizing antibodies demonstrated viral circulation among multiple bat species in Guatemala. The presence of bat RABV in rural communities requires new strategies for public health education regarding contact with bats, improved

laboratory-based surveillance of animals associated with human exposures, and novel techniques for modern rabies prevention and control. Additionally, healthcare practitioners should emphasize the collection of a detailed medical history, including questions regarding bat exposure, for patients presenting with clinical syndromes compatible with rabies or any clinically diagnosed progressive encephalitis.

Introduction

Bats (Order: Chiroptera) have been implicated as hosts and reservoirs for numerous emerging infectious diseases, and are considered one of the most relevant groups of mammals in the study of disease ecology [1]. Guatemala is home to some of the world's richest bat biodiversity, with over 104 extant species [2-4]. As one representative global disease detection site established by the U.S. Centers for Disease Control and Prevention, enhanced rabies surveillance and pathogen discovery over the past five years targeting bats has facilitated the discovery of numerous novel viral and bacterial agents [5-8]. Considering the diversity and zoonotic potential of pathogens detected to date, the most pressing zoonotic threat from bats in Guatemala is *Rabies virus* (RABV), the only *Lyssavirus* documented in the New World [9]. Rabies is defined clinically in humans that present with an acute progressive encephalitis dominated by hyperactivity or paralytic syndromes that eventually deteriorate towards coma and death in nearly 100% of cases [10].

Rabies epizootiology is well appreciated in countries with an established laboratory-based surveillance network. Combined with molecular epidemiology, enhanced and passive surveillance are used to define the geographic distribution associated with RABV variants, infer the temporal and spatial spreads associated with diverse reservoir hosts, identify spillover

infections into humans, and to devise relevant prevention and control based upon such information [11-13]. Generally, RABV can be divided into two major clades: one comprising variants associated with carnivores around the globe, and another containing variants associated with bats, raccoons and skunks in the New World. In Latin America, RABV is classically recognized as two broadly distinct epizootiological forms or ‘cycles’, one ‘urban’, in which dogs may serve as the primary reservoir host and vector, and the other as a so-called rural or ‘sylvatic’ cycle, involving wildlife [13].

RABV transmitted by vampire bats (*Desmodus rotundus*) represents the most apparent economic and public health threat associated with bats [14]. Vampire bats exist only in Latin America, and range from the Tropic of Cancer in Mexico, to the Tropic of Capricorn in Argentina and Chile [15]. Vampire bats include three genera, and within each, a single species derives all nutrition from feeding on the blood of other vertebrates. A consequence of this unique biological adaptation is that vampire bats are highly effective at transmitting RABV to a wide diversity of mammals, primarily livestock, but also humans, if preferred prey are not widely available [16]. To date, human rabies fatalities have been associated with RABV spillover from frugivorous and insectivorous bats. However, the burden of mortality appears much less in these taxa than the impact associated with the common vampire bat [17-19].

Laboratory-based surveillance is recommended by the World Health Organization in all RABV enzootic countries. Accurate ongoing prevalence measures are essential in rabies prevention and control, to estimate the burden of disease, to monitor trends to evaluate the effectiveness of case intervention, and to ensure appropriate management of outbreaks [20]. The surveillance system for rabies in Guatemala is passive, where samples from suspected human and animal cases are collected, sent, and tested by the direct fluorescent antibody (DFA) test at

one of two laboratories: the Laboratorio Nacional de Salud in Villa Nueva (main facility), or Laboratorio de Ministerio de Agricultura, Ganadería y Alimentación (MAGA) in Quetzaltenango. The number of reported human rabies cases has decreased over the past two decades. Dogs remain the primary RABV vector, and are associated with approximately 73% (44 of 60) of human cases from 1994 to 2012. However, 23% (14 of 60) of these cases were due to an unspecified or unknown exposure [21,22]. In the U.S., numerous reports link insectivorous bats to the majority of human rabies cases without a history of conventional exposure to RABV [23]. During the same time period in Guatemala, despite detection of over 200 cases of rabies in livestock, and suspected association of such cases with vampire bat rabies, there were no reported cases in bats.

Why RABV has not been detected in Guatemalan bats is unclear, considering RABV has been detected in the majority of bats species tested throughout North America [24]. Very few bats are tested based upon the existing passive surveillance system, and monoclonal antibody or genetic characterization of RABV variants infecting humans, domestic animals and wildlife is not performed. Given the nocturnal and somewhat cryptic nature of bats, transmission dynamics are difficult to study in natural populations, and many critical gaps remain for a basic understanding the epizootiology of different RABV variants maintained in specific bat taxa. In the present study, we report results from field studies conducted in Guatemala from 2009-2011. The objective of the study was to test whether enhanced surveillance would: (1) complement passive surveillance, specifically regarding the presence of RABV in bats, and (2) extend information on the geographic distribution of RABV circulation among bats to assess the public and veterinary health risks associated with bats in Guatemala.

Materials and Methods

Ethics statement

All animals were captured and handled in accordance with national guidelines (Guide for the Care and Use of Laboratory Animals) [25]. Protocols for animal capture and use were approved by the CDC Animal Care and Use Committee (USA) protocol number 1843 and 2096, and the Animal Care and Use Committee of the Universidad del Valle de Guatemala (Guatemala).

Enhanced surveillance sampling

Guatemala was selected as one major comparative New World study location as part of the U.S. Centers for Disease Control and Prevention's (CDC) Global Disease Detection (GDD) program among ten international locations. The objective of the CDC GDD program is to develop and strengthen global capacity to rapidly detect, accurately identify, and promptly contain emerging infectious threats that occur internationally. Nineteen field sites for sampling bats in Guatemala were selected on the basis of historical outbreaks of rabies, contemporary national surveillance data, known or suspected vampire bat depredation upon human populations, or neurological illness reported in livestock. Figure 1a illustrates the geographical distribution of field sites used in the present study. Bats were collected using mist nets set near fruit trees, confined livestock, or near the entrance of caves. Nets were opened between 19:00 to 0:00, and checked every 30 minutes. Bats were removed from mist nets, placed individually in cloth bags, and transported to a nearby temporary field station, where they were sedated by a 0.05- to 0.1mg intramuscular injection of ketamine hydrochloride, oral/fecal swabs obtained, and terminal blood samples were collected by cardiac puncture under heavy anesthesia. Following euthanasia, bats were identified to the level of species following a key for bats of Costa Rica [26]. Standard morphological measurements were also collected (e.g. gender, age, mass, and forearm length). A complete

necropsy was then performed on all bats, and samples were stored immediately on dry ice in the field, and maintained thereafter at -70°C in the laboratory at the Universidad del Valle de Guatemala until shipment to the CDC Rabies Laboratory in Atlanta, GA. Carcasses were banded and fixed in 10% buffered formalin for several days, then permanently transferred to 70% ethanol, for archival purposes.

Direct fluorescent antibody testing

Brain impressions prepared from frozen bat tissues were fixed in acetone at -20°C, and RABV antigens were detected by the DFA test, using fluorescein isothiocyanate (FITC)-labeled monoclonal antibody (mAb) conjugate (Fujirebio Diagnostics, Inc., Malvern, PA, USA), as described [27].

Serology

The presence of RABV neutralizing antibodies (rVNA) was determined by the rapid fluorescent focus inhibition test (RFFIT) or a modified micro-RFFIT test on sera collected from ten field sites [28,29]. The rVNA titers of individual bats were calculated by the Reed-Muench method, and were converted to international units (IU/mL) by comparison to a standard rabies immune globulin (SRIG) control containing 2 IU/mL [30]. The SRIG titer was generally higher in the micro-RFFIT test compared to the standard RFFIT. For the objective of this study, positive rVNA titers (≥0.06 IU/mL) were defined by at least 50% neutralization of the RABV challenge virus dose (50 focus forming doses) at a 1:5 dilution (RFFIT) or 1:10 dilution (micro-RFFIT). Final titers less than 0.06 IU/mL were considered negative for the presence of rVNA for the purposes of this investigation.

Antigenic characterization

Antigenic characterization was performed by indirect immunofluorescence using eight mAbs directed against the RABV nucleoprotein (N) antigens (C1, C4, C9, C10, C12, C15, C18, C19), supplied by EMD Millipore Corporation (Billerica, MA, USA), as previously described [31]. Positive reactivity pattern results were analyzed using previous described antigenic variant patterns [32,33].

Virus isolation

Virus isolation was attempted on oral and fecal swabs stored in growth medium (MEM-10), and organs, obtained from DFA-positive bats. Homogenates (10% w/v) were prepared in MagnaNA lyser tissue homogenizer tubes containing 1.4-mm (diameter) ceramic beads (Roche Applied Science, Penzberg, Germany), using 1.0 mL of MEM-10 as a diluent. The entire solution from oral and fecal swabs was used to inoculate cells for isolation. For virus recovery, 100uL of test inoculum was added to 1mL of MEM-10 containing $5x10^6$ mouse neuroblastoma cells (MNA) in a T-25 tissue culture flask (Corning, NY). Tissue culture flasks were incubated at 0.5% CO_2 at 37ºC for 72 hours. All cultures were sub-passaged a minimum of four times. For infectivity assessments, Teflon-coated four well slides were seeded with 30uL of MEM-10 containing $0.5 x 10^6$ cells per mL, and incubated in a humid chamber at 0.5% CO_2 at 37ºC for 24 hours. The slides were then rinsed with phosphate-buffered saline (PBS 4550), and fixed in cold acetone at -20ºC for one hour. RABV antigens were visualized by use of the DFA test, using optimal working dilutions of FITC-labeled anti-RABV mAb conjugate (Fujirebio Diagnostics, Inc., Malvern, PA, USA) after each passage.

RNA extraction, reverse transcription, and PCR

Total RNA was extracted from fecal swabs and organ tissues of RABV-infected bats using TRIZol reagent (Invitrogen, Carlsbad, CA, USA). Fecal swabs were stored in 1ml of MEM-10 in the field, and 200µl of the swab suspension was mixed with 1 ml of TRIzol for RNA extraction. Primers were selected within the coding region of the N gene and the initial reaction was performed with sense primer 1066F, GARAGAAGATTCTTCAGRGA (positions 1157-1173), which was also used for reverse transcription and antisense primer 304B, TTGACGAAGATCTTGCTCAT (positions 1514-1533). The hemi-nested reaction was performed with sense primer 1087F, GAGAARGAACTTCARGA (positions 1157-1173). The glycoprotein (G) gene was amplified as two overlapping fragments using primer combinations umf2/994b and 760f/308b, as previously described [32]. All positions are given according to the Street Alabama Dufferin RABV strain genome sequence (GenBank accession number M31046). The RT-PCR was performed as described elsewhere [34]. Positive hemi-nested results were confirmed by nucleotide sequencing. RT-PCR products were purified with Wizard® PCR Preps DNA Purification System (Promega, Madison, WI, USA), according to the manufacturer's recommendations and sequenced in forward and reverse directions using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit, version 1.1 on an ABI3770 sequencer (Applied Biosystems, Carlsbad, CA, USA).

Phylogenetic analysis

The complete N and G sequences were assembled and translated to amino acid sequences using the Bio Edit program [35]. The dataset was supplemented with complete and partial gene

sequences available from GenBank and aligned in ClustalX [36]. Table S1 describes details of all sequences used in this study, including accession numbers, country of origin, year, and specimen source. The GTR+I+G model were selected based on the Bayesian factor and Akaike criterion evaluated in MEGA, version 5.1. No molecular clock evaluation was implemented. The analysis was performed using the Bayesian skyline population prior, and two independent Markov Chain Monte Carlo (MCMC) runs were performed with 1,000,000 iterations each. The results were combined in Log Combiner, and the resulting maximum clade credibility (MCC) tree generated with Tree Annotator using 20% for burn in and visualized in Fig Tree, version 1.4.0 [37].

Statistical analysis

All statistical analyses were performed using JMP version 9.0.2 or SAS version 9.2 (SAS Institute Inc., Cary, NC). The 95% confidence intervals (CI) were calculated for proportions of seropositive bats by species and location of capture. A nested mixed logistic model was used to test the effect of trophic guild on seroprevalence. Species nested within trophic guild was treated as a random effect and trophic guild was treated as a fixed effect. As only a single sanguivorous species was tested, we also compared the model outputs with a data set excluding vampire bats. The antibody prevalence between sex were also compared using χ^2 tests, and the level of significance was evaluated at α=0.05.

Results

Geographic distribution of bat sampling

During 2009-2011, a total of 672 bats of 31 species were collected from Guatemala. Bats were collected during 2009 (n=220) Table S1; 2010 (n=135) Table S2 and 2011 (n=317) Table S3. Table 1 provides the details on the cumulative frequency of capture by species. Among all captures, 56.3% were male. The most frequently captured species was *D. rotundus* (n=200; 30%), followed by the Jamaican fruit bat, *Artibeus jamaicensis* (n=128; 19%) (Table 1).

Serologic evidence of RABV circulation in bats

From sera available for testing (n=398), 28 bats demonstrated detectable rVNA for an antibody prevalence of 7% (95% CI 5-10%). Seroprevalence was highest for insectivorous species (21%), followed by omnivorous (8%) and sanguivorous (9%) taxa. The proportion of rVNA seropositive bats varied significantly across trophic guilds in a complete data set ($F_{3,20}$=4.65, p=0.04; Species (Diet) = 3.4 x 10^{-21}) and a data set without vampire bats ($F_{2,20}$=6.97, p=0.005; Species (Diet) = 4.4 x 10^{-19}). In both models, pairwise contrasts revealed a significant difference between frugivorous and insectivorous bats. Insectivorous bats were 8.5 times as likely to be seropositive compared to frugivorous bats. No other pairwise contrasts in rVNA seroprevalence between trophic guild levels were significant. Among capture locations, the proportion of seropositive bats was highest for Naranjo (21%), followed by El Jobo (19%), and El Penate (11%). Table 2 provides the details for individual bat species sampled by location and trophic guild. Antibody prevalence between sexes was similar (3.8% of males, 3.3% of females)(χ^2 = 0.08, *P*=0.77). Species composition of bats captured varied across sites (Table 2).

Detection of RABV and antigenic typing

By the DFA test, RABV antigens were detected in the brain from two common vampire bats. The bats were collected in El Pumpo, near the Pacific Coast in the Department of Santa Rosa, and Palo Seco, near the border with Mexico in the Department of San Marcos (Figure 1b). The antigenic reaction pattern derived from the brain specimens were consistent with RABV antigenic variant V3 associated with *Desmodus rotundus* (Table 3). Upon capture, one bat demonstrated signs consistent with RABV CNS infection. Additionally, this bat was dehydrated, appeared in poor physical condition, and had evidence of several bite-like wounds to its body.

Isolation of RABV and detection of viral RNA by hemi-nested reverse transcription-PCR

RABV was isolated in MNA cells 24h after inoculation from the kidney of one bat, and from oral swabs of both rabid bats. Additional sub-passages revealed the presence of RABV in the spleen of one bat, and the kidney and lung for both bats. RABV was not isolated from the heart, liver, intestine, and fecal swabs. Viral RNA was detected by hemi-nested RT-PCR in all specimens examined, except the liver of Bat 321. The results of RABV isolation and nucleic acid detection from tissues of rabid bats are presented in Table 4.

GenBank accession numbers

In total, two RABV G sequences and two RABV N sequences were generated from the brain tissues of infected bats in this study and deposited into GenBank (accession numbers: KF656696-99).

Molecular relationships between RABV associated with vampire bats in Latin America

The N gene sequences of both viruses were 100% identical, whereas the G sequences differed in one non-synonymous substitution ($302_{T,S}$). The viruses were most similar to one of the lineages of vampire bat rabies viruses circulating in Mexico, and were relatively distant from the lineages circulating in South America, with the exception of Columbian lineages. The most phylogenetically related viruses were described previously from Chiapas, Tabasco and Veracruz states of eastern Mexico [38]. The only available vampire bat RABV N gene sequence from El-Salvador (FJ228492) also clustered within this lineage (Figure 2, G gene reconstruction not shown).

Discussion

Given the critical ecological importance of bats, especially in tropical regions, novel strategies are necessary for the prevention and control of bat-associated zoonoses. To our knowledge, this is the first report and isolation of RABV from bats in Guatemala. In this study, the prevalence of rabies among all collected bats was low (0.3%), even among vampire bats (1%). The two vampire bat RABV isolated were related phylogenetically to viruses associated with vampire bats in the eastern states of Mexico and El Salvador, which is not unexpected given their geographic proximity. Rabies epizootics and phylogenetic clusters of RABV circulating in vampire bat populations are relatively constrained in space [39,40]. Previous phylogeographic studies suggested that vampire bat rabies likely originated in the territory of Mexico [38]. If true, these viruses may have been introduced to Guatemala and El Salvador via eastern Mexico. The detection of rVNA from bats in this study demonstrated RABV exposure among multiple species of bats in Guatemala. The overall rVNA prevalence of 7% was similar to other bat RABV surveillance studies conducted in Peru (10.3%), Grenada (7.6%) and Trinidad (12.8%), but less

than the 37% detection in Colima, Mexico [42-44]. Seroprevalence among collection sites ranged from 0 to 21% among all bats, and 0 to 25% for *D. rotundus,* respectively. Our results are concordant with previous studies in demonstrating that a substantial fraction of apparently healthy bats have detectible rVNA, indicating previous exposure to RABV, and suggesting clearance of peripheral infection without clinical disease [41-43]. The presence of rVNA only demonstrates prior exposure to RABV antigens, and does not provide information about the timing, intensity, or frequency of infection [44]. Furthermore, rVNA negative test results do not guarantee a lack of exposure, and may vary according to the selected cutoff value used for the serology test, as reviewed by Gilbert et al. [45]. At a population level, the rVNA seroprevalence data described here provide information about the cumulative exposure history among all bats collected for each field site.

In experimentally infected bats, rVNA are typically generated only among survivors, and bats that succumb to infection may only seroconvert during late stages of clinical disease [44]. Similar observations have been made regarding rVNA seroconversion in human rabies patients [46]. Combined with antigen detection results, rVNA seroprevalence data can be used to elucidate infection dynamics in bat populations. Detection of RABV excretion can provide meaningful insights for identifying potential transmission pathways that can inform the structure of disease dynamic models. In this study, the highest levels of virus were consistently detected from oral swabs, kidney and lung tissue. These results support primary secretion by oral routes, while suggesting a possible pathway in urine (via the kidneys), which warrants further investigation.

Strategies to control RABV transmitted by vampire bats in Latin America have relied historically on population reduction methods, either by non-specific destruction of roosts or by

anti-coagulants applied to cattle or individual bats. When applied to the bats, it is suspected that treated bats then return to a roost where the anti-coagulant paste is spread to other conspecifics through allogrooming. Culling of vampire bats is thought to benefit agriculture in the short-term by alleviating bat bites on livestock. However, there is little evidence that this method actually targets rabid bats, and the apparent positive effect of culling on seroprevalence, combined with demographic and behavioral responses, may actually increase the proportion of susceptible bats [40,47]. A recent report also suggests that it may be more economically beneficial to support pre-exposure vaccination of cattle, than rely on vampire bat culling [48].

Rabies surveillance at the national level in Guatemala falls under the jurisdiction of the Ministry of Public Health and Social Welfare [49]. Rabies control efforts in Guatemala are focused primarily on mass vaccination of domestic dogs, which has led to a significant decrease in human rabies cases, though it remains unclear whether current efforts can achieve ultimate elimination of canine rabies [21,50]. Indirect vaccination of vampire bats with recombinant RABV vaccines have proven immunogenic and effective in experimental infection models of *D. rotundus*, though this strategy has not been tested in the field [51]. Despite possibly reducing RABV infection of cattle, this strategy would not eliminate the behavior or health and economic consequences (e.g. secondary bacterial infections) of vampire bat depredation on cattle. Interventions at the human-bat interface should be directed at decreasing the risk of human exposure to bats by improved educational campaigns on the risk of rabies associated with bats and by bat-proofing dwellings [52]. Routine laboratory-based RABV surveillance is necessary to properly evaluate any intervention strategy and should include the submission of all suspect human and animal cases. For Guatemala, overcoming cold chain and transportation barriers should be a priority to increase the proportion of tested suspect cases. To further elucidate the

relative impacts of the urban or sylvatic cycles on public health and agriculture, routine typing of all positive rabies cases should be implemented. Rapid exchange of information between sectors involved in human and animal rabies surveillance and control is essential.

Unfortunately, despite significant progress in the prevention and control of rabies, once clinical signs manifest in humans the case fatality rate approaches 100%, even with intensive supportive care [46]. However, appropriate post-exposure prophylaxis, including immediate washing/flushing and disinfection of the wound and prompt administration of RIG, and modern cell-culture vaccines according to recommended vaccination schedules assures prevention of rabies if bitten by a rabid bat [53]. Fortunately, modern cell culture vaccines are readily available for both humans and animals in Guatemala, and national authorities should provide pre-exposure prophylaxis guidelines for persons or animals with regular exposure to bats or other potential wildlife reservoirs (e.g. carnivores).

As the first focused rabies study in Guatemala, gaps were evident in our study. Limitations to the current study included a lack of more focal seasonal sampling, a broader spatial scale, due to restriction to the southern portion of the country, and a need for greater engagement of the Ministries of Health and Agriculture in a One Health context. The presence of bat RABV in rural communities likely requires new strategies for joint public health and veterinary education and outreach, increased availability for diagnostic laboratory testing in remote areas, and continued enhanced surveillance for rabies prevention and control, as suggested for other developing countries [54,55]. Additional studies at the human-bat interface would be useful to obtain information on demographic characteristics (e.g. age, gender, education) of persons exposed to bats, circumstances of bat exposures (e.g. bites, scratches, skin contact), actions taken following exposure, and knowledge of bat-borne zoonoses. Previously,

based upon ignorance or inaction, preventable human rabies cases have occurred from bat exposure [56-58]. Given the implications when such recommendations are not operative, localities with a high risk for exposure to bats, and bat-borne zoonoses, should be targeted in laboratory based surveillance activities for the evaluation of robust, long-term prevention and control strategies.

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Supporting Information

Table S1. Bats collected for rabies testing from eight field sites in Guatemala, 2009.

Table S2. Bats collected for rabies testing from two field sites in Guatemala, 2010.

Table S3. Bats collected for rabies testing from nine field sites in Guatemala, 2011.

Table S4. List of sequences included in the data set from Central and South American vampire bat RABV.

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Table 1. Bats collected for rabies testing from 19 field sites in Guatemala, 2009-2011.

Family	Species	Frequency (%)
Molossidae		
	Molossus sinaloae	2 (0.3)
Mormoopidae		
	Pteronotus davyi	20 (3.0)
Noctilionidae		
	Noctilio leporinus	1 (0.1)
Phyllostomidae		
	Artibeus jamaicensis	128 (19.0)
	Artibeus lituratus	35 (5.2)
	Artibeus phaeotis	10 (1.5)
	Artibeus toltecus	2 (0.3)
	Carollia brevicauda	2 (0.3)
	Carollia castanea	2 (0.3)
	Carollia perspicillata	30 (4.5)
	Carollia sowelli	2 (0.3)
	Centurio senex	1 (0.1)
	Chiroderma salvini	6 (0.9)
	Chiroderma villosum	1 (0.1)
	Desmodus rotundus	200 (29.8)
	Glossophaga soricina	47 (7.0)
	Lasiurus ega	1 (0.1)
	Macrophyllum macrophyllum	2 (0.3)
	Micronycteris microtis	25 (3.7)
	Phyllostomus discolor	14 (2.1)
	Platyrrhinus helleri	18 (2.7)
	Sturnira lilium	96 (14.3)
	Sturnira ludovici	2 (0.3)
	Trachops cirrhosus	1 (0.1)
	Uroderma bilobatum	5 (0.7)
	Vampyressa pusilla	2 (0.3)
	Vampyrodes caraccioli	1 (0.1)
Vespertilionidae		
	Eptesicus fuscus	5 (0.7)
	Myotis elegans	6 (0.9)
	Myotis keaysi	1 (0.1)
	Myotis nigricans	4 (0.6)
	Total	**672 (100)**

Table 2. Bat sera tested for rabies virus neutralizing antibodies (positive/tested) from ten collection sites in Guatemala, 2009-2011.

Species	**Agüero**	**El Jobo**	**El Penate**	**Finca San Julian**	**La Viña**	**Los Hilos**	**Los Tarrales**	**Montañas Azules**	**Naranjo**	**Salacuim**	Subtotal	95% CI
Frugivorous											**0.03 (6/199)**	0.01-0.06
Artibeus jamaicensis	(0/22)	(0/6)		(0/17)	(1/4)	(0/1)		(0/4)	(0/4)	(0/2)	0.02 (1/60)	0.00-0.09
Artibeus lituratus	(0/2)		(1/1)	(0/3)			(0/2)	(0/3)		(0/4)	0.07 (1/15)	0.01-0.30
Artibeus phaeotis								(0/1)			0.0 (0/1)	-
Artibeus toltecus	(0/1)						(0/1)				0.0 (0/2)	-
Carollia castanea							(0/1)		(0/1)		0.0 (0/2)	-
Carollia perspicillata	(0/6)	(1/11)	(0/1)	(0/3)		(0/3)	(0/3)	(1/2)			0.07 (2/29)	0.02-0.22
Centurio senex										(0/1)	0.0 (0/1)	-
Chiroderma salvini				(0/1)							0.0 (0/1)	-
Platyrrhinus helleri	(0/1)			(0/2)			(0/2)	(0/10)			0.0 (0/15)	
Sturnira lilium	(0/15)	(1/4)	(0/5)	(0/9)	(0/3)	(0/2)	(0/9)	(0/14)	(1/1)	(0/4)	0.03 (2/66)	0.01-0.10
Sturnira ludovici					(0/1)		(0/1)				0.0 (0/2)	-
Uroderma bilobatum	(0/1)			(0/2)							0.0 (0/3)	-
Insectivorous											**0.21 (8/38)**	0.11-0.36
Vampyressa pusilla										(0/2)	0.0 (0/2)	-
Eptesicus fuscus	(0/3)							(0/1)			0.0 (0/4)	-
Macrophyllum macrophyllum	(0/2)										0.0 (0/2)	-
Molossus sinaloae	(0/2)										0.0 (0/2)	-
Myotis elegans		(0/1)							(1/5)		0.17 (1/6)	0.03-0.56
Myotis keaysi								(0/1)			0.0 (0/1)	-
Myotis nigricans	(0/1)	(1/1)						(0/1)			0.33 (1/3)	0.06-0.79
Pteronotus davyi						(0/1)			(6/19)		0.30 (6/20)	0.15-0.52
Omnivorous											**0.08 (6/73)**	0.04-0.17
Glossophaga soricina	(0/8)	(2/6)		(0/1)	(0/1)	(0/1)	(1/5)	(0/8)	(0/5)	(0/2)	0.08 (3/37)	0.03-0.21
Micronycteris microtis		(1/3)				(0/22)					0.04 (1/25)	0.01-0.20
Phyllostomus discolor							(2/10)				0.20 (2/10)	0.06-0.51
Trachops cirrhosus										(0/1)	0.0 (0/1)	-
Sanguivorous												
Desmodus rotundus	(0/22)	(2/10)	(0/2)	(0/5)	(0/6)		(1/4)	(0/5)	(2/13)	(3/21)	**0.09 (8/88)**	0.05-0.17
Total	0.0 (0/86)	0.19 (8/42)	0.11 (1/9)	0.0 (0/43)	0.07 (1/15)	0.0 (0/30)	0.11 (4/38)	0.02 (1/50)	0.21 (10/48)	0.08 (3/37)	**0.07 (28/398)**	
95% CI	-	0.10-033	0.02-0.44	-	0.01-0.30	-	0.04-0.24	0.00-0.10	0.12-0.34	0.03-0.21		0.05-0.10

Table 3. Antigenic patterns of two bat rabies virus isolates from Guatemala

	Patterns of reaction (N-mABs)								Antigenic Variant
	C1	C4	C9	C10	C12	C15	C18	C19	
Bat 258	-	+	+	+	+	-	-	+	*Desmodus rotundus* (V3)
Bat 321	-	+	+	+	+	-	-	+	*Desmodus rotundus (*V3)

Table 4. Cell culture isolation and primary/ hemi-nested (nRT-PCR) results from rabid bat specimens collected in Guatemala. Sequences from positive RNA detection in this table were not published in Genbank.

	Bat 258						**Bat 321**					
	Virus Isolation				**RNA detection[1]**		**Virus Isolation**				**RNA detection**	
Specimen Source	Passage 1	Passage 2	Passage 3	Passage 4	Primary	Hemi-nested	Passage 1	Passage 2	Passage 3	Passage 4	Primary	Hemi-nested
Fecal swab	-	-	-	-	-	+	-	-	-	-	-	+
Kidney	+	+	+	+	+	+	-	+	+	+	-	+
Spleen	-	-	+	+	+	+	-	-	-	-	-	+
Heart	-	-	-	-	-	+	-	-	-	-	-	+
Liver	-	-	-	-	+	+	-	-	-	-	-	-
Lung	-	+	+	+	-	+	-	+	+	+	+	+
Intestine	-	-	-	-	+	+	-	-	-	-	-	+
Oral Swab	+	+	+	+	NT[2]	NT	+	+	+	+	NT	NT

[1]Primary (primers 1066:304), Hemi-nested (primers 1087:304)
[2]NT, not tested

Figure 1. Map of selected field sites, Guatemala 2009-2012. (A) Circles indicate the location and circle size is proportional to the number of bats collected. (B) Geographic distribution of seropositive bats collected and the location of two infected vampire bats (*D. rotundus*) during enhanced surveillance.

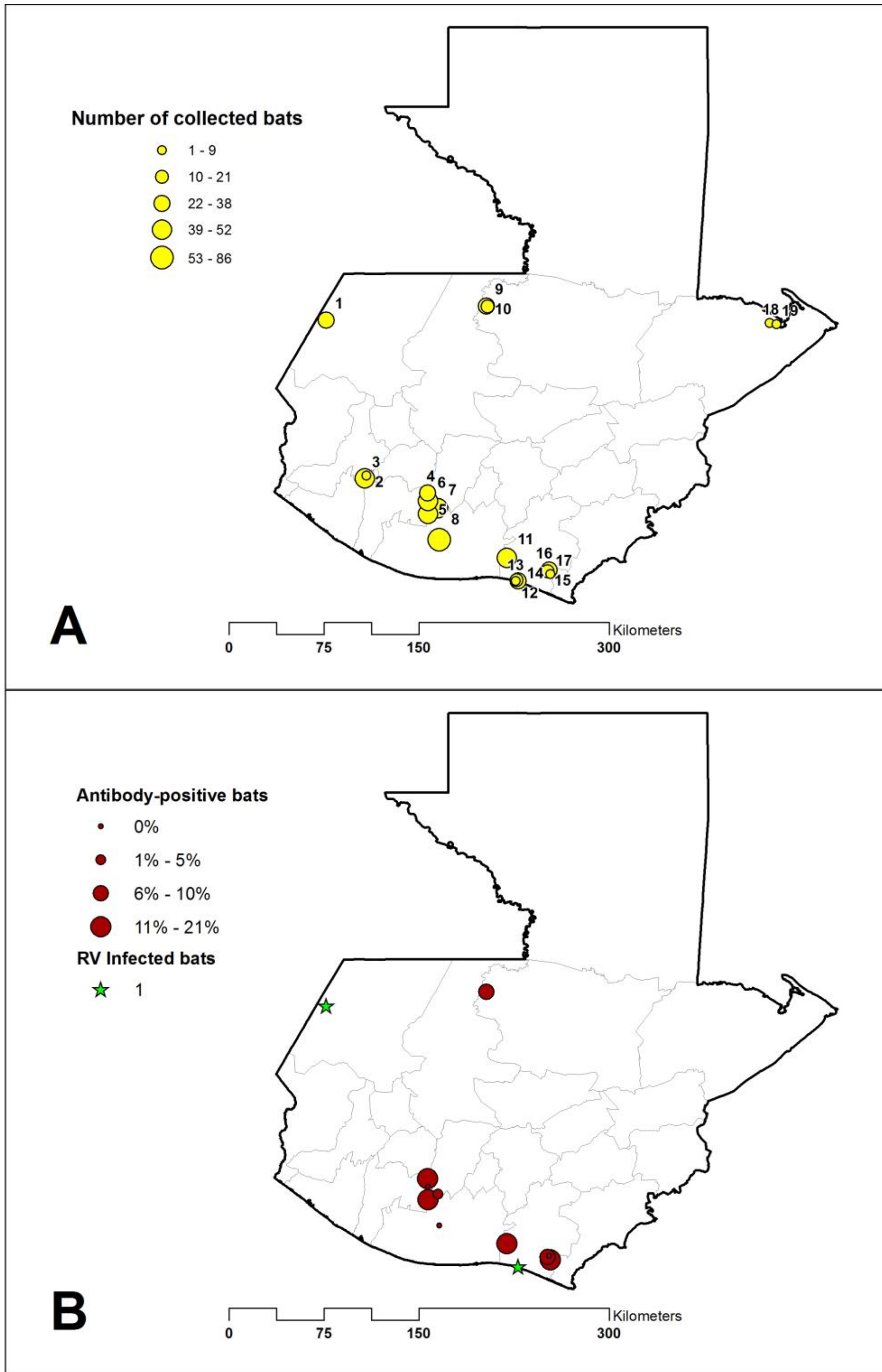

Figure 2. Maximum clade credibility tree of vampire rabies viruses based on the nucleoprotein (N) gene sequence. Posterior probabilities are shown for key nodes. Mexico dog RABV was used as an out-group. Novel sequences identified during this study are shown in red.

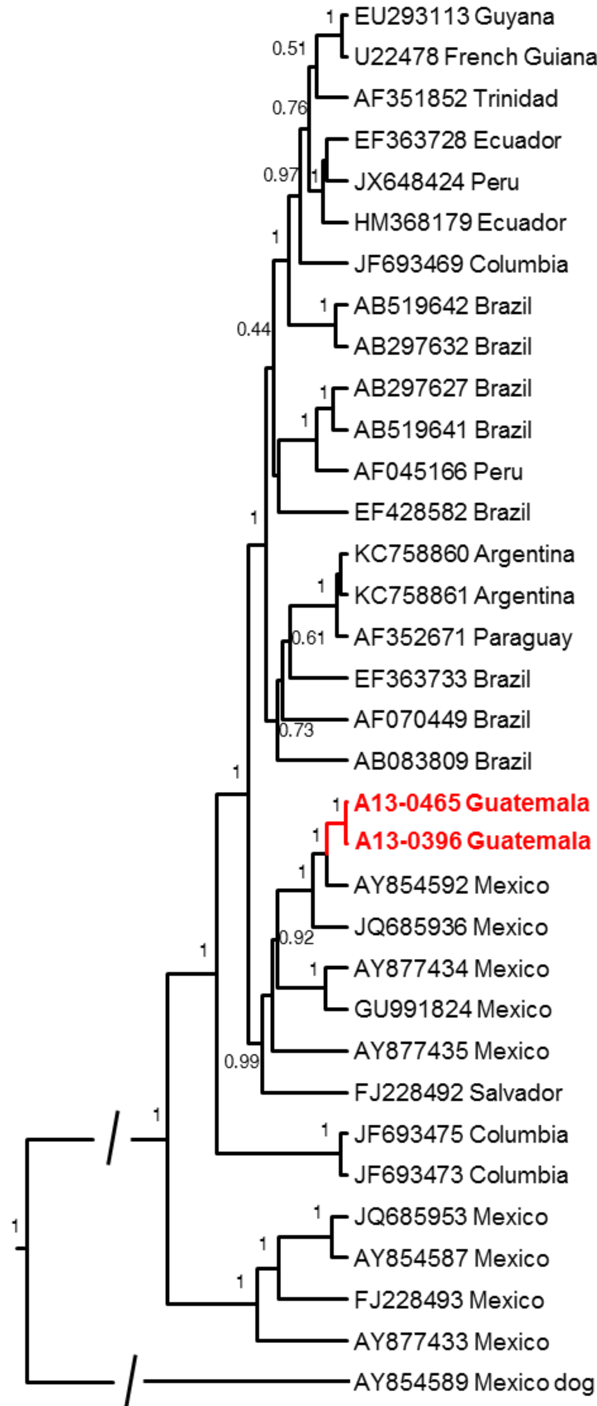

Table S1. Bats collected for rabies testing from eight field sites in Guatemala, 2009.

Species	**El Jobo**	**El Penate**	**La Viña**	**Los Hilos**	**Los Tarrales**	**Montañas Azules**	**Naranjo**	**Salacuim**	Subtotal
Artibeus jamaicensis	6		4	1			4	2	17
Artibeus lituratus		1			2			4	7
Artibeus toltecus					1				1
Carollia castanea					1		1		2
Carollia perspicillata	11	1		3	3				18
Centurio senex								1	1
Desmodus rotundus	10	2	6		4		13	21	56
Glossophaga soricina	6		1	1	5		5	2	20
Micronycteris microtis	3			22					25
Myotis elegans	1						5		6
Myotis nigricans	1								1
Phyllostomus discolor					10				10
Platyrrhinus helleri					2				2
Pteronotus davyi				1			19		20
Sturnira lilium	4	5	3	2	9	1	1	4	29
Sturnira ludovici			1		1				2
Trachops cirrhosus								1	1
Vampyressa pusilla								2	2
Total	42	9	15	30	38	1	48	37	**220**

Table S2. Bats collected for rabies testing from two field sites in Guatemala, 2010.

Species	**Agüero**	**Montañas Azules**	Subtotal
Artibeus jamaicensis	22	4	26
Artibeus lituratus	2	3	5
Artibeus phaeotis		1	1
Artibeus toltecus	1		1
Carollia perspicillata	6	2	8
Desmodus rotundus	22	5	27
Eptesicus fuscus	3	1	4
Glossophaga soricina	8	8	16
Macrophyllum macrophyllum	2		2
Molossus sinaloae	2		2
Myotis keaysi		1	1
Myotis nigricans	1	1	2
Platyrrhinus helleri	1	10	11
Sturnira lilium	15	13	28
Uroderma bilobatum	1		1
Total	86	49	**135**

Table S3. Bats collected for rabies testing from nine field sites in Guatemala, 2011.

Species	**Don Israel**	**Don Neto, El Pumpo**	**Finca Don Hugo**	**Finca Guadalupe**	**Finca Las Conchas**	**Finca Las Pavas**	**Finca San Julian**	**Palo Seco**	**Finca El Pumpo**	Subtotal
Artibeus jamaicensis		12	2	10	5	1	17	18	20	85
Artibeus lituratus	3	2	2	6			3	1	6	23
Artibeus phaeotis		2		3		1			3	9
Carollia brevicauda				2						2
Carollia perspicillata					1		3			4
Carollia sowelli		2								2
Chiroderma salvini		1	3	1			1			6
Chiroderma villosum				1						1
Desmodus rotundus		3	24	30	1	24	5	10	20	117
Eptesicus fuscus						1				1
Glossophaga soricina	1	2	1	3			1		3	11
Lasiurus ega									1	1
Myotis nigricans									1	1
Noctilio leporinus									1	1
Phyllostomus discolor		4								4
Platyrrhinus helleri			1	1			2		1	5
Sturnira lilium		8	9	11			9		2	39
Uroderma bilobatum				1		1	2			4
Vampyrodes caraccioli				1						1
Total	4	36	42	70	7	28	43	29	52	**317**

TableS4. List of nucleoprotein sequences included in the data set from Central and South American vampire bat RABV.

Sequence ID	Country	Year	Specimen Source
A13-0396 (KF656697)	Guatemala	2012	Vampire bat
A13-0465 (KF656696)	Guatemala	2012	Vampire bat
AB083809	Brazil	1998	Cattle
AB297627	Brazil	2002	Neotropical fruit bat
AB297632	Brazil	2002	Vampire bat
AB519641	Brazil	1998	Great fruit-eating bat
AB519642	Brazil	2000	Vampire bat
AF045166	Peru	1996	Human
AF070449	Brazil	unknown	Vampire bat
AF351852	Trinidad	1995	Vampire bat
AF352671	Paraguay	1994	Vampire bat
AY854587	Mexico	1996	Vampire bat
AY854589	Mexico	1990	Dog
AY854592	Mexico	2000	Cow
AY877433	Mexico	1990	Cow
AY877434	Mexico	1995	Horse
AY877435	Mexico	1993	Cow
EF363728	Ecuador	2005	Human
EF363733	Brazil	2005	Human
EF428582	Brazil	2006	Vampire bat
EU293113	Guyana	1990	Dog
FJ228492	Salvador	2002	Human
FJ228493	Mexico	2003	Cow
GU991824	Mexico	2002	Vampire bat
HM368179	Ecuador	2007	Cow
JF693469	Columbia	2002	Horse
JF693473	Columbia	1996	Cat
JF693475	Columbia	1995	Human
JQ685936	Mexico	2009	Vampire bat
JQ685953	Mexico	2009	Vampire bat
JX648424	Peru	2002	Horse
KC758860	Argentina	1996	Cow
KC758861	Argentina	2001	Cow
U22478	French Guiana	1994	Dog

Chapter 4

Comparative pathogenesis of experimental rabies in the red fox (*Vulpes vulpes*)

Abstract

Each variant of rabies virus (RABV) occupies a unique ecological niche. The opportunity for re-emergence of rabies in meso-carnivores and the likelihood of sustained transmission of RABV in different species is influenced in part by host and variant-specific factors. Such attributes are critical when conducting risk assessments of the epizootiological potential of specific viruses in different hosts for management considerations. In an attempt to identify potential differences in susceptibility, pathogenesis, neurovirulence and neurotropism of major US RABV variants associated with carnivores and bats, experimental infections were conducted in red foxes (*Vulpes vulpes*). In this study, 30 red foxes were divided into six groups of five animals, and each group was inoculated bilaterally into the gastrocnemius muscle with a standardized dose of an antigenically distinct RABV variant (silver-haired bat; NYC dog; coyote; raccoon; north central skunk; south central skunk). Immunohistochemical and immunoflourescent techniques were used to assess relative antigen distribution and intensity throughout sections of brainstem, cerebellum, hippocampus, and cerebrum. No differences in overt susceptibility were detected, with 97% (29/30) mortality observed among infected foxes. However, time after infection to clinical presentation varied significantly by variant. Foxes infected with a canine RABV variant had the shortest incubation periods. Clinical signs were diverse and not specific to the RABV variant used for infection. The distribution of RABV antigens was significantly different throughout brain sections for the hippocampus and cerebrum, respectively. Transmission potential varied by group: infectious virus was recovered from the

submandibular salivary glands of only one fox infected with a skunk RABV variant, and all foxes (n=5) infected with a gray fox RABV variant. No infectious virus was recovered from the salivary glands of foxes infected with a bat, canine, raccoon, or most skunk RABV. This study confirms the observation that red foxes are highly susceptible to heterologous RABV infection. These data also support the concept that spillover infections of biologically relevant North American RABV among red foxes may be transient or dead ends, but dependent upon the isolate, dose, and route, as well as animal density, could result in a sustained transmission when epizootiological conditions are ideal.

Introduction

Rabies is caused by a lyssavirus infection of the central nervous system, and is characterized clinically by acute progressive encephalomyelitis. The *Lyssavirus* genus comprises some of the most virulent, neurotropic viruses yet discovered, including many variants of the type species *Rabies virus*. Antigenic and genetic studies have provided evidence that each principle RABV reservoir species maintains a unique rabies virus (RABV) variant, which is suggestive of virus adaptation to host physiology and species-specific population dynamics (Smith, 1989; Smith et al., 1992; Streicker et al., 2010). Adaptations to different hosts have been demonstrated to some extent by *in vivo* experiments addressing disease susceptibility and virus excretion. Adaptation of a RABV variant to its principle host appears to be indicated by the host's increased susceptibility to infection with that particular variant and a high frequency of viral excretion into their saliva.

The most common mode of RABV transmission is through the bite of an infected animal, and therefore, virus adaptations that result in frequent and concentrated viral excretions are

conducive for virus propagation and disease transmission. Furthermore, to be sustained in a population, a RABV variant must either exploit normal mechanisms of social interaction between its host and susceptible individuals or it must promote infective contact with these individuals by altering host physiology and behavior (Wandeler et al., 1994). Though these associations between host species and RABV variants make intuitive sense, evidence for specific virus adaptations is lacking and there have been few studies to characterize the pathogenesis, dynamics of viral shedding and clinical presentation of multiple RABV variant infections of a single species.

The pathogenic capacity of a RABV variant is affected by virulence factors associated with its ability to invade and colonize its natural reservoir host, and produce disease. The regulation of RABV glycoprotein (G) is one notable virulence factor concerning pathogenesis. Viral RABV glycoprotein mRNA expression directly correlates with virion uptake, successful infection of the central nervous system (CNS), and viral dissemination, and inversely correlates with the rate of virus replication and subsequent expression levels of RABV proteins (Chopy et al., 2011). Preservation of the cellular functions involved in virus transport and minimal RABV gene expression levels of antigenic viral protein likely delay apoptotic activity and other host immunological responses during initial infection.

How a RABV variant manages to induce a specific disease syndrome in a particular host remains unknown. Proper adjustment is constraining virus variability and impeding adaptation to new hosts. This notion is supported by the observation that virus variation in nature is not as prominent as expected from an RNA virus (Wandeler et al., 1994). Host susceptibility is suggested to be influenced by unique attributes to various species, and likely influenced by individual factors, such as immunological status, and age (Casals, 1940). Typically for rabies,

cross-species infections occur sporadically, and produce a single fatal spillover event, however, secondary transmissions have been observed as well (Kuzmin et al., 2012; Leslie et al., 2006). In nature, the transmission of RABV generally requires that infected animals develop clinical signs of rabies with concurrent excretion of virus in saliva. Differences in clinical presentation associated with carnivore or bat RABV variants in human cases suggest a link between viral genetic variability and pathogenesis (Udow et al., 2013). Successful manifestation of clinical signs via central nervous system (CNS) infection, and subsequent RABV transmission to other organisms is suggested to reflect variation in viral dissemination within the host.

The goal of this study was to further our understanding of RABV pathogenesis following spillover infection with RABV variants not adapted to the natural reservoir. We identified variations in RABV pathogenesis, host-susceptibility, neurovirulence and neurotropism via experimental infections in red foxes (*Vulpes vulpes).* The red fox was selected as an experimental model following review of the national rabies surveillance database, indicating that raccoons are the most frequently reported rabid animals, however, humans are more likely to be bitten by a rabid fox (Blanton et al., 2011; Yousey-Hindes et al., 2011). The red fox has the widest geographical range, and occupies the greatest spectrum of habitats of any carnivore, and red foxes have expanded their northern limits (higher altitudes and latitudes) during the 20th century throughout most of the central and western United States (Fichter and Williams, 1967; Hersteinsson and Macdonald, 1992; Macdonald and Bacon, 1982). In an urban population of red foxes, those that exhibit dispersive behavior are more susceptible to injury, particularly bite wounds on the muzzle, lower jaw, and cheeks from territorial fighting with other foxes, providing ample opportunity for RABV infection (Fox, 1969; Soulsbury et al., 2008).

Materials and Methods

Experimental Animals

Captive-bred red fox kits (*Vulpes vulpes*; < 6 months; n=30) used in this study were purchased from a commercial vendor (Ruby's Fur Farm, New Sharon, IA). All animals were individually identified with microchips and ear tattoos. Foxes were divided into six groups of five, and held in quarantine for 6 months prior to experimental procedures. During quarantine, and throughout the experiment, foxes were housed in individual kennel runs (6m x 1.6m x 2m) in an Animal Bio-Safety Level-2 (ABSL-2) facility (Lawrenceville, GA). Animals were provided 240-330g of carnivore and omnivore chow daily and *ad libitum* access to water. For routine blood collection and inoculation, foxes were anesthetized intramuscularly with Telazol® (8mg/kg; Fort Dodge Animal Health, Fort Dodge, IA, USA).

Blood Collection and Serology

Blood samples were collected from the jugular or brachiocephalic vein into 10 ml Vacutainer tubes (Becton Dickinson, Franklin Lakes, New Jersey, USA). Blood samples were collected for baseline status (Day 0) and on Day 7 and Day 14 post inoculation (p.i.) were centrifuged at 1,000 g for 12 min at 4°C. Sera (1-2 ml) were collected and stored at -20ºC until testing. Prior to testing, sera were thawed and heat-inactivated at 56ºC for 30 min. The presence of RABV neutralizing antibodies (rVNA) was determined by the rapid fluorescent focus inhibition test (RFFIT) (Smith et al., 1996). The rVNA titers of individual foxes were calculated by the Reed-Muench method (Reed and Muench, 1938), and converted to international units (IU/mL) by comparison to a standard rabies immune globulin (RIG) control containing 2 IU/mL. Positive

rVNA titers were defined by at least 50% neutralization of the RABV challenge virus dose (50 focus forming doses) at a 1:5 dilution (i.e., ≥0.06 IU/mL). Titers less than 0.06 IU/mL were considered negative for the presence of rVNA.

Rabies Virus inoculum

Primary RABV isolates of Silver-haired bat (LnRV, GenBank accession number JQ686001); Coyote (COY, GenBank accession number KC791861); Raccoon (RAC, GenBank accession number JQ685980); North central skunk (NCSK, GenBank accession number JQ685987); South central skunk (SCSK, GenBank accession number KC791840 and KC791839) variants were obtained from relevant enzootic localities. Briefly, the virus inocula were derived from submandibular salivary glands collected at necropsy, homogenized with sterile alundum (Fisher Scientific, USA) in a mortar and pestle to prepare a 10% suspension in diluent containing 2% inactivated fetal bovine serum (Hyclone, Atlanta GA) and PBS (CDC formulation 4550, Atlanta GA). One animal per isolate was used to derive source material, except for the SCSK isolate which was pooled from two infected skunks. The homogenates were clarified, and the supernatants were collected and stored at -80°C prior to characterization. The NYC Dog (GenBank accession number KC791861) represents multiple-passage material of the salivary glands of dogs inoculated peripherally, and was kindly provided by USDA Center for Veterinary Biologics (Ames, IA). Infectivity titers for all isolates were calculated by the mouse inoculation test and expressed as median mouse intracerebral lethal doses ($MICLD_{50}$/ml) using the Reed-Muench formula (Koprowski, 1996; Reed and Muench, 1938). Prior to fox inoculation, the inocula were diluted in MEM-10 to ensure equivalent challenge doses across isolates.

Experimental Infection

Each group of five foxes was inoculated bilaterally into the gastrocnemius muscle with a standardized dose (1 $MICLD_{50}$) of a different RABV isolate. Animals were observed daily p.i., and upon display of two or more clinical signs of RABV infection, animals were anesthetized (~9mg/kg Telazol®, Fort Dodge Animal Health, Fort Dodge, IA, USA) and euthanized (120mg/kg-3mL Bethanasia-D Special®, Schering-Plough Animal Health Corp., Union, NJ, USA). Post-mortem necropsy was conducted with all foxes for the collection of submandibular salivary glands (SMSG) and CNS tissue specimens. Surviving animals were euthanized on day 60 p.i.

Detection of rabies virus antigen

Representative samples of CNS tissue were collected from brain stem, cerebrum, hippocampus and cerebellum regions. A portion of tissues were flash-frozen and stored on dry ice, whereas the other portion of tissues were immersion-fixed in 10% neutral buffered formalin, embedded in paraffin wax, sectioned at 5 µm, and stained with hematoxylin and eosin. Rabies diagnosis was made by direct fluorescent antibody test (DFA) on brainstem tissue, as described (Dean et al., 1996), using FITC-labeled antibody conjugate (Fujirebio Diagnostics, Inc., Malvern, PA, USA). Immunohistochemistry was performed on CNS tissues of rabies positive animals for detection of RABV antigen as previously described (Hamir et al., 1995; Hamir et al., 1996) using murine polyclonal and monoclonal (no. 802-2) rabies virus specific antibodies with the HistoMark® Biotin Streptavidin-HRP systems (KPL, Gaithersburg MD, USA). Assessments of RABV antigen distribution of each tissue were graded by two readers independently. Where disagreement occurred, the respective cases were re-examined. Each section was graded from 0

to 4 according to the amount and distribution of antigen, as previously described (Bingham and van der Merwe, 2002) and modified as follows: 0, no antigen throughout section; 1, antigen low, one or more antigen particles in fewer than 50% of microscope fields; 2, antigen moderate, one or more particles in fewer than 100% of fields, but more than 50% of fields; 3, antigen abundant, one or more particles in each field, but not in all fields ; 4, antigen very abundant in 100% of fields.

Virus titration in mice

Isolation was attempted from submandibular salivary gland (SMSG) homogenates by mouse inoculation via intracerebral (i.c.), and intramuscular (i.m.) routes. Virus titrations were performed in 3-week old mice ICR/CD1 mice (Charles River Laboratory, Wilmington, MA, USA). Groups of five mice each were tested across 5 serial 10 fold dilutions per salivary gland specimen (total N=750). Mice were inoculated i.c. with 0.03 ml test material and checked twice daily for 30 days as described previously (Koprowski, 1996). For all positive SMSG specimens via i.c. route, an undiluted (neat) peripheral inoculation was performed into the right gastrocnemius muscle, in a total volume of 0.05 ml. Acutely moribund mice were euthanized by CO_2 immersion. Brain tissue was collected at necropsy and examined by DFA for rabies diagnosis (Dean et al., 1996). The highest dilution infecting mice was considered the endpoint of the mouse inoculation test (MIT). The $MICLD_{50}$ per ml were calculated as previously described (Reed and Muench, 1938).

RT-PCR

Total RNA was extracted from salivary glands and CNS tissues of RABV-infected foxes using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Samples of salivary glands were homogenized in 1ml of MEM-10, and then 200ul of the suspension was mixed with 1ml of TRIzol. Brain tissues were homogenized in 1ml of TRIzol. RNA extraction was performed according to the manufacturer's instructions. The RT-PCR and hemi-nested RT-PCR were performed as described elsewhere (Kuzmin et al., 2003). Standard primer combinations were used for reverse transcription and PCR amplification of the complete glycoprotein (G) gene (Ellison et al., 2013). Amplification of two overlapping gene fragments within the coding region of the G gene resulted in complete sequences for most samples; however, some samples required additional hemi-nested reactions. Detection of viral RNA in SGs by hemi-nested reverse transcription-PCR was performed as previously described (Ellison e*t al., In review*). The RT-PCR products were purified and subjected to direct sequencing with Big Dye terminator kit, version 1.1 (Applied Biosystems). The sequencing products were processed on an ABI 3730 DNA Sequencer (Applied Biosystems). Complete G gene sequences were assembled and compared to challenge virus sequences using the Bio Edit program (Hall, 1999). To evaluate phylogenetic origin of the RABV variants included in the study (Figure 1, the collapsed variant phylogenetic tree was modified with permission and previously described by Kuzmina et.al 2013), the Bayesian analysis was implemented in the BEAST, v. 1.6.1 under the GTR+G+I model, and visualized in Fig Tree, v. 1.3.1 (Drummond and Rambaut, 2007).

Data analysis

Kaplan–Meier survival analysis and Log Rank (Mantel–Cox) test were used to evaluate variation in incubation period among RABV variants. Mean rVNA titers were compared on day 7pi and

day 14pi across RABV variants by nonparametric comparisons using Kruskal-Wallis one-way ANOVA on ranks. The change in mean rVNA titer from day 7pi and day 14pi were compared on across RABV variants by nonparametric analysis of variance (ANOVA) on ranks. Histopathologic scores were analyzed using Kruskal-Wallis one-way ANOVA on ranks, with post-hoc tests by the Dunn method for joint ranking. All statistical analyses were performed using JMP v.9.0.2 (SAS Institute Inc., Cary, NC). $p < 0.05$ was considered as significant.

Results

Experimental infection

No differences in overt susceptibility were detected, with 97% (29/30) mortality observed among experimentally infected foxes (Table 1). Time to clinical presentation varied significantly by isolate ($\chi^2=40.8$, $p<0.05$) (Figure 1), and 20% (n=6) of foxes progressed rapidly without display of any clinical signs. All foxes that succumbed, or were euthanized during the observation period, tested positive for rabies by DFA. Foxes infected with a NYC dog variant had the shortest mean incubation period (13.8 days) and foxes infected with the raccoon variant had the longest mean incubation period (23.6 days; Table 1). Diverse clinical signs were observed during the observation period including behavioral (aggressiveness, odd vocalization, and lethargy) and CNS (abnormal eye movement, paralysis, head tilt, tremors, and ataxia) features, which did not appear to be specific to the RABV variant used for infection (data not shown). One fox inoculated with a LNRV RABV variant survived until Day 60 p.i. and no RABV antigen was detected by DFA.

RABV shedding in salivary glands

Virus was isolated from only one of five foxes infected with a SCSK RABV variant, but was recovered from all foxes (n=5) infected with a Coyote RABV variant (Table 2). All RABV isolates derived from the SMSG were lethal by IC route also killed mice peripherally. No infectious virus was recovered from the SMSGs of foxes infected with bat, dog, raccoon, or most skunk RABV variants. However, RABV RNA was detected in SMSG homogenates from foxes infected with a RAC and SCSK variant.

RABV neutralizing antibodies in experimentally infected foxes

RABV neutralizing antibodies were detected on day 7 p.i. from 36% (11/30) of foxes; however, statistically significant differences associated with challenge virus variants were not detected at this time point (χ^2=10.26, p=0.07). At day 14 p.i., 52% (15/29) of the foxes had detectible rVNA, and titers were significantly different across variants (χ^2=11.68, p<0.05). There was a significant variant effect for the mean change in rVNA titer from day 7 to day 14 (χ^2=11.57, p<0.05). The level of rVNA detected in foxes infected with a NCSK RABV was higher at day 14 p.i. compared to SCSK, RAC, and NYC dog RABV variants (Figure 3).

RABV Antigen distribution in the CNS

The greatest distribution of RABV antigen was detected in the brainstem across all groups (Figure 3). Distribution of RABV antigens varied significantly throughout sections of the cerebrum (χ^2=15.32, p <0.05) and hippocampus (χ^2=19.68, p <0.05) across RABV variants (Figure 4). No significant difference was detected between variant and antigen distribution in the cerebellum (χ^2=9.28, p =0.10), and brainstem (χ^2=9.46, p=0.09).

Discussion

This study is the first to examine RABV pathogenesis following inoculation with a standardized dose of antigenically distinct RABV variants in the red fox. In this study, examining differences among homologous and heterologous RABV variants were used to assess pathogenesis; time to clinical presentation; and viral dissemination in the CNS. In previous experimental infections in red foxes the incubation period was generally inversely proportional to the dosage of virus inoculated (Sikes, 1962). In this study the susceptibility of red foxes to different variants of RABV was high, with only one fox surviving experimental infection. This contrasts other studies reporting low susceptibility of silver foxes to other lyssaviruses (EBLV-1 and EBLV-2) when inoculated via i.m. route (Picard-Meyer et al., 2008). The interpretation of serologic response in rVNA suggesting antigenic difference should consider the influence of the challenge virus used for the assay. Higher heterologous than homologous titers and individual variations against antigen raised against the same sera should be considered qualitatively (Horton et al., 2010). In experimental infection studies conducted before the advent of monoclonal antibodies for antigenic typing, the variant information is not available, precluding any direct comparisons to this study.

In previous pathogenesis studies in salivary glands from which RABV was recovered, the amount of virus was often greater than that from the brain of the same animal (Parker and Wilsnack, 1966). In our study the highest amount of virus was recovered from foxes infected with coyote RABV variant. RABV G protein determines viral dissemination in the brain, and mediates fusion of the viral envelope with endosomal membranes to release the infections RNP core into the cytosol for viral transcription and replication (Morimoto et al., 1992; Yan et al., 2002). After replication, RABV is transported along the spinal cord to the brain where it infects

neurons in almost all brain regions. In the CNS, RABV spreads throughout the neurons via the synaptic junctions (Ugolini, 1995; Wickersham et al., 2007). These results demonstrate that RABV does not infect all structures of the brain equally, and support rabies diagnostics current recommendations of the use of brainstem as the most reliable structure for detecting RABV viral antigen (Figure 5) (Meslin et al., 1996; OIE, 2012) .

Previous studies have linked RNA virus genetic variability to changes in viral pathogenesis, influencing host placidity during infection, and to the acquisition of mechanisms required for a host switch (Brodeur, 2012). The RNA viruses are notorious for rapidly generating genetically diverse populations during a single replication cycle, and the implication of these mutant spectra could be involved in cross-species transmission. Such an ability to quickly generate highly diverse populations before encountering a selective pressure, rather than in response to it, is likely a critical trait that itself has been fine-tuned by evolution. RABV populations confront a multitude of selective pressures with only five viral proteins expressed.

RABV populations demonstrate geographic variability, dominated by spatiotemporal separation and compartmentalization of their diversity based on the reservoir species (Bourhy et al., 2008; Streicker et al., 2010). One amino acid substitution suggested as a marker of pre-adaptation of bat RABV to cause effective infection in carnivores is the $242_{S,T}$ in the G ectodomain (Kuzmin et al., 2012). The amino acid in this position was significant for pathogenicity in the Nishigahara strain, as the parental virus with A_{242} was pathogenic in a mouse model, whereas a mutant with S_{242} was attenuated (Takayama-Ito et al., 2006). Another study provided evidence that A_{242} increases virus spread between infected cells (Ito et al., 2010). Only two amino acid substitutions were detected in this study, one synonymous, and other non-

synonymous (Table 2). No other studies on amino acids in these positions have been described to date.

Red foxes have flexible social systems, being solitary, forming pairs or living in groups under different ecological conditions (Nowak, 1999). Being a highly opportunistic species that adapts to a multitude of different environmental conditions and food supplies, red foxes have progressively invaded the cities and suburbs of many urban areas (Willingham et al., 1996). In such a capacity, red foxes could be viewed as a sentinel for RABV spillover events. Successful spill-over infections and host shifts depend on the host phylogeny; such that virus circulation is a balanced equilibrium influenced by the genetic and antigenic properties of the pathogen, pathobiology of infection on the individual level, and ecologic properties of the host on the population level (Rupprecht et al., 2011).

Understanding basic host susceptibility and pathogenesis associated with any etiological agent is necessary if we are to demarcate its 'reservoir- host' status. For example, numerous maladaptive events may be overlooked if this is inferred incorrectly, or passively which perhaps explain why spillover of certain novel, non-host adapted pathogens into another species may in fact- result in a proverbial dead end road (i.e. the death of the host before secondary transmission), e.g. not likely the major natural reservoir host. By understanding the mechanisms of emergence, outbreak management plans can be developed and risk mitigation processes can be implemented. Once identified, risk reduction strategies can be implemented through public health outreach and education (Smith and Wang, 2012).

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Figure 1. Bayesian tree of RABV G sequences analyzed in the present study. Phylogenetic lineages that contain more than one sequence are collapsed. Posterior probabilities are shown for key nodes. Lineage abbreviations: LN, *Lasionycteris noctivagans* bats; SCSK, south-central skunk; MXSK, skunk RABVs from Mexico; NCSK, north-central skunk; AZFX, Arizona gray fox; TXFX, Texas gray fox; CVS, challenge virus standard (laboratory strain); CASK – California skunk; BCS – Baja California skunk; PR – Puerto-Rico; SE- South East; PV, ERA, SAD – rabies vaccine strains.

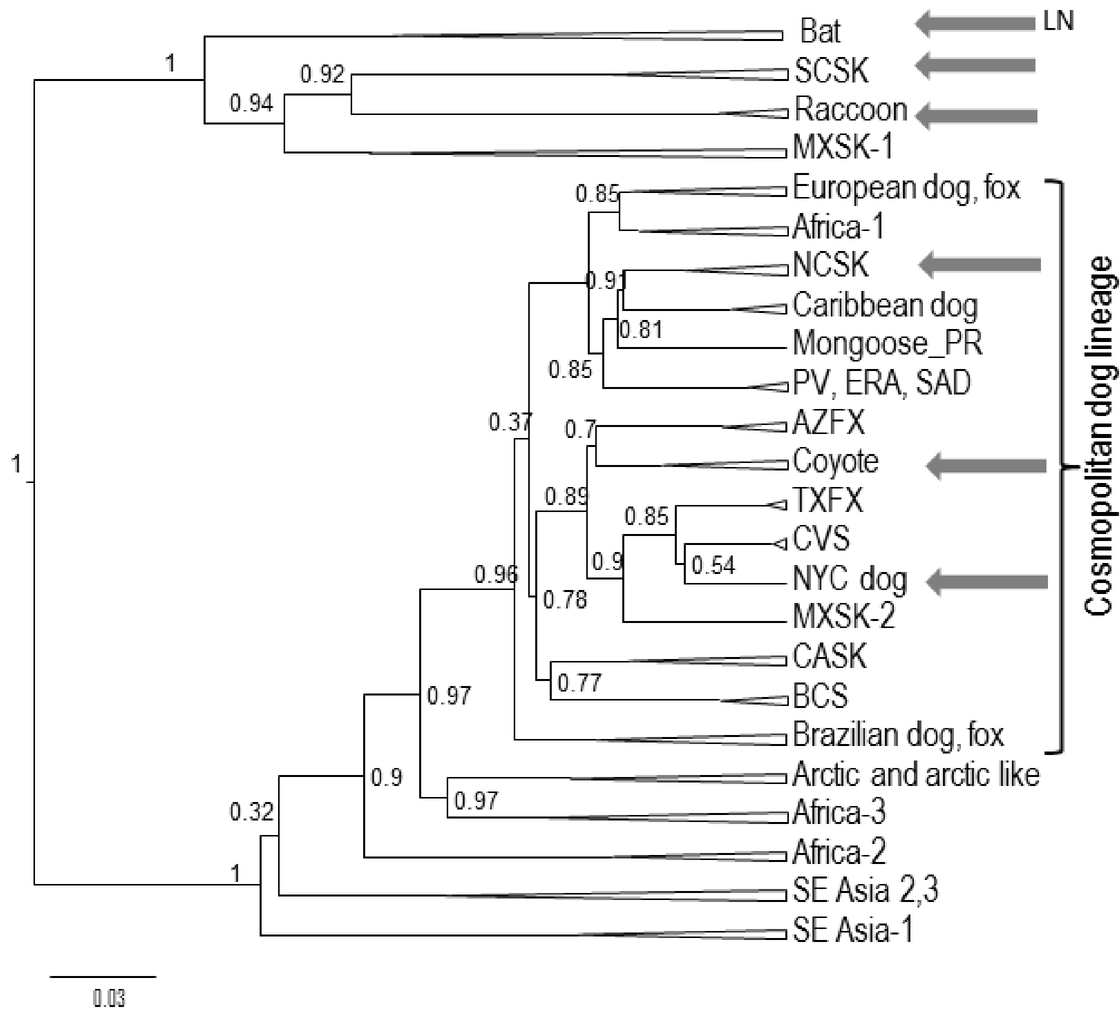

Figure 2. Kaplan–Meier survival analysis to evaluate variation in incubation periods across RABV variants.

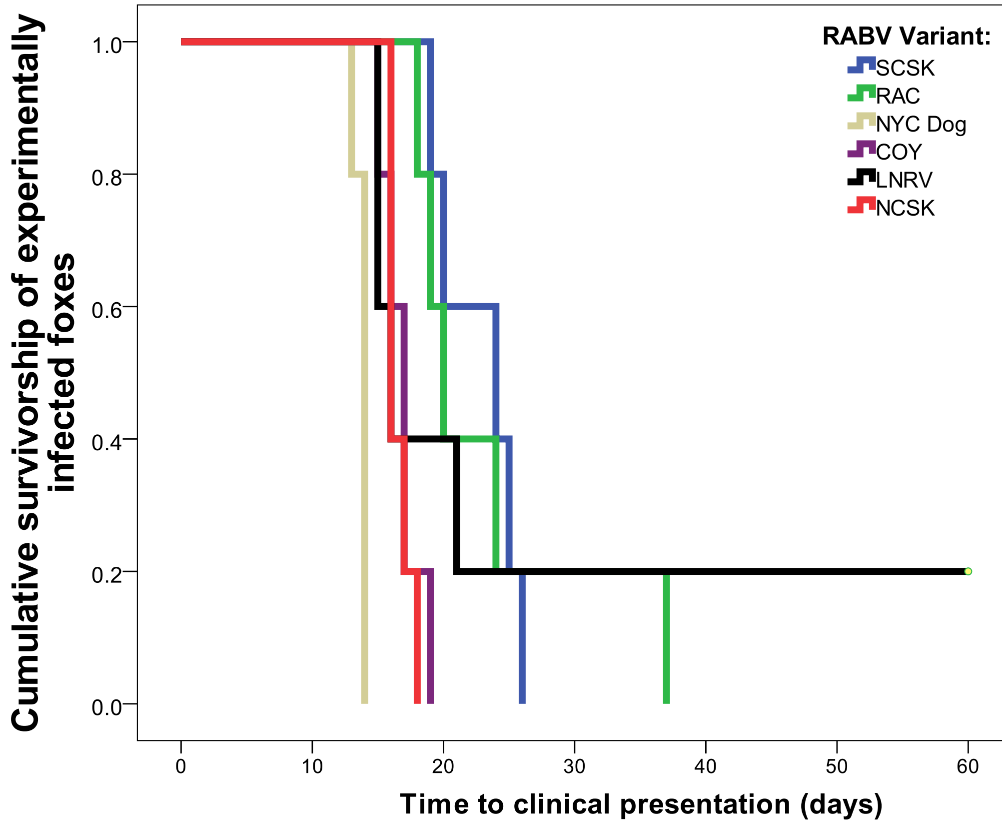

Figure 3. Mean rVNA response from experimentally infected foxes at day 7 and 14 p.i.

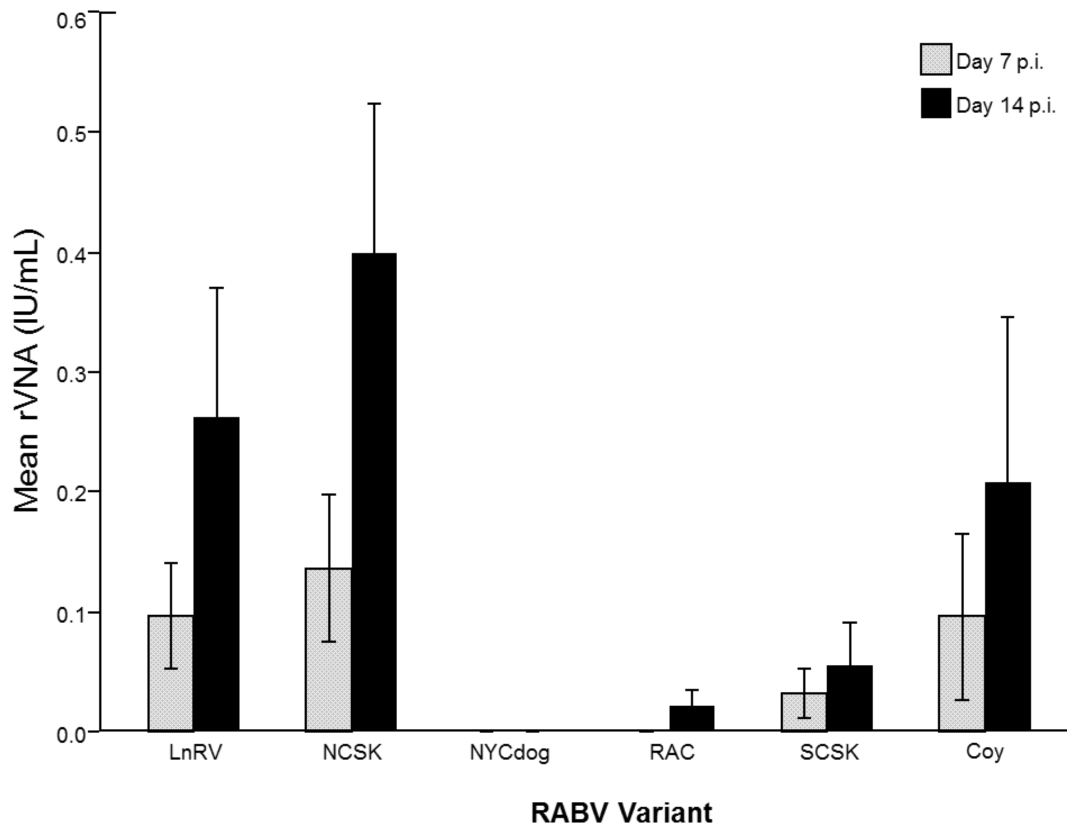

Figure 4. Mean distribution of RABV antigens varied throughout brain sections across RABV variants. Levels not connected by same letter indicate a significant difference ($p<0.05$).

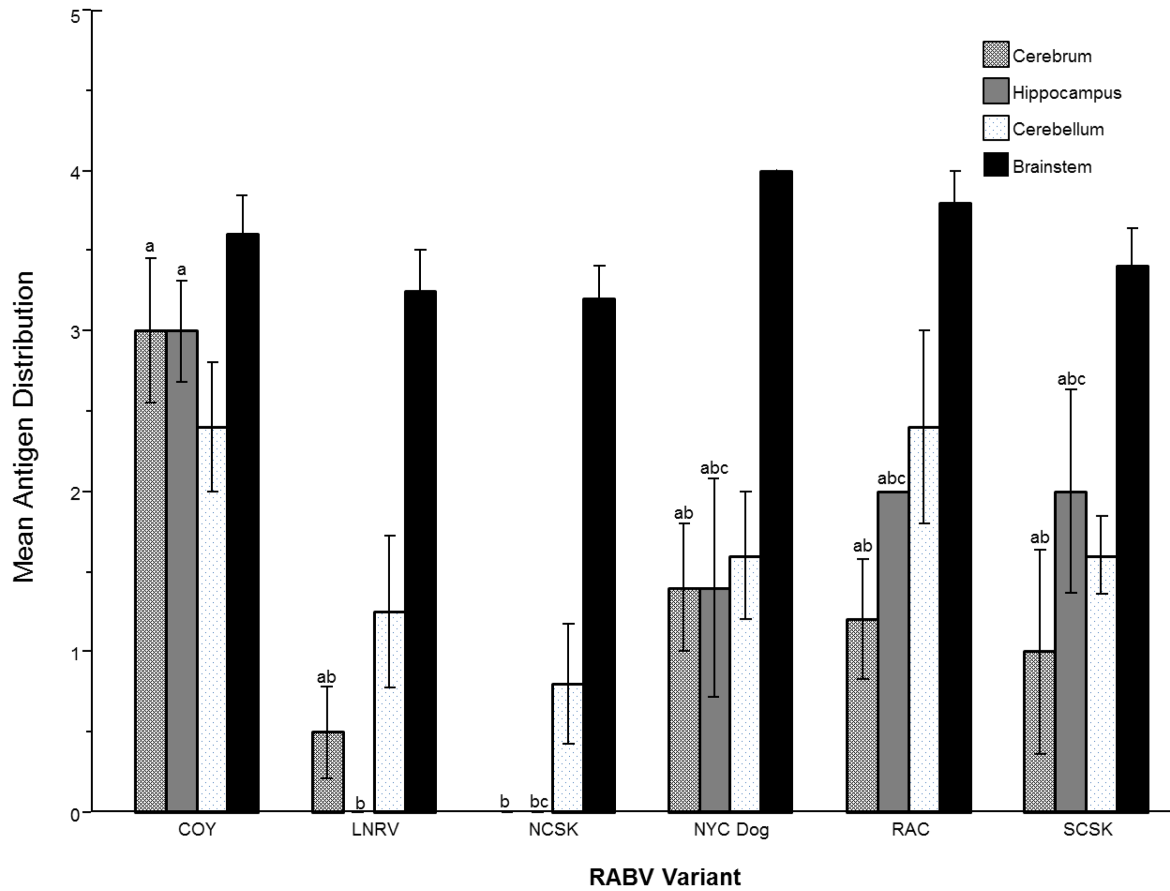

Figure 5. Representative micrographs of CNS histopathology and RABV antigen distribution (20x, 100x insert).

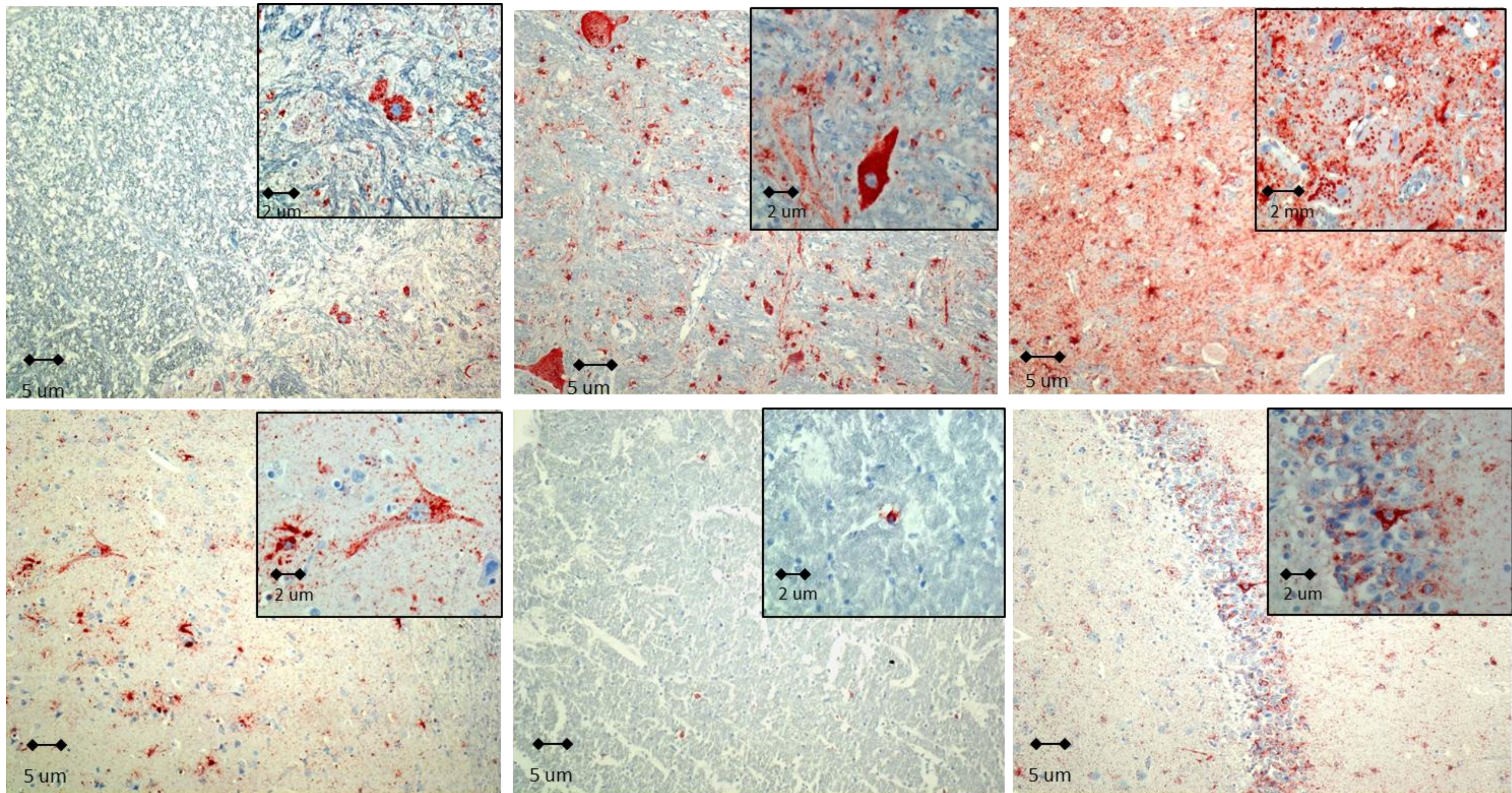

Table 1. Survival following experimental RABV infection in red foxes by variant

		Incubation Period				
RABV Variant	**No. of Survivors**	**Average**	**Std. Error**	**95% Confidence Interval**	***f*-test**	**p-value**
SCSK	0/5	22.8	1.39	(20.1 - 25.5)	1.21	0.91
RAC	0/5	23.6	3.50	(16.7 - 30.5)	7.7	0.13
NYC Dog	0/5	13.8	0.20	(13.4 - 14.2)	39.76	**<0.05**
TXFX/Coyote	0/5	16.8	0.66	(15.5 - 18.1)	3.65	0.24
LNRV	1/5	16.8	1.41	(13.9 - 19.6)*	ref	
NCSK	0/5	16.6	0.40	(15.8 - 17.4)	9.94	**0.05**
Overall	**1/30**	**18.4**	**0.90**	**(16.6 - 20.3)**		

*Estimation excludes survivor (group size for ref=4)

Table 2. RABV isolated from SMSGs by mouse inoculation test and pairwise comparison of nucleotide and amino-acid sequence identities of experimentally infected foxes with referenced challenge virus. Percentage of RABV nucleotide sequence identity for glycoprotein and intergenic region (G-L).

RABV Variant	SMSG virus isolated (MIT)	$MICLD_{50s}$/mL (log_{10})	Presence of RNA in SMSG	Nucleotide (%)	Nucleotide substitution	Amino acid (%)	Amino acid substitution
SCSK	(1/5)	4.0	(1/5)	100*	-	100	-
RAC	(0/5)	-	(1/5)	99.90	432 T→C and 1486T→C**	99.81	I377L
NYC Dog	(0/0)	-	(0/5)	99.95	660C→A, 1105A→G ***	99.81	R350G
Coyote	(5/5)	3.1 - ≥5.0	(5/5)	100	-	100	-
LNRV	(0/5)	-	(1/4)	100	-	100	-
NCSK	(0/5)	-	(0/5)	100	-	100	-
Overall	(6/30)	3.1 - ≥5.0	(8/30)				

*- % identity with respective challenge source
** - both substitutions were presented in one sequence (Fox 20)
*** - one substitution in each sequence (Fox 16 and Fox 8, respectively)

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Chapter 5

Summary and Conclusion

Rabies virus (RABV) virulence is a difficult patho-physiological trait to predict, and remains a fundamental issue underlying the epizootiology of rabies. Studies in optimal virulence demonstrate that traditional short-term interventions attempting to eradicate a pathogen often have long-term effects on virulence, as a pathogen rapidly evolves in response to artificially imposed constraints on transmission and replication [1,2]. Indeed, a pathogen that minimizes immune detection also minimizes the immune response of the host, which in some cases can be more taxing on immediate host fitness than merely the presence of a pathogen or antigen [3]. Fundamentally, there exists an equilibrium point regarding pathogenesis, and variation on the virulence axis, towards higher or lower virulence, will result in lower fitness for the variant and thus will be selected against [4]. The trade-off in the host is balanced between minimizing its immune response and clearing the infection. Reflecting this spectrum and recognition that RABV is enzootic in all New World bat populations thus far examined, I investigated whether bat RABV variants exhibit variable pathogenicity, and if these variants exhibited virulence factors influencing host susceptibility. I evaluated several mechanisms of virulence associated with a RABV variant's ability to infect and replicate in a host under natural and experimental infection scenarios.

By rapidly diagnosing zoonotic infections in animals, outbreak management plans can be developed and risk mitigation processes can be implemented. However, the diagnosis of rabies remains a challenge in the absence of laboratory testing, which requires postmortem examination of the infected brain. Despite this difficulty, the experiments contained herein, in addition to other recent studies have identified physiological correlates among specific viral and host

components providing prognostic value in linking disease etiology and pathogenesis with the clinical manifestation of disease [5-7]. In Chapter 2, I determined the normal infrared thermographic profile in the captive big brown bat (*Eptesicus fuscus*); and investigated the hypothesis that a facial surface temperature increase, as determined by the measurement of temperature using infrared thermography, precedes the visible onset of clinical signs of RABV CNS infection (determined by measurement of pre- and post-inoculation facial temperatures) among experimentally infected individuals. Contrary to my initial prediction of an increase in facial temperature, following experimental infection 62% of rabid bats had detectable facial temperature decreases compared with pre-inoculation baseline values.

This study observed an association between experimental RABV infection and a decrease in facial temperature. However, it is not known if RABV directly caused the change in facial temperature. In order to rely on facial temperature as a tool to detect RABV infected bats in the field, the next step would be to determine whether RABV directly causes a change in facial temperature. For this, an additional experiential RABV infection in big brown bats is required to address the precise timing of changes in facial and body temperature (e.g. stages of clinical manifestation) in relation to RABV pathogenesis. The thermographic data obtained from the experimentally infected bats that become rabid should be analyzed for differences among the disease progression intervals of pre-inoculation, pre-prodromal, prodromal, and clinical in a one-factor repeated-measures data structure, with bats as a random subject effect and the disease progression intervals as the repeated measure. If marked differences in the thermographic images of the experimentally infected bats that demonstrate clinical signs of rabies is observed, the data should be analyzed as a mixed linear model; with *a priori* linear contrasts applied to the thermographic pattern compared with 1) images of the same bat prior to infection, 2) bats

infected but not yet demonstrating clinical signs, and 3) with any bat that fails to develop rabies to specifically test whether each succeeding state of disease demonstrates a lower average facial temperature than the preceding stage.

Many host-pathogen interactions have an environmental component that affects transmission patterns. As torpid hibernators, bats can adjust to a wide range of temperatures and reduce their body temperature by 1–2 °C above the ambient temperature, thus drastically decreasing their metabolic rates. The adaptive value of this state is to conserve energy, although hibernating animals arouse periodically and their body temperature metabolic rate returns to normal for a period of usually less than 24 hours [8,9]. Such a slight decrease in temperature (e.g. 37°C to 34°C) –has a marked *in vitro* effect on the infectivity of a bat RABV variant—with ten times more cells being infected at lower temperatures [10]. The effect of temperature has several implications concerning infectivity and membrane fusion activity of bat RABV variants. For bats, torpor induces leukopenia, and during this period lower levels of lymphocyte proliferation and antibody production can be observed, which is beneficial in terms of energy conservation [11]. An appropriate immune response operates most efficiently within narrow temperature confines, constrained by the unique properties of an individual's physiology. With regard to bats, the immune response to viral antigens includes production of complement-dependent, complement-independent, neutralizing, and non-neutralizing antibodies, depending on the immunodominant epitopes of the pathogen [12]. If thermographic changes are indicated, this technique could enhance surveillance and detection of RABV in bats by providing a sign indicative of rabies when other signs are not present (e.g. increase aggression, acute weight loss, atypical vocalizations, ataxia, or paresis). Thermographic signatures (e.g. warmer or cooler body surface or nose temperatures as compared to others in the roost) may be observed when

examining a day or night roost. This would provide an opportunity to estimate rabies prevalence in a natural roost, without having to remove a subsample of the population for testing. Currently, additional research is warranted to determine the utility of infrared thermography to address the precise timing of changes in facial and body temperature (e.g. stages of clinical manifestation) in relation to RABV pathogenesis under field conditions.

Fossil evidence indicates that vampire bats have inhabited the Americas since the Pleistocene period some 2.5 million years ago [13]. Presently, haematophagous bats exist only in Latin America, ranging from the Tropic of Cancer in Mexico to the Tropic of Capricorn in Argentina and Chile. The limiting factor for the range of the vampire bat is the winter temperature—vampire bats cannot live in areas where the temperature drops below 15°C [14]. The common vampire bat *Desmodus rotundus*, roosts in caves with other bat species, and both auto-grooming and allo-grooming are common conspecifics –vampires also display altruistic feeding behaviors by regurgitating blood meals and sharing with others within the roost that have not fed—facilitating RABV intraspecies maintenance [15]. The common vampire bat feeds principally on livestock, but also other mammals, including humans, when preferred preys are not widely available providing ample opportunity for cross-species RABV transmission.

A pathogen that successfully invades a new host without triggering an immediate immune response will be able to allocate the majority of its resources towards replication, while a pathogen that is easily recognized by the host's immune system may be more strongly selected for immune resistance, thus possibly compromising its reproductive rate. Serum fractionation of normal bat serum confirmed that bat IgM IgG and IgA are homologous to corresponding to human immunoglobulins [16,17]. For RABV, the glycoprotein protein is the only viral antigen shown to induce virus-neutralizing antibody (rVNA) [18]. Purification of IgG from RABV-

seropositive bats tested in neutralization assays demonstrates rVNAs *in vitro* inhibition of viral replication [19]. Within this context –and considering that bat RABV has been documented in neighboring localities—in Chapter 3, I examined serological indicators of RABV exposure, and described patterns of seroprevalence among a diverse Neotropical bat community in Guatemala. In this study, the prevalence of RABV among all collected bats was low (0.3%), even among vampire bats (1%). Notably, the proportion of seropositive bats varied significantly across trophic guilds ($p<0.05$), suggestive of complex intraspecific compartmentalization underlying RABV perpetuation. This study also adds to several reports indicating that a substantial fraction of apparently healthy bats have detectible rVNA –suggesting previous exposure to RABV—but the infection failed to progress to clinical disease [19-21]. However, rVNA negative test results do not guarantee a lack of exposure, and are critically dependent on the cutoff value used for the test [22]. These results demonstrate that seroprevalence (as a proxy of RABV infection pressure) is significantly associated with trophic guild, but not sex, and suggests that bats are a potential reservoir for RABV in Guatemala. Considering that RABV is enzootic in most populations of bats, these observations suggest that this phenomenon is widespread, perhaps universal, among diverse New World bat species.

As with any virus there is a minimum infectious dose required for initiating a progressive infection, and that dose is influenced by the genetics of the host and the pathogen. The detection of RABV excretion provides meaningful insights for identifying potential transmission pathways that inform the structure of disease dynamic models. In naturally infected vampire bats examined in Chapter 3, the highest levels of virus were consistently detected from oral swabs, kidney and lung tissue. The results support primary secretion by oral routes, while suggesting a possible pathway in urine (via the kidneys), which warrants further investigation. As the first focused

rabies project in Guatemala, gaps were evident in this study. Limitations included a lack of more focal seasonal sampling, a broader spatial scale, and a need for greater engagement of the Ministries of Health and Agriculture to improve routine laboratory based surveillance. Given the nocturnal and elusive nature of bats, transmission dynamics are difficult to study in natural populations, and many gaps remain for a more complete understanding of the epizootiology of RABV in bats in Guatemala. In light of these findings, it is necessary to seek medical attention, including post-exposure rabies vaccination –for anyone who may have come in contact with a bat in Guatemala—particularly if the bat is unavailable for laboratory testing.

The ability to evade the immune system is not equal among RABV variants, and infection resulting from certain RABV isolates may result in remarkable CNS pathology [23,24]. In support of variation in RABV pathogenicity, several studies conclude that bat-associated RABV variants (when compared with carnivore RABV variants) are associated with a longer incubation period, an ability to replicate at lower temperatures and infect non-neuronal cell types, and a more rapid rVNA response –there is even a case report documenting human survival associated with bat rabies—in the literature [25-27]. These unique pathobiological features are suggested to increase the transmissibility of bat RABV variants, poising a greater risk for sustained transmission following spillover into a non-reservoir host. Awareness of clinical differences between bat and carnivore RABV variants following spillover has implications for suspecting a diagnosis –particularly in humans presenting with an unspecified acute encephalitis—of rabies and may provide insights into potential differences in pathogenesis associated with the RABV variant [5]. Approximately one-third of domestic human rabies cases are diagnosed postmortem. Because of the rarity and lack of clinical and pathologic recognition of RABV infection –complicated by the variable clinical presentation of rabies (e.g. furious or

paralytic)—the disease may go undiagnosed [28-30]. Strikingly, more than 1000 persons die annually of unexplained encephalitis in the United States [28,31].

The mechanisms leading to increased host susceptibility may be a result of immune suppression and evasion, or lack of available rVNA in the periphery. Within in this framework – in Chapter 4—I investigated the hypothesis that an experimental spillover infection with a bat RABV variant results in different pathobiological features (e.g. the cellular location of viral antigen, as determined immunohistochemically in the CNS) and transmission potential (e.g. shedding of infectious virus via the salivary glands) than those associated with a carnivore-adapted variant. While differences in inflammatory patterns have been described in paralytic and furious syndromes –others have hypothesized that rabid humans with a prolonged incubation period may have slowed viral replication—resulting in a protracted prodrome period. This seems unlikely due to the extent and severity of parenchymal inflammation observed in the spinal cord, which is unusual for most human RABV infections [27,32,33].

In Chapter 4, I compared five biologically relevant North American RABV variants in experimentally infected foxes, and tested the effect of the variant on survival within and across isolates. In this study no differences in overt susceptibility were detected, with 97% mortality observed among experimentally infected foxes. Kaplan–Meier survival analysis and Log Rank (Mantel–Cox) test revealed the time to clinical presentation varied significantly by isolate ($p<0.05$). Foxes infected with a dog variant had the shortest mean incubation period (13.8 days) and foxes infected with the raccoon variant had the longest mean incubation period (23.6 days). Moreover, 20% of foxes progressed rapidly without display of any clinical signs. Here, serology provided a useful tool to monitor production of rVNA and potential immune-mediated viral clearance from the CNS. At day 14-post inoculation, 52% of foxes demonstrated detectible

rVNA, and titers were significantly different across variants ($p<0.05$). The level of rVNA detected in foxes infected with a north central skunk RABV was higher at day 14 p.i. compared to south central skunk, raccoon, and dog RABV variants. There was also a significant variant effect for the mean change in rVNA titer from day 7 to day 14 ($p<0.05$). This finding is congruent with other reports describing a prolonged incubation period associated with a raccoon RABV variant –phylogenetically closer to bat than carnivore RABV variants—responsible for multiple clusters of human transplant recipient deaths following a solid organ transplant derived from a rabid donor infected with a raccoon RABV variant [34].

RABV is commonly transmitted by bite from a rabid animal, after transport from the site of entry to the CNS it replicates to high amounts, and during the end stage of clinical disease centrifugal spread of the virus to non-neuronal tissues (e.g. salivary glands) is required for subsequent transmission [35]. The amount of virus that introduced by the bite or scratch of a bat is negligible compared to the level of exposure due to the bite inflicted by a carnivore, such as a raccoon, coyote, skunk or dog. The results in Chapter 4 support the theory that red foxes are susceptible to bat RABV variants. In this study, transmission potential varied by group: infectious virus was recovered from the submandibular salivary glands of a single fox infected with a skunk RABV variant, and all foxes infected with a carnivore RABV variant. The precise amount of RABV introduced by bite or non-bite exposures necessary for a productive infection and lethal disease remains unknown. Moreover, this study provided experimental data to support the concept that spillover infections of biologically relevant North American RABV among red foxes –even a carnivore variant—may be transient or dead ends. The lack of viral dissemination to the salivary glands is a major factor preventing sustained transmission, and the generation of

more virulent variants. However, dependent upon the isolate, dose, and route, as well as animal density, could result in a sustained transmission when epizootiological conditions are ideal.

For this research, novel tools and approaches were developed for quantifying and understanding the balance between individual host infection and immunity and viral pathogenesis and epizootiology associated with bat RABV variants. As with other etiological agents associated with bats, a greater appreciation is needed at the individual host–pathogen level, predicated with the diversity of outcomes from the variables of variant, dose, route and host population. Taken together, these results raise several fundamental questions: For example, a scenario emerges whereby a fox infected with a bat RABV variant inevitably encounters a rabid skunk infected with its respective host adapted variant. Co-infection with similar variants of other viruses in the Order: *Mononegavirales* provide a novel environment for genetic assortment –creating progeny viruses with varying host susceptibility—responsible for global influenza epidemics and the occasional pandemic in humans [36-38]. The potential for co-infection with two or more RABV variants within a single host clearly requires further investigation.

Enhanced pathogen surveillance in humans and animal populations –coupled with virus characterization and pathobiologic assessment—stimulates further research necessary to identify which variants may cause the next pandemic. Such functional studies will likely result in significant insights into host–virus relationships, the implications of which will have impacts on the development of novel therapeutics for other species and the ability to predict viral spillover events [39,40]. The findings in Chapters 2 and 3 contribute to our understanding regarding the epizootiology and pathogenesis associated with bat RABV variants, and reveal a carefully balanced complex suite of interactions functioning to promote virus replication and spread within

a host while either directly or indirectly disrupting host physiology. The specific modifications in the RABV glycoprotein gene through adaptation –shifts binding preferences to specific receptors expressed throughout diverse mammalian hosts—is fundamental to virulence, variant range expansion, and interspecies perpetuation of bat RABV variants. By comparison, Chapter 4 highlights that RABV immunogenicity differs between variants, and transmission potential is related to a variant's ability to evade the host's immune system. Fundamentally, the more competent the host immune system, the less chance there is for the virus to survive [41].

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