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Experimental infection of Jamaican fruit bats (Artibeus jamaicensis) with Tacaribe virus

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Experimental Infection of Jamaican Fruit Bats (*Artibeus jamaicensis*) With Tacaribe Virus

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ABSTRACT

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Virtually nothing is known about the immunology and virus-host interactions of bats (order Chiroptera). The discovery of bats as reservoirs or potential reservoirs of many important human and veterinary pathogens such as ebolaviruses and Nipah and Hendra viruses, has recently sparked an interest in bat immunology. Bats may become persistently infected with viruses without signs of pathology, while human infections often lead to severe disease and death. It is unknown how this type of virus-host interaction is established in bats. This project began to examine the immune systems and virus-host interactions in two species of bats. Tacaribe virus was isolated from two species of *Artibeus* bats in the early 1960s and belongs to a group of arenaviruses that cause the South American hemorrhagic fevers. Tacaribe virus is not known to cause natural human infections. It would be highly unusual for Tacaribe virus to be hosted by *Artibeus* bats, as it would be the only arenavirus, for which the host is known, that does not have a rodent host. Discovery of the specific immune characteristics that allow bats to become persistently infected with many pathogens may offer medically important information for the treatment of human infections. Cytokine genes from Seba's short-tailed fruit bat (*Carollia perspicillata*), were cloned, sequenced, and compared to orthologous mammalian sequences. It was hypothesized that bat cytokine genes are similar to the cytokine genes of other mammals. Jamaican fruit bats (*Artibeus jamaicensis*) were experimentally infected with Tacaribe virus and it was hypothesized that they would become persistently infected; however, this did not occur. These results

indicate that bat cytokine genes are highly conserved with respect to other mammalian cytokine sequences, and that Jamaican fruit bats do not become persistently infected with Tacaribe virus.

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LIST OF ABBREVIATIONS

Abbreviation	Term
ANDV	Andes virus
BLAST	Basic local alignment search tool
BSL	Biosafety level
CD	Cluster of differentiation
cDNA	Complimentary deoxyribonucleic acid
CMV	Cytomegalovirus
CO_2	Carbon dioxide
CPE	Cytopathic effect
CSU	Colorado State University
CTL	Cytotoxic lymphocyte
DNA	Deoxyribonucleic acid
EBV	Epstein Barr Virus
ELISA	Enzyme linked immunosorbent assay
FAT	Fluorescent antibody test
FBS	Fetal bovine serum
GM-CSF	Granulocyte macrophage-colony stimulating factor
HCPS	Hantavirus cardiopulmonary syndrome
HF	Hemorrhagic fever
HRP	Horseradish peroxidase
IFA	Immunofluorescent antibody

IFN	Interferon
Ig	Immunoglobulin
IHC	Immunohistochemistry
IL-10	Interleukin-10
IL-23a	Interleukin-23a
IN	Internasal
IPTG	Iopropyl β-D-1-thiogalactopyranoside
LCMV	Lymphocytic choriomeningitis virus
MAPV	Maporal virus
NIH	National Institutes of Health
PAGEIA	Protein-A/G horseradish peroxidase enzyme-linked immunosorbent assay
PCR	Polymerase chain reaction
Poly I:C	Polyinosinic:polycytidylic acid
RING	Really interesting new gene
RNA	Ribonucleic acid
RT	Reverse transcriptase
SAHF	South American hemorrhagic fever
SARS	Severe acute respiratory syndrome
SARS-CoV	Severe acute respiratory syndrome coronavirus
SC	Subcutaneous
SNV	Sin Nombre virus
STAT	Signal transducers and activators of transcription
TCRV	Tacaribe virus
TGF-β	Transforming growth factor beta
TLR	Toll-like receptor

TNF	Tumor necrosis factor
TOPO	Topoisomerase
UNC	University of Northern Colorado
UPGMA	Un-weighted pair group method with arithmetic mean
VEEV	Venezuelan equine encephalitis virus
VZV	Varicella zoster virus
WNS	White nose syndrome
WNV	West Nile virus

CHAPTER I

INTRODUCTION

Bats (order Chiroptera) represent nearly one fifth of all known species of mammals [1], yet many aspects of their biology are poorly understood [2]. The ecology and general biology of bats has been well studied, but there is relatively little known about bat immune systems or the immune responses of bats to infectious agents. For many years, rabies virus has been the most studied microbe of bat disease ecology [3-5] [2]. It is only more recently that bats have been identified as reservoirs or potential reservoirs of other important human and veterinary pathogens [2, 6]. Among these are SARS CoV-like coronaviruses [7-9], Nipah and Hendra paramyxoviruses [10, 11], lyssaviruses [12, 13], and Marburg and ebolaviruses [14, 15].

In some instances, viruses may cause persistent infection without producing recognizable pathology. The establishment of persistent infections with little or no signs of pathology could have contributed to bats being underappreciated as reservoirs of many viruses [2]. Understanding how bat immune systems engage viruses or how many viruses evade sterilizing host immune responses in bats will require further investigation. It is suspected that the ability of bats to become persistently infected with viruses could be due to distinct differences in bat cytokine genes and the expression of immune genes during infection.

Characteristics of bat immune systems have not been previously studied because methods to do so did not exist. Another problem with studying virus-host interactions in bats and some viruses they are thought to host, is that many of these

viruses are potential human pathogens that must be handled at biosafety level 3 or 4 (BSL3 or BSL4) [2]. Increased biosafety precautions make experimentation substantially more difficult.

This project was initiated to develop immunologic and virologic methods in bats, currently absent in infectious disease research. This project has largely been modeled after work that had previously been conducted on a hantavirus/deer mouse project [16-19]. Hantaviruses are rodent- or shrew-borne viruses, and some have the ability to cause serious illness in humans. Sin Nombre virus (SNV) is a hantavirus that is hosted by deer mice (*Peromyscus maniculatus*), which become persistently infected with SNV without developing pathology. However, human infections with SNV and other hantaviruses can cause hantavirus cardiopulmonary syndrome (HCPS). This illness is a cytokine-mediated immunopathology [20-24]. Some researchers are investigating possible differences between deer mouse and human immune responses to the viruses [17, 25]. It is thought that deer mice and SNV have coevolved resulting in the evolution of virus-host coexistence by allowing the virus to establish a persistent infection without causing disease or immunopathology in the host. If the mechanisms by which this interaction takes place can be identified, they may offer a potential solution for avoiding the immunopathology that is experienced with human infections [17, 21, 25]. Thus, a similar rationale underpins this study on bats and their viruses.

The examination of bat immune systems and immune responses is important on several levels. First, immunology, virus-host interactions, and disease ecology is an area of bat biology that has received little attention. Such examination could offer information about a previously unexplored area of bat biology.

Second, there is high probability that the examination of bat immune systems might offer important biomedical information about how bats avoid severe disease and immunopathology that develops in humans. It is important to characterize the genetics

and activity of bat immune responses as well as the complex virus-host interactions that occur to better understand and control the emergence of viruses that are known or suspected to be hosted by bats [26].

This project has focused on examining immune genes and virus-host interactions in Seba's short-tailed fruit bats (*Carollia perspicillata*) and Jamaican fruit bats (*Artibeus jamaicensis*). Captive colonies of these bat species held at the University of Northern Colorado were used in these studies.

A project to clone and characterize cytokine genes of Seba's short-tailed fruit bat was initiated to begin the process of characterizing bat immune systems. Cytokines are hormone-like proteins that are used to mediate local immune responses. Actions of cytokines can be inflammatory [23, 27, 28], anti-inflammatory [29], or can act to modulate the development and actions of immune cells and tissues [30-32]. Characterization of bat cytokine genes is an important step in understanding the immunological relationship between viruses and their bat hosts.

If bats act as reservoirs of specific viruses, the establishment of persistent infections would seem likely. An investigation of the interaction between Jamaican fruit bats and Tacaribe virus was initiated to begin identifying the underlying mechanisms that govern non-pathogenic infections of reservoirs. Tacaribe virus (TCRV) is a New World arenavirus that is not known to cause natural human infections and can be manipulated at BSL2 [33, 34]. Other New World arenaviruses include Junín, Machupo, Sabia, Guanarito, and Chapare viruses, which cause Argentine hemorrhagic fever (HF), Bolivian HF, Brazilian HF, Venezuelan HF, and an as yet unnamed hemorrhagic fever respectively [35-38]. These New World arenaviruses are extremely virulent in humans and experimental work with them must be performed at BSL4.

Other New World arenaviruses such as Tacaribe, Oliveros, and Pichinde viruses are not human pathogens [38, 39]. All arenaviruses, for which the reservoir is known,

are hosted by rodent species [40]. Previously, researchers believed that TCRV had a unique non-rodent host species [33].

Tacaribe virus, was originally isolated from two species of *Artibeus* bats. On two separate occasions, TCRV was isolated from captured Jamaican fruit bats; however, it is unknown if this species is a reservoir of TCRV, or if infections were due to incidental infection [33, 34]. This finding is of interest for several reasons. If bats were found to be the host of TCRV, it would be the first arenavirus to have a non-rodent host. Second, TCRV may establish persistent infections in Jamaican fruit bats, and examination of this interaction may help identify characteristics of bat immune responses that allow for the establishment of persistent infections without the development of pathology. A third aspect that is attractive about TCRV is that it is a New World arenavirus that can be manipulated at BSL2 making it easier to manipulate during experimentation. It is also possible that TCRV could be used as a disease model for South American hemorrhagic fevers (SAHF). Development of a Tacaribe arenavirus infection model has recently been suggested using interferon (IFN) receptor deficient mice [41, 42]. Results from the work presented in this dissertation suggest that TCRV infection in Jamaican fruit bats could be used as a disease model (Unpublished results).

Specific Aims And Hypotheses

The first aim of this project was to clone and sequence immune genes and immune-related transcription factors from Seba's short-tailed fruit bat. Cloning and characterization of immune genes from this bat species has not previously been done. Developing these types of immunologic methods will allow the characterization of bat immune systems.

Specific aim 1: To determine if bat immune genes, specifically cytokine genes, are similar to orthologous mammalian sequences, and to begin characterizing chiropteran immune systems. Seba's short-tailed fruit bats from the University of Northern Colorado were used in this project. Spleens were collected from euthanized bats and used for ribonucleic acid (RNA) extraction, complimentary deoxyribonucleic acid (cDNA) synthesis and amplification of cytokine genes. Cytokine-specific primers were used to amplify the genes.

H1: Chiropteran cytokine gene sequences are similar to orthologous mammalian sequences.

The second aim of this project was to determine if Jamaican fruit bats can become persistently infected with Tacaribe virus (TCRV) and to examine tissue tropism. Persistent infections would be expected if Jamaican fruit bats were the natural reservoir.

Specific aim 2: To determine if Jamaican fruit bats can become persistently infected with TCRV. Jamaican fruit bats were experimentally infected with TCRV at the biosafety level (BSL) 3 unit at Colorado State University. Blood samples were collected for virus isolation, and neutralizing antibody tests. Oral and rectal swabs were collected to determine if virus shedding occurred. Collected tissues were used for polymerase chain reaction (PCR), virus isolation and immunohistochemistry (IHC) to determine virus tropism.

H2: Jamaican fruit bats are reservoirs of Tacaribe virus**.**

The third aim of this study was to develop a rapid field test for detection of TCRV specific antibody. This is not a hypothesis driven experiment, but rather work to develop an immunoassay that is greatly needed for research with TCRV. A rapid field-test for TCRV would allow for quick identification of naturally infected animals in wild populations, as well as confirmation of infection and seroconversion of experimentally infected animals.

Specific aim 3: To develop a serological test by cloning and expressing an antigen of the TCRV nucleocapsid that will be recognized by TCRV-specific antibodies. The nucleocapsid gene was cloned and placed into an expression vector. Protein expression and purification could allow the nucleocapsid antigen to be used in an enzyme immunoassay, such as a protein A/G horseradish peroxidase conjugate.

The findings from this research project indicate that Seba's short-tailed fruit bat cytokine genes are highly conserved with respect to orthologous mammalian cytokine genes. Results also indicate that Jamaican fruit bats do not become persistently infected with TCRV, and that they likely are not the natural reservoir of TCRV. A large portion of the TCRV nucleocapsid gene has been cloned into an expression vector, and it is believed that expression of this protein will be possible. This study is an important step toward the general characterization of bat immune systems, the development of immunologic methods in bats, as well as a step toward developing an immunological model for host-pathogen interactions in bats.

CHAPTER II

REVIEW OF LITERATURE

General Bat Ecology

Bats (Order Chiroptera) comprise nearly one fifth of the approximately 5416 documented species of mammals. Bats inhabit all continents of the world except Antarctica, and they are important assets to their ecosystems [2, 43]. Bats are unique because they are the only mammals that exhibit true powered flight, and they are able fly and forage at night [43, 44]. Bats exhibit a wide range of body sizes, with the smallest bat weighing only 1.5 grams and the largest bat weighing as much as 1.5 kilograms [43, 44] and some species may live as long as 30 years. Bats employ a wide variety of roosting behaviors and mating systems and typically produce one to two offspring per year. Some bats enter torpor (a shortened form of hibernation) to conserve energy when resources are limited or temperatures are cold, while others migrate [43-45].

Bats are divided into two suborders; Microchiroptera, which is comprised of 17 families and approximately 1030 species [1], and Megachiroptera, which is comprised of one family (Pteropodidae) and approximately 186 species [1]. The suborder Microchiroptera encompasses the echolocating bats. While many of these bats are insectivorous, there are diverse feeding ecologies seen within this suborder, which includes bats that feed on fruit, nectar, pollen, blood, small vertebrates, insects, and arthropods [43, 45]. Bats in the family Phyllostomidae exhibit many of these diverse feeding ecologies [43]. The work presented in this dissertation focused on two species of bats from the suborder Microchiroptera and the family Phyllostomidae. Bats in the

suborder Megachiroptera are known as the Old World fruit bats or flying foxes, and eat mostly fruit and nectar. Megachiropterans do not utilize complex echolocation, but instead rely on vision and olfaction to locate food sources [43].

The diverse feeding ecologies of bats play several important ecological roles. Species of insectivorous bats aid in the control of insects [46, 47]. Fruit and pollen-eating species of bats are important in the pollination and seed dispersal of many plant species [48, 49]. In some cases, the interaction between bats and plants can be specific. Jamaican fruit bats have been found to be valuable seed dispersers of fig seeds in Panama [49]. Orii's flying fox (*Pteropus dasymallus inopinatus*) has been found to be an important seed disperser of several native plant species on Oki-nawa-jima Island, Japan. In this case, Orii's flying fox may be the only seed disperser on the island that is capable of moving the large fruits produced by some plants [50]. The pollination and seed dispersal of plants by bats is beneficial to plant species by providing a means for plants to maintain their populations [48-50]. It has also been found that bats may play an important role in re-seeding fragmented or disturbed areas of forests [51]. These pollination and seed dispersal activities are beneficial to fruit-eating mammals including humans by providing them with food sources [49]. Bats also produce guano which has important uses in agriculture and pharmaceuticals [2, 52].

Bat Immunology

Although bat ecology and general bat biology are areas that have been extensively researched, many aspects of bat biology and physiology are poorly understood. Specifically, bat immunology and disease ecology have previously received little attention [2, 6]. This significant area of bat biology needs to be examined to fully understand how bats interact with the ecosystem.

In some cases, it is possible that bat populations could be experiencing disease and mass-mortality from infection with microorganisms. An example of this is white nose

syndrome (WNS), which has caused severe declines in some bat populations in North America [53]. It is thought that WNS is caused by infection with the fungus *Geomyces destructans* [53, 54]. Symptoms include fungal growth on the nose, ears, and wing membranes, leading to severe physiological dysfunction [53, 55]. Bats have long been studied as reservoirs of rabies virus, which has occasionally been documented causing mortality in bat populations [5]. However, this is not always the case as bats sometimes appear to be persistently infected with rabies without developing symptoms [5, 56].

While rabies research in bats has received much attention and there is extensive literature available on bats and lyssaviruses [2, 5], it is only within the past decade that bats have been discovered as reservoirs or potential reservoirs of many other important human and veterinary pathogens [2, 8, 10, 14, 57]. Among the list of viruses known or suspected to be hosted by bats are lyssaviruses other than rabies [12, 13], ebolaviruses [14, 58], Marburg virus [15, 59], Nipah virus and Hendra virus [10, 11], and Severe acute respiratory syndrome coronavirus (SARS CoV)-like viruses [7-9].

In some cases, it appears viruses may establish persistent infections in bats without causing pathology. This is intriguing because human infections with many of these viruses can cause severe disease and death. Whether considering a bat pathogen, or a virus that may establish a persistent infection in bats without pathology, little is known about bat immune systems or virus-host interactions in bats. It is necessary to investigate this portion of bat physiology, biology and ecology to fully understand all aspects of bat biology. Although examining bat immune systems is essential to a comprehensive understanding of bat biology, the examination of bats as reservoir hosts to viruses can be somewhat controversial. This is because identifying bats as reservoirs of virulent human and veterinary pathogens may led to their persecution and eradication [60]. This reaction could be contraindicated, as loss of biodiversity has also been proposed as a mechanism by which infectious diseases

emerge and spread [61]. Just as human population growth and encroachment into previously uninhabited territory are drivers of disease emergence, loss of bats as hosts to many of these viruses may led to increased numbers of human and veterinary outbreaks [61-63]. For these reasons, and the ecological importance of bats, examination of bat immunology and virus-host interactions while protecting them from persecution must be conducted [60]. Examining the immune systems of bats as well as the virus-host interactions that occur may clarify some aspects of bat biology and offer insight as to how persistent infections are accomplished. This could provide important information about how to treat human infections caused by many deadly viruses.

Researchers have begun to examine some of the biological characteristics of bats with respect to their ability to act as reservoirs of many viruses [60, 64]. Characteristics that have been identified as potential factors influencing the ability of bats to act as reservoirs or sources of emerging diseases include the use of torpor, the ability to fly long distances, genetics of immune genes, and the regulation of immune genes during infection [64, 65]. This review will discuss virus-host interactions, immunologic methods that exist for examining these interactions, what is known about bat immunology, and viruses that have been isolated from species of bats. This review also aims to explain the need for examining bats as reservoirs and how the research project presented in this dissertation approaches this need.

Persistent Infections

An examination of the relationship that exists between a virus and its natural host is essential to understanding all aspects of a virus' ecology. In many cases, a reservoir host of a virus can become persistently infected and not develop disease [22, 66, 67]. A virus must have a susceptible host to infect to persist in nature. If a virus were to cause serious pathology or kill the natural host, it would not be advantageous for the virus or host.

On the other end of the spectrum, spillover infections into humans or other species often cause serious disease. Humans and other non-reservoir species are usually dead-end hosts [22, 66, 67].

It is not fully understood how persistent infections occur in host species, but research has been successful in identifying some of the underlying mechanisms. Researchers have investigated several aspects of virus-host interactions including activity and regulation of immune genes and immune cells during infection, virus tropism, viral evasion strategies, and viral replication strategies [66, 68]. Some of this work has been performed in cell culture, while other researchers have explored experimental infections. Coevolution between virus and host has also been suggested as a potential reason for which viruses do not cause disease in the natural reservoir [67]. Examples of interactions where persistent infections are established in the natural reservoir include hantaviruses and arenaviruses and their rodent hosts. Human infections with many of these viruses led to serious disease and possibly death.

Hantaviruses

In North America, the deer mouse can become persistently infected with Sin Nombre virus (SNV) (Family *Bunyaviridae*, genus *Hantavirus*) without signs of pathology [22]. Deer mice can be persistently infected for long periods of time, and the virus can be spread and maintained from one population of deer mice to another [69]. Infected deer mice can shed infectious virus in urine, feces, and saliva. When humans come in contact with infectious products, the virus is contracted through the lungs, leading to hantavirus cardiopulmonary syndrome (HCPS) or hemorrhagic fever with renal syndrome [20, 21, 25, 70]. This interaction has led researchers to investigate the underlying molecular and cellular mechanisms that allow for the accomplishment of persistent infections in the rodent host.

Arenaviruses

Arenaviruses are another example of viruses that cause persistent infections in the host species, yet cause serious disease in spillover infections. Arenaviruses, for which the host is known, are rodent-borne viruses [67, 71, 72]. Arenaviruses such as Junín, Machupo, and Guanarito viruses cause South American hemorrhagic fevers (SAHF) in humans, and are hosted by the drylands vesper mouse (*Calomys musculinus*), the large vesper mouse (*Calomys callosus*), and the short-tailed cane mouse (*Zygodontomys brevicauda*), respectively [38, 67, 71]. Persistent, yet asymptomatic infections are established in the rodent hosts [67, 73]. Experimental inoculations of the short-tailed cane mouse with Guanarito virus have shown that persistent infections are established and that the cane mouse is the natural reservoir of Guanarito virus [73]. Infected rodents shed virus in secretions including urine, feces, and saliva, and when humans come in contact with these secretions, serious disease can ensue [38]. Researchers have examined the interaction between some arenaviruses and the rodent hosts in much the same way that the interaction between SNV and the deer mouse was investigated. This research has led to the discovery of some of the mechanisms by which persistent infections occur in rodents [74-77].

It appears that interactions between many viruses and the natural hosts may offer information about avoiding the development of serious disease in human infections. For this reason, it is important to investigate how these interactions occur. For some viruses, researchers have developed immunologic methods to investigate the complex virus-host interaction.

Methods Used To Examine Persistent Infections

Hantaviruses

Cytokine genes encode polypeptides that are largely responsible for mediation of the immune response. Cytokines are chemical signals that can be secreted or released from several different types of immune cells to mitigate different microbial threats. Many researchers have focused on cloning and characterization of the cytokine genes as a way of examining the immune systems of host species [78-80]. This allows for the examination of the immune system on the genetic level and for comparison to other mammalian cytokine gene sequences. Cytokines such as granulocyte macrophage-colony stimulating factor (GM-CSF), tumor necrosis factor (TNF), interleukin-10 (IL-10), and interleukin-23a (IL-23a) have specific activities in the immune response and can mediate immune cell stimulation, inflammation and immune cell chemotaxis, down-regulation of the immune response, and the initiation of the adaptive immune response [29-31, 81, 82].

In SNV infection in deer mice, researchers have been investigating the mechanisms by which persistent infections are accomplished by characterizing deer mouse immune genes and examining immune responses during infection. Deer mouse cytokine genes have been cloned, sequenced and compared to orthologous mammalian sequences in an attempt to identify any possible differences in the genetic sequences. The results of these projects largely agree that deer mouse cytokine genes are highly conserved with other mammalian cytokine genes [19, 80]. With no identifiable differences between the genetics of the immune genes, researchers believe that the difference in disease progression could lie within the regulation of cytokines and the behavior of immune cells during infection [17, 23, 80].

Bone marrow and T cell culture parameters have been developed for the deer mouse [18, 83]. These methods have allowed for the detailed examination of the cellular and molecular characteristics of immune genes and cells of the deer mouse [18, 83]. These methods have been helpful in the characterization of deer mouse T cell responses and development of other immune cells in response to SNV. More recent studies on the deer mouse and SNV using these methods have found that regulatory T cells expressing the cytokine TGFβ1 may be important for down-regulation and control of immunopathology in the deer mouse [17].

Arenaviruses

Methods that have been used to investigate arenavirus infections in rodent hosts are very similar to the methods described for SNV and the deer mouse. Many studies examining persistence of arenaviruses in rodents are performed using lymphocytic choriomeningitis virus (LCMV), which was the first arenavirus to be discovered [75, 84]. A study performed in 1936 utilized experimental infections of white lab mice and wild mice to describe the mode of transmission of LCMV in mouse populations and the length of persistence of viral infection [75]. Results from this experiment indicated that infectious virus was shed in the urine and nasal secretions. Researchers also found that mice were typically infected before or shortly after birth with the route of exposure likely being the nasal mucosa [75]. The length of persistence in infected individuals was observed to be as long as 15 months. It was concluded that an immunocompromised status, like that of a neonate, is necessary for LCMV to evade clearance by the immune system and establish a persistent infection [75].

Virus tropism of persistent arenavirus infection was examined using LCMV and in situ hybridization [84]. Neonatal mice were experimentally infected with LCMV and were euthanized after establishment of persistent infections. Tissue samples were fixed in buffered formalin and placed in paraffin blocks. Tissue sections were then sliced and

placed onto slides for examination. In situ hybridization was performed using a ^{35}S-labeled DNA probe that was complementary to a portion of the S segment of the LCMV genome. Hybridization was then visualized by exposing the slides to high-resolution film. Film was then developed and slides were counter-stained with haematoxylin and eosin to visualize, and were observed using microscopy. The results of this study indicated that virus was present in many tissues of infected mice including the brain, lung, liver, kidney, pancreas, thyroid, thymus, lymph nodes, testes, bladder, and salivary glands [84]. This study and others performed before it showed that mice infected with LCMV develop persistent viremia [84, 85].

More recent studies with LCMV have focused on specific mechanisms that allow LCMV to evade a sterilizing host immune response. A study performed in 1997 examined the use of neutralizing antibody to prevent the establishment of persistent LCMV infections in mice [86]. The results showed that neutralizing antibodies did prevent the development of persistent infections in immunocompetent and immunocompromised mice, including neonates. These results further support the findings that arenaviruses must establish persistent infections in rodent hosts when they are young and not yet immunocompetent [86].

Animal models

Another approach to examining the difference between viral infection of the natural reservoir and spillover infections is to examine an animal model that exhibits similar symptoms to human disease. Hantaviruses like SNV and Maporal virus (MAPV) are asymptomatically carried by the natural rodent reservoirs. However, researchers found that when MAPV was experimentally inoculated into Syrian golden hamsters *(Mesocricetus auratus)* it caused pathology similar to the disease that develops in human cases of HCPS [21]. This insight has allowed researchers to examine MAPV infection of golden hamsters as a disease model for human HCPS [21]. A disease

model has also been developed using Andes virus (ANDV) in the Syrian golden hamster [25]. An animal disease model offers researchers the ability to experimentally examine the interaction that occurs during acute infection to identify factors that contribute to disease progression.

Viral evasion strategies

Infectious agents that have the ability to persist in host species without pathology generally have genes that allow them to evade host immune responses. Some of the viral genes that help viruses persist can suppress the immune functions of specific cells by causing the blockage of antigen presentation [87]. Other viruses are able to produce proteins that allow cytokine escape by binding and inactivating host cytokines[87]. Some viruses also use cytokine and chemokine mimicry by producing proteins that closely resemble host cytokines and chemokines, but the viral proteins do not have the same biological activity [87-90]. By subverting host immune responses, viruses are able to avoid clearance and persist in the host. Examining viruses for viral evasion strategies is allows for characterizing the virus-host interaction that leads to persistent infections.

As was described for persistent arenavirus infections in mice, some viruses avoid immune clearance by infecting immunocompromised individuals [86]. Also, the nucleoprotein of LCMV and other arenaviruses can be antagonistic of the type I IFN response [74]. In this interaction, arenavirus nucleoproteins were found to block interferon response factor-3 (IRF-3), a transcription factor that leads to signaling events and eventually type I IFN production in infected cells. This has been suggested as a mechanism by which arenaviruses evade host immune responses and are able to establish persistence [74]. Similar findings have been made in New World hantaviruses such as SNV and ANDV. The nucleocapsid proteins and glycoprotein precursors of these viruses were found to inhibit IFN-β induction and the downstream signaling pathways [91].

In cell culture, Hendra and Nipah viruses have been identified as encoding proteins that interfere with STAT1 transcription factor. This viral evasion strategy allows the viruses to disrupt signaling events leading to the release of type I IFN which is important in cellular immune responses [92, 93]. This finding is of particular interest in terms of bat immunology, as specific species of megabats have been identified as reservoirs of these viruses [94, 95].

Virus-host coevolution

Research has indicated that many viruses and their reservoir hosts have coevolved over time [38, 67, 96]. Interactions over millions of years could account for infection without disease.

Phylogenetic studies of viruses and host species have been conducted for arenaviruses and hantaviruses. The results of these analyses show a very tight association between specific viruses and hosts [38, 67, 71, 97]. Phylogenetic trees constructed for the New World arenaviruses show noticeable similarity to the phylogenetic trees constructed for rodents found in the same geographic region. These results support the hypothesis that viruses and the natural hosts have been interacting and cospeciating for many years [67].

Presence of viral gene segments in host genomes are thought to represent an ancient interaction between virus and host that, over time, led to the incorporation of viral genes into host genomes. This field is referred to as paleovirology [96]. Phylogenetic studies and next generation gene sequencing methods have been used to study the possible coevolution of filoviruses and mammals.

Phylogenetic tree construction allowed researchers to identify possible paleoviral elements in small mammals, including bats, insectivores, and rodents [96]. While these types of studies do not explain specific mechanisms employed to establish persistent infections, they do help reveal that the ability to act as reservoir to a virus without

developing disease is not an easily or quickly acquired characteristic. Understanding the history of virus-host interactions might help characterize the complexity of the relationship.

Immunologic Methods In Bats

Characteristics of bat immune systems and the mechanisms by which viruses establish persistent infections in bats have not been extensively studied because methods to do so have only recently been developed. In many cases, methods that currently exist to characterize immune responses in other mammals may be useful in examining bats, but likely need to be optimized for use in bat species. Using methods that have previously been established for immunologic research in other mammals is the approach that some researchers have taken to bat immunology.

Due to the enormous amount of research that has been performed on rabies and related lyssavirus infections in bats, there are well-developed methods for detecting rabies infection and examining the virus-host interaction that occurs [12, 56, 98, 99]. Methods for detecting other infections in bats could be developed by using methods similar to those used to diagnose rabies, and in some cases, these tests have already been developed [57, 100-102]. Some of the methods available to detect viral infection in bats include immunofluorescence and immunohistochemistry, neutralizing antibody tests, enzyme linked immunosorbent assay, and PCR [12, 98, 99].

Immunofluorescence and immunohistochemistry

Accurately testing for some viral infections such as rabies and related lyssaviruses may require tissue collection from the organism. Tissues can be examined for the presence of viral nucleocapsid using techniques such as direct immunofluorescence assay (IFA) or fluorescent antibody tests (FAT) [12, 99, 103, 104]. Histopathological and cytological examinations can also be performed on tissue samples

to detect rabies. These methods may require fixed tissue sections or cell culture, and may vary in sensitivity. A study performed in 2003 tested the efficiency of FAT, histopathology and cytological examinations for the detection of rabies [105]. The authors prepared identical tissue samples for each method, and compared the ability of each to accurately detect rabies virus. The study concluded that FAT is the most sensitive technique followed by histopathology and then cytological examination [105].

Plaque assays can also be conducted to detect virus presence. Plaque assays include the use of cell culture to detect the formation of plaque units due to activities of virus. This technique is only useful if the virus is known to show cytopathic effect (CPE) in cell culture [106]. While methods such as IFA, FAT, and plaque assays are useful in different situations, they may take a considerable amount of time to complete.

Enzyme-linked immunosorbent assay

Many researchers have begun to develop and use specific immunoassays such as enzyme-linked immunosorbent assays (ELISA) [15, 107] This type of test is used to identify specific antibodies to virus in serum samples (indirect ELISA) or the presence of viral antigen (sandwich ELISA). One challenge to indirect ELISAs is that it is necessary to develop virus specific antigens for each virus species. In these tests, viral antigen is prepared and coated onto the wells of microtiter plates. Tests for many viruses have previously been developed for detection of antibody in humans or other animals. Developing many of these tests for use in bat research may require modification by producing anti-bat immunoglobulins, which would be used as secondary detection antibodies [15, 107].

ELISAs have been developed to detect the presence of lyssavirus-specific antibodies in collected serum samples of bats [12]. The use of ELISA can make screening processes and discovery of previously undetected viral infections much

simpler and rapid than other methods [14, 59, 101, 108]. In 2001, a random tissue and serum sampling of bats found in restaurants of Cambodian provinces was performed. Serum, organs, and tissue samples were investigated using IFA, FAT, and ELISA [12]. This study was the first to identify anti-lyssavirus neutralizing antibodies in the serum of Cambodian bats. This project was a random sampling and was not performed on bats showing symptoms of rabies infection. Bats sampled in this project were seemingly healthy, and therefore the authors conclude that the bats may be reservoirs of the detected lyssavirus. The authors suggest that without previously causing mortality or disease in bats, it could that this lyssavirus strain was previously undiscovered [12]. Subsequently, researchers have developed similar tests for detecting viral infection of other viruses in bats including Nipah virus, ebolaviruses, and Marburg virus [14, 15, 100, 101, 107-109].

While ELISAs can be useful for detection of virus-specific antibody or viral antigen, the length of time required to complete these assays can be extensive. Some ELISAS may require up to 5 hours to complete. This makes rapid screening of mammalian populations in the field difficult [16, 110]. More recent developments have made it possible to perform rapid detection of antibodies to SNV during field studies. Development of such rapid immunoassays allows for much faster identification of infected rodents in the field without requiring transport of samples to a lab [16]. It is likely that similar tests can be developed for use in screening both wild and experimentally infected bats for presence of virus-specific antibody. The development of a rapid field immunoassay for Tacaribe virus, which was isolated from the Jamaican fruit bats in Port of Spain Trinidad (Unpublished observations) [33], is a current activity of this dissertation work.

Bactericidal activity and T-cell mediated responses

Less specific tests including the measurement of bactericidal activity, and T cell mediated responses to mitogenic challenge have also been employed to examine bat immune systems [111]. Using these methods, one study aimed to examine the innate and adaptive immune responses of wild caught bats in relation to the type of roost that was utilized. In this study, female Brazilian free-tailed bats (*Tadarida brasiliensis*) were caught and blood samples were taken. Whole blood samples were diluted and mixed with bacterial samples, which were then plated out on trypticase soy agar plates. After the plates were incubated, bactericidal load was calculated from colony counts. The fewer bacterial colonies that were present after incubation indicated increased bactericidal activity present in the blood sample. Captured bats were also examined for T cell mediated immune activity by administering subcutaneous injections of phytohaemagglutinin to stimulate leukocyte agglutination. Adaptive immune function was measured by quantifying the amount of swelling that occurred at the site of infection after a 12-hour period. Results indicated that there was a difference in the bacterial killing ability and the T cell responses of bats at different roost sites. The researchers concluded that immune responses and susceptibility to disease may be linked to roosting ecology of bats [111].

Polymerase chain reaction and gene sequencing

PCR is useful for detecting presence of virus in tissue samples and other collected samples such as swabs, and real time PCR can be performed to quantify viral load. While other techniques such as ELISA can be useful for detecting viral antigen or virus-specific antibody, PCR techniques offer the possibility of gene sequencing to confirm presence of virus. PCR requires viral RNA extraction, cDNA synthesis, and amplification using virus specific primers. After amplification, gene sequencing should

be performed to confirm identity of viral genes. In several studies, total RNA was extracted from serum samples or organ samples of bats and screened by PCR for the presence of infectious agents. This type of screening not only allows for the evaluation of experimentally infected animals, but also the identification of reservoirs in broad screening projects. This has led to the discovery of bats as potential reservoirs of novel astroviruses, a reovirus, and a novel GBV-like flavivirus [112-114]. Other studies have detected Marburg virus and Nipah virus in organs of sampled bats using similar methods [100, 115].

Next generation sequencing technologies such as pyrosequencing have been used to detect the presence of bat-borne viruses in collected guano samples [116]. Pyrosequencing technology sequences genes as DNA synthesis takes place by detecting DNA polymerase activity with chemiluminescent enzymes. In one study, bat guano samples were collected underneath bat roosts in Texas and California and viral nucleic acids were extracted using QIAamp viral RNA extraction kits. Random primers were then used on RNA samples to synthesize cDNA libraries and amplify genes. Amplified PCR products were resolved on a 2% agarose gel and gel purified. PCR products were then sequenced using pyrosequencing technology. Sequence contigs were made using Sequencher software and basic local alignment search tool (BLAST) analyses were performed. The BLAST results indicated that a large number of viral genomes were present in the guano samples. The majority of detected viral sequences were similar to eukaryotic viruses that infect insects. The second largest group of viral sequences showed similarity to viruses that infect plants and fungi. The third largest group of viral sequences showed similarity to viruses that infect mammals. While none of the detected viral sequences showed similarity to any known human pathogen, some viral sequences were identified from the Picornaviridae, Adenovridae, Poxviridae, Astroviridae, and Coronaviridae viral families. It was concluded that many of the viruses

identified in this study likely reflect the diet of the insectivorous bats that were screened. PCR and gene sequence analysis can be helpful in large screening projects performed to identify any possible viruses in species of bats [116].

In some cases, virus may be shed through the urine of infected bats, and the capture and extraction of urine from bats without requiring euthanasia can be quite time consuming [117]. An easier way of collecting bat urine samples was developed in 1999 while examining pteropid species of bats for occurrence of Nipah virus. The method includes the use of large plastic sheets that are placed on the ground below the entrance to the roosting place of bat populations. The plastic sheet collects the urine samples, which then may be collected with sterile swabs for viral RNA extraction and PCR [117]. Multiple samples can be collected using this method, but it is possible that samples from many different bats will be pooled together. This method may be applicable in detecting viral infections in other bat species infected with other viruses.

The methods described above were used as a way to detect viral infection in bats and other mammals. While the ability to detect current or past infection with viruses is an important part of understanding disease ecology in bats, it is also necessary to examine the characteristics and activity of bat immune systems.

As some of the more recent immunologic research in bats has shown, it is possible that characteristics of bat immunology can be investigated using modified methods that are commonly used in mammalian immunology [2]. In the past decade, there have been advancements made in the field of bat immunology, and future research may further foster this progression.

Characterization Of Bat Immune Genes and Antibodies

Bat cytokine genes

As was previously discussed, the examination of immune genes and related transcription factors has helped researchers gain information about how the immune systems of other mammals behave during infection [17]. Examination of bat immune genes and transcription factors may help identify possible components of bat immune systems that allow for viral persistence. Methods used to characterize the immune system of deer mice and the virus-host interaction that occurs between deer mice and SNV have been modified to examine the immune systems of bats [80]. Several cytokine genes from Seba's short-tailed fruit bat have been cloned and characterized using these methods (Unpublished results). The methods and results of these studies will be discussed in chapter three of this dissertation.

Bat immunoglobulin genes

Other studies have focused on the cloning and sequencing of bat immunoglobulin (Ig) genes [118-120]. It was found that bat Ig genes are highly conserved when compared to other mammalian Ig gene sequences [119]. Although no significant sequence differences were identified, two studies have found that bat Ig genes have a surprising amount of sequence diversity in the variable heavy chain repertoire [118, 119]. It was suggested that this increased diversity in the variable heavy region of bat Ig may be associated with the ability of bats to act as reservoirs of viruses. It was also suggested that coevolutionary relationships between bats and viruses could be a reason for this diversity [118].

Bat cluster of differentiation 4

The sequence of bat cluster of differentiation 4 (CD4) T cell receptors has also been examined using PCR and sequencing analysis [120]. Spleen samples were

collected from Egyptian fruit bats (*Rousettus aegyptiacus*) and total RNA was extracted. cDNA synthesis was followed by PCR using CD4 specific primers. Amplified PCR products were then sequenced. Bat CD4 sequence alignment with human, monkey, mouse, cat, dog, rabbit, and pig CD4 sequences indicated that bat CD4 is conserved in relation to other mammalian CD4. A few differences were identified between the bat CD4 and human, monkey, and mouse CD4 sequences. These differences included a lack of a cysteine residue, along with an 18 amino acid long insertion in the bat sequence. The cysteine residue is involved in disulfide bond formation in the CD4 receptor. The lack of this cysteine residue could possibly interfere with the structure and function of the CD4 receptor, but the authors did not consider these differences to be of great importance because cat, dog, and pig CD4 sequences also lack the same cysteine residue. The overall conclusion was that bat CD4 is highly similar to other mammalian CD4 [120].

Bat signal transducers and activators of transcription

STAT1 (signal transducers and activators of transcription factor-1) is a transcription factor involved in signaling events associated with the type I IFN response. One study examined STAT1 of the Egyptian fruit bat using cloning and sequencing techniques. The activity of bat STAT1 was also investigated in cell culture using immunofluorescence staining and confocal microscopy [121]. Results from these studies indicate that the sequence of bat STAT1 is conserved with respect to other mammalian STAT1 sequences, and that the function of bat STAT1 also appears to be the same as other mammalian STAT1 [121].

The results from cloning and sequencing projects indicate that bats have immune genes and related transcription factors that are very similar to other mammals. As in the case of other virus-host interactions, including the deer mouse and SNV, these results

have led researchers to suspect that the differences between bat and human infections with many viruses may lie in the regulation of immune genes during infection. Some researchers have focused attention on cell culture and experimental infections to investigate this possibility.

Bat interferon and primary cell culture

Some studies have examined the expression of bat interferon (IFN) genes in cell culture. In these studies, researchers investigated bat IFN-α and IFN-β gene expression from primary kidney and lung cells of the Egyptian fruit bat [122]. The authors were interested in examining any differences in interferon expression or antiviral behavior between bats and humans. Genomic DNA was extracted from fresh bat liver samples and was used in the initial sequencing of the bat IFN-α and IFN-β genes. The authors then examined expression of IFN mRNA from bat primary kidney and lung cells using polyinosinic:polycytidylic acid (poly I:C) treatment to induce IFN expression from the cells. Poly I:C is known to interact with toll-like receptor (TLR) 3 and induce signal transduction events in response to antigens. Toll-like receptors are an important part of activating the innate immune system. Supernatant containing bat type I IFN was collected and placed into primary kidney and lung cell cultures to examine expression of interferon in response to treatment with bat type I IFN. It was found that bat primary kidney and lung cells behaved differently in response to the treatment with bat type I IFN media. It appears that up-regulation of type I IFN will induce bat kidney cells to produce more IFN, but this was not seen in the bat lung cells. The authors believe that characterization of bat antiviral mechanisms is important in determining how some animals, including bats, can act as asymptomatic carriers of infection. [122]

Methods used to examine filovirus infection using a line of primary bat cells has recently been developed [123]. The cell line R06E was developed from fetal Egyptian

fruit bat cells [124]. These fruit bats have recently identified as a reservoir of Marburg virus. In this study, the R06E cell line was examined for the potential to be used in experiments with Marburg virus. The researchers found that the R06E cell line was highly susceptible to filovirus infection and they believe that use of these cells will be beneficial in gaining information about how filoviruses interact with the natural reservoir. This study suggests that the development of cell lines from bat species may be useful in examining the virus-host interaction *in vitro* [123].

Experimental Infections

As was previously mentioned, experimental infections can also provide important information about virus-host interactions. Experimental infections have been employed for many viruses and the host species, and are also being used to examine the interaction between bats and viruses that have been detected in or isolated from them. A few examples include experimental infections with rabies, henipaviruses, and filoviruses [125-127]. These studies will be discussed in later sections of this review.

Future methods

Other methods for characterizing bat immune systems and immune responses that will require more work to develop include bone marrow and T cell culture, and the development of better diagnostic techniques. Bone marrow cell and T cell culture parameters have been established in non-model rodents including the deer mouse [18, 83]. It is possible these methods could be adapted and used to characterize the immune responses of bats. The need for continued development of immunologic methods in bats is reflected in the number of viruses that have been isolated from them.

Bats As Reservoirs

Rabies and related lyssaviruses

Historically, lyssaviruses, such as rabies, are commonly found in bat species throughout the world. Rabies and rabies-like lyssaviruses have been the most studied

bat viruses to date [128, 129]. Not only have lyssaviruses been identified in bats, but there have also been documented spillover infections in other mammal species such as foxes (*Vulpes vulpes*), skunks (*Mephitis mephitis*), martens (*Martes foina*) and even humans. Susceptible species include almost all warm-blooded animals [99, 103, 104, 130].

Rabies and related lyssaviruses are in the family *Rhabdoviridae* and the genus *Lyssavirus.* In this genus, there are seven known genotypes including rabies virus, Australian bat lyssavirus, European bat lyssavirus 1, European bat lyssavirus 2, Duvenhage virus, Lagos bat virus, and Mokola virus [5, 98, 131]. Bats are important vectors for lyssaviruses with six out of the seven known genotypes having been documented various species of bats [5]. Rabies virus has a worldwide distribution and is found in a wide range of wild and domestic mammalian species including bats [130, 131]. Australian bat lyssavirus was found in fruit bats and was identified after causing human cases of encephalitis [132]. European bat lyssaviruses 1 and 2 have been found in insectivorous species of bats as well as other small wild mammals [99, 103, 104, 131]. Lagos bat virus has been found in cats, dogs, and shrews, as well as bats [131]. Mokola virus was first isolated out of as species of shrew, and has been found in several cases of domestic animal infections [131].

Human infections with rabies can usually be traced to physical contact, such as a bite, from an infected bat or other mammal [133]. Cases of exposure where no contact was experienced are very rare [134]. Natural infection with rabies causes death in the majority of human infections, but rabies-specific neutralizing antibodies have been detected in seemingly healthy bats [3, 56]. However, rabies and related lyssaviruses are known to cause mortality in bat populations [5]. The mechanisms by which some bats develop disease while others do not are unknown. The identification of bats as reservoirs of these viruses, and the inconsistency in the outcome of bat infections with

them, have inspired some researchers to screen bat populations to identify infected individuals that may not be exhibiting symptoms. This type of approach has led to the discovery of lyssavirus infection of bats in restaurants in Cambodia [12]. Experimental infections of bats with rabies and related lyssaviruses have also been conducted to examine the role of bats as reservoirs.

Big brown bats (*Eptesicus fuscus*) have been experimentally infected with Eurasian bat lyssavirus strains to determine viral pathogenesis and tissue tropism of the virus [127]. The authors of this study found that lyssavirus strains show tissue tropism mainly in neuronal tissues with some virus being shed in the salivary glands up to 5 days prior the onset of symptoms. The bats also showed no virus transmission to other uninfected cage mates [127]. Another study used rabies virus variants to experimentally infect Jamaican fruit bats to define disease progression and tissue tropism. Bats were inoculated with different strains of rabies virus and monitored for symptoms. Moribund bats were euthanized and tissue samples were examined using immunoperoxidase-staining techniques. The authors found that disease progression in bats infected with different virus variants was very similar in most cases [133].

Researchers have also performed experimental infections with rabies virus in bats to examine the ability of bats to either develop disease, or clear the virus [56]. In one study, big brown bats were experimentally infected three times. After each round of infections, bats either cleared the virus and survived, or developed disease and died. Bats that cleared the virus after each infection were re-infected. The results indicated that repeated exposure and infection with rabies virus in bat populations might contribute to a reduced susceptibility to rabies. It was also suggested that the dose of virus that bats are exposed to might play a role in the outcome of infection with lower dose exposure possibly acting to immunize populations [56].

While rabies and related lyssaviruses have been known as bat viruses for many years, other viruses have been discovered in bats only more recently. Due to these discoveries, studies with these viruses in bats are relatively new.

Henipaviruses

Hendra and Nipah viruses are in the family *Paramyxoviridae* and the genus *Henipavirus*. They are closely related and cause severe febrile encephalitis in humans with a high mortality rate [135]. An outbreak of Hendra virus was first identified in 1994 in Hendra, Australia when 21 horses and two humans began showing symptoms of severe respiratory disease and fever with a high fatality rate [136]. Upon first examination, the virus was named equine morbillivirus, but was later named Hendra virus. Nipah virus, was identified in Malaysia in late 1998 when domestic pigs and eventually humans developed neurologic disease and fever [94]. At the time of the first outbreaks of these viruses, the natural reservoir and route of exposure was unknown. Sequencing studies provided viral classification and subsequent serological surveys that followed identified the reservoir species of these viruses [94, 136].

After the initial outbreak of Hendra virus in 1994, researchers set out to identify the natural reservoir of the virus. It was hypothesized the virus was hosted by a wild mammal species and, thus, trapping programs were initiated. Serum samples from many species were screened for the presence of antibody specific to Hendra virus, and eventually several seropositive species of Australian flying foxes (genus *Pteropus*) were identified [95]. Virus isolation was accomplished on RK13 cell cultures, and sequencing confirmed Hendra virus infection. IFA staining showed that cell cultures were infected with Hendra virus, and serology detected Hendra virus-specific antibody in serum samples. These results indicated that certain species of flying foxes found in Australia are the natural reservoir for Hendra virus [57]. Experimental Hendra virus infections were performed on species of pteropid bats and results showed that bats became

infected with the virus, and seroconverted. Histopathology of collected organ samples showed limited pathology in bat tissues, and positive immunostaining of placental tissue indicated that horizontal transmission of Hendra virus in bats is possible [137].

After the initial outbreak of Nipah virus in 1998, and its identification as a paramyxovirus similar to Hendra, researchers began to search for the natural reservoir [94]. Because Hendra virus had been isolated from species of pteropid bats, investigators focused more attention on species of bats found in Malaysia. Serologic studies sampled many species found in the area of the outbreak, and in five species of bats Nipah-specific neutralizing antibodies were detected [108]. Isolation of Nipah virus from urine samples and partially eaten fruit was performed using Vero cells, and confirmed using IFA and virus sequencing. Vero cells, which are African green monkey kidney epithelial cells, are typically used for virus isolation studies because they have a disrupted type I IFN response making virus propagation easier. The results from the Nipah virus study indicated that island flying foxes are one of the natural hosts of Nipah virus [10].

Another 2002 study surveyed many species of Cambodian bats and found only flying foxes to test seropositive for Nipah-specific antibody by ELISA. Serum and urine samples were collected and used for ELISA and for virus isolation which was successful in Vero E6 cells [100]. Vero E6 cells are a closely related lineage of Vero cells. In the case of Hendra and Nipah viruses, pteropid species of fruit bats have been identified as the natural reservoir. More recently, experimental infections of pteropid bats with Nipah virus were performed. Similar to the results of experimental Hendra virus infections, it was found that the bats seroconverted, developed subclinical infection, and were intermittently shedding virus in urine. These results suggest that pteropid bats are reservoirs of Nipah virus [102].

Coronaviruses

Coronaviruses can cause respiratory and gastrointestinal diseases in humans and other mammals. Severe acute respiratory syndrome coronavirus (SARS CoV) emerged in 2002 in China [138]. The reservoir of the virus was unknown when the outbreak of SARS first occurred. However, palm civets (*Paguma larvata*) and other animals sold in live animal markets were implicated as reservoirs [8, 138]. Surveillance studies on wildlife populations in China eventually led to the discovery of SARS CoV-like coronaviruses in bats [7, 8, 139, 140]. A SARS CoV-like virus was discovered in Chinese horseshoe bats during a study in 2004 and 2005 [7]. Several species of animals were captured during this study, and blood, anal swabs, and nasopharyngeal swabs were collected. Samples were tested for coronavirus RNA using PCR and sequencing. Three different coronaviruses were identified. With this discovery found only in bats, the authors suggested that bats with roosting behavior be closely monitored for transmission of coronavirus-like disease into human populations [7]. Studies surveying for the presence of SARS CoV-like viruses have also been performed in North America [9], South America [141], northern Europe [142], and the Philippines [126]. Each of these studies was successful in the detection of coronaviruses in bat species.

Filoviruses

Ebolaviruses and Marburg virus belong to the family *Filoviridae* and can cause outbreaks of hemorrhagic fevers in Africa. [15]. The source of outbreaks of Ebolaviruses and Marburg virus were previously unknown. Following an outbreak of Ebola Zaire in 1995, researchers performed experimental infections of plants and animals with Ebola Zaire virus. It was found that species of insectivorous bats and fruit-eating bats developed high titer viremia upon infection.

While this did not provide evidence that bats could be possible reservoirs of filoviruses, it did show susceptibility [143]. More recent serologic evidence has suggested that ebolaviruses and Marburg virus may be hosted by species of fruit bats [14, 59].

A study performed between 2001 and 2005 tested many species of mammals for ebolavirus infection [14]. The authors of this study were able to detect ebolavirus-specific IgG in the serum of three bat species. Viral RNA was also detected in organ samples using viral gene amplification by PCR. Each species of bat that was determined to be positive for ebolavirus infection has a home range that spans the areas where previous ebolavirus outbreaks (in human populations) have been encountered in Africa. These results suggested that bats are a candidate for the natural reservoir of ebolaviruses [14].

Another study tested bats in the rainforests of Angola for Marburg virus [59]. The authors believed that an outbreak of Marburg virus in Angola in 2005 came from bats living in the rainforests of Gabon. Tissue samples were collected from 10 bat species and examined for viral RNA using PCR. Serum samples were also collected and tested for antibody to Marburg virus. Of the 10 species examined, only one species was PCR positive for Marburg infection. These results suggested that the Egyptian fruit bat (*Rousettus aegyptiacus*) may be the natural reservoir of the virus and that filoviruses may exhibit host species specificity [59].

Other studies have been successful in identifying that Marburg and ebolaviruses both circulate in populations of Egyptian fruit bats in Gabon [101]. In a study performed in 2009, isolation of Marburg virus was accomplished from Egyptian fruit bats on several occasions. Results of this study indicated that long-term virus circulation occurs in fruit bat populations [107]. A similar study performed in 2010, showed long-term survival of a tree-roosting fruit bat (*Eidolon helvum*) that had been naturally infected with an

ebolavirus and Lagos Bat virus. These data indicated that this bat species may also act as a reservoir for these viruses [144]. As was previously discussed, a close coevolutionary relationship may account for the lack of disease progression in bats with filoviruses [96].

Flaviviruses

West Nile virus (WNV), widely know as a bird virus, can cause serious disease in some mammals. WNV-specific antibodies were identified in bats in 1999 in the U.S [145]. Experimental infections with WNV had previously been limited to birds [146], but a study in 2005 examined the ability of North American bats to serve as amplifying hosts for WNV [106]. The authors also examined infected bats for clinical signs of disease. Serum samples were collected from wild species and tested using a blocking enzyme immunoassay to assess natural infection rate. Experimental infections were performed at BSL3 using bats that were captured in the field. Bats were observed for symptoms of disease and were euthanized on days 1 through 6 and day 14 and 40 post infection. Serum samples were collected and tested for neutralizing antibodies using plaque-reduction neutralization tests. Seven big brown bats showed viremia during the experiment while other species showed no signs of infection. Oral swabs showed no viral RNA and virus isolation was unsuccessful. The authors of this study concluded that North American species of bats only developed low-level viremia for a short period of time. While these species can become infected with WNV, they are unlikely amplifying hosts of the virus [106].

A more recent discovery was the detection of a novel flavivirus in Old World fruit bats [112]. The authors of this study began a wide survey of bat species in attempt to identify known or possibly unknown microorganisms. Serum samples were collected from several Indian flying foxes (*Pteropus giganteus*) in Bangladesh, and screened using pyrosequencing technology. The authors were able to identify a novel Flavivirus, GBV-

D. The abbreviation GB came from the initials of a surgeon who was acutely infected with hepatitis. Experimental inoculations of tamarins (*Saguinus abiatus*) with serum from the surgeon led to hepatitis [147, 148]. In the 1990s, Flaviviruses GBV-A and GBV-B were identified from infected tamarins after several virus passages [147, 148]. GBV-D virus was the first GBV-like virus to be detected in species of bats [112].

Astroviruses

Astroviruses can cause gastroenteritis in many species of mammals including humans. Recently, astroviruses have been detected in species of bats in China [114]. During a study in Hong Kong, nine species of bats were sampled on two occasions and tested for presence of astrovirus. The first sampling took place in different wild habitats in Hong Kong, and the second sampling took place in a cave roost. Bats were captured, identified, and oral and rectal swabs were collected in viral transport medium. Viral RNA was extracted from the swabs and PCR was performed using astrovirus specific primers. Cloning and sequencing allowed the authors to perform a phylogenetic analysis of the astroviruses obtained from the swabs. It was found that 46% of collected rectal swabs were positive for astrovirus and 8% of the oral swabs were positive. Out of the nine species of bats examined, astroviruses were detected in seven. Phylogenetic analysis of the bat astroviruses showed that 72 out of 77 bat astrovirus genes cluster together. This discovery denoted a novel group of astroviruses. The authors suggest that mark and recapture studies be used to examine the possibility of viral persistence [114].

Herpesviruses

Herpesviruses are a group of DNA viruses that can cause latent infections in humans and other mammals. Examples include cytomegalovirus (CMV), Epstein-Barr virus (EBV), and varicella zoster virus (VZV). CMV is classified as a betaherpesvirus, EBV is classified as a gammaherpesvirus, and VZV is classified as an alphaherpesvirus.

The first discovery of a bat-related herpesvirus was in 1996 when CMV was

encountered in the submandibular glands of two little brown bats (*Myotis lucifugus*) [149]. Tissue sections of little brown bat salivary glands were being examined by electron microscopy when CMV particles were discovered. This was the fourth mammalian order to be discovered with CMV in the salivary glands [149]. Other studies have utilized PCR to survey species of bats for presence of herpesviruses.

In 2007, seven gammaherpesviruses and one betaherpesvirus were discovered in eight bat species in Germany using a herpesvirus PCR assay. Healthy, moribund, and dead bats were collected and used in the study. Lung tissues and other organ samples were collected and examined using histopathology and PCR. Virus was detected using primers that were specific for herpesvirus DNA polymerase. Sequence alignments and phylogenetic analyses showed that the newly discovered bat herpesviruses were related to known gammaherpesviruses, but are novel [150].

Due to the discovery of novel herpesviruses in bats, other projects were initiated to screen additional species of bats for presence of herpesviruses. Alphaherpesviruses have been detected in phyllostomid and pteropid bats in Cambodia and Madagascar [151]. Throat swabs were collected from several species of bats and herpesviruses were detected using PCR and sequencing analysis. This study was the first to identify alphaherpesviruses in bats, as well as herpesviruses in bats of the Pteropodidae and Phyllostomidae families [151].

Novel gammaherpesviruses and novel betaherpesviruses have since been discovered in bats the Philippines and Japan using direct sequencing technology and a rapid determination system of viral RNA sequences [152, 153]. Bats are now considered natural reservoirs of herpesviruses [151].

Other bat viruses

Three viruses have been detected in Seba's short-tailed fruit bats. These include an unspecified betaretrovirus, Venezuelan equine encephalitis virus (VEEV) and a group

1 coronavirus [141, 154, 155]. It is unknown if the short-tailed fruit bat is a reservoir for these viruses, or if infection is incidental. VEEV was first isolated in Venezuela during an equine outbreak in the 1930s [155]. VEEV has the ability to cause severe symptoms in horses and humans. VEEV-specific antibodies had previously been discovered in *Artibeus* bats in Guatemala [156]. After this discovery, studies were conducted to examine natural and experimental infections of several species of Neotropical bats with VEEV variants. It was found that a species of *Carollia* bats (*C. subrufa*) developed high titer viremia with no apparent pathology [155, 157, 158]. This, again, suggests that bats may become persistently infected with virus and serve as a maintenance host.

Bacteria And Other Microbes

While bats are reservoirs or potential reservoirs for many viruses, they are also known to interact with other types of microbes. In some cases they become acutely infected or may act as reservoirs of bacterial and fungal microorganisms.

Leptospirosis

Leptospira is a urine-borne bacterial pathogen that has the ability to cause leptospirosis in humans and other mammals. Complications from this disease include an enlarged spleen, jaundice and inflammation of the kidneys. A serological study of Australian flying foxes was conducted to identify any exposure of these bats to leptospiral species of bacteria [159]. This survey of leptospiral infection in pteropid bats was prompted by an increase in the number of reported human infections. Serologic results from this study found that not only are flying foxes sufficient hosts for Leptospira, they may play an important roll in the establishment of new serotypes of bacteria, as well as the natural transmission cycle of this bacteria in Australia [159].

Histoplasmosis

Histoplasmosis is a fungal disease caused by *Histoplasma capsulatum*. It has the ability to cause the human illness known as acute pulmonary histoplasmosis [160].

Histoplasmosis is often associated with bats due to the contraction of the disease after visiting caves [161, 162]. This fungus has been isolated from samples of soil as well as bat guano in caves and rural work places [163]. Presence of *Histoplasma capsulatum* was also detected in the intestinal tract of a Hairy slit-faced bat (*Nycteris hispida*) [163]. Although the natural reservoir for this fungus is not known these isolations indicate that bats do interact with this fungus and could be aiding in the transmission of this disease [161-163].

White nose syndrome

White-nose syndrome (WNS) is a fungal disease that is negatively affecting some bat populations. The fungus *Geomyces destructans* is thought to cause WNS. The fungus grows in the epidermis and dermis of the nose, ears, and wing membranes of hibernating bats [53-55]. Hibernating bats infected with the fungus present with an accumulation of white fungal growth on the muzzles and wing membranes, but there is no inflammation associated with the infection [53, 55]. Examination of bat tissues shows that this fungus penetrates hair follicles and sebaceous glands and causes serious damage to the wing membranes [55, 164]. Infected bats have been observed using fat reserves at a much faster rate and coming out of hibernation in the middle of winter [53, 54, 165]. Researchers have placed the fungus in the *Geomyces* genus using gene sequencing and phylogenetic methods [54]. Isolation of this fungus from collected bat tissues has been accomplished on dextrose agar. Laboratory propagation and investigation of the fungus indicates that it grows best at low temperatures such as those that may be found in hibernation chambers of bats. It has been suggested that bats may also have the disadvantage of experiencing decreased immune activity during hibernation [54].

WNS was first discovered in 2006 in New York State and has since caused the death of many thousands of bats [165]. White nose syndrome has been observed to

spread quickly and has now been detected in bat populations in other northeastern areas of the U.S. and Canada [165]. White nose syndrome is clearly a pathogen to bats and if the spread of this disease is not stopped, the effects could be catastrophic for some bat populations. As previously discussed, bats play an important role in the ecosystems in which they live, and severe declines in bat populations could cause dramatic changes in the ecosystem [53, 165]. More research is needed to characterize the interaction between bats and this fungus, and to identify possible ways to avoid devastation of even more bat populations.

Arenaviruses

While it is not surprising that bats may be the reservoir of some viruses, it is intriguing that an arenavirus, Tacaribe virus (TCRV), has only been isolated from bats [33]. This is because arenaviruses, for which the hosts are known, are rodent-borne viruses. Isolation of TCRV from *Artibeus* species of bats has led many to believe that these bats are the natural reservoir. If this were confirmed, TCRV would be the only arenavirus to have a non-rodent host.

Arenaviruses belong to the family *Arenaviridae* and the genus *Arenavirus.* The name comes from the Latin word *arena*, which means “sand” or “grains of sand.” This refers to the appearance of ribosomes inside of arenavirus virions [166]. Arenaviruses are spherical in shape, enveloped and have a single-strand, ambisense RNA genome [167]. Arenaviruses have two gene segments, the large (L) segment and the small (s) segment. The large gene segment encodes an RNA-dependent RNA polymerase and a zinc-binding protein called RING finger protein, Z [35]. The small segment encodes the nucleocapsid protein and a glycoprotein precursor [35, 168, 169].

Arenaviruses are assigned to two categories, the Lassa virus complex (Old World), and the Tacaribe virus complex (New World). The Lassa virus complex includes Lassa virus, found in Africa, which is the etiologic agent of Lassa fever in humans,

Lymphocytic Choriomeningitis virus, which can cause aseptic meningitis in humans, and Lujo virus, which causes hemorrhagic fever in humans [35, 37, 75, 84, 170]. The Tacaribe virus complex includes Junín, Machupo, Sabia, Guanarito, and Chapare viruses, which cause Argentine hemorrhagic fever (HF), Bolivian HF, Brazilian HF, Venezuelan HF, and an as yet unnamed hemorrhagic fever respectively [35-38]. Some of these viruses are endemic to the area of the world after which they were named [171]. TCRV is also included in this group, but is unique in the sense that it is not known to cause natural human infection. There have been several laboratory-acquired infections reported, which only caused mild febrile illnesses. Also, out of many serologic tests performed on bat handlers in Trinidad, only one tested positive for neutralizing antibody to TCRV [34].

Arenaviruses can establish persistent infections in cultures of mammalian cells as well as rodent hosts [40, 67, 71, 77, 172]. Many arenaviruses can infect mammalian cell culture with little to no noticeable cytopathic effect [173]. It is largely unknown how persistence is established in cell culture or in the mammalian hosts, but is has been suggested that the presence of interfering particles (IP) that are released by the host cells are involved in establishing persistent infections. IP are viral particles that are missing some or all of the viral genome [77, 174]. The nucleoprotein of many arenaviruses is antigenic, and also acts as an antagonist of the type I interferon response [74]. The exception to this is that TCRV does not inhibit activation of beta interferon or IRF 3 in infected cells [74]. It has also been suggested that macrophages may be the target for virus replication for some arenaviruses such as Junín virus. Virus replication was greatly slowed in macrophages of mice that had been previously vaccinated against Junín virus [173]. These aspects could contribute to establishment of persistent infections.

Isolation of Tacaribe virus

In the early 1960s, Tacaribe virus was isolated from two species of *Artibeus* bats (*A. jamaicensis* and *A. lituratus*), during a rabies surveillance program in Port of Spain, Trinidad [33]. Research was conducted at the Trinidad Regional Virus Laboratory (TRVL). The project examined diseased bats that had been brought in by residents of the area, as well as bats that were netted in different areas around the island of Trinidad.

Jamaican fruit bats are in the suborder Microchiroptera and the family *Phyllostomidae*. These bats are found in tropical regions of South America, the Caribbean Islands, the Florida Keys, and Central America. On average, adult Jamaican fruit bats weigh 42 gm and have a wingspan of 45 cm. Average body length is 10 cm [175]. Jamaican fruit bats are fruit-eating generalists and fig specialists [49, 175]. They exhibit harem-breeding behavior and females typically produce one or two offspring per year. Gestation is approximately 150 days and young bats are carried by the mother for about 40 days after birth [175].

During research with TCRV, virus isolation was successful using the original collected bat tissues that had been homogenized and injected into suckling mice. The strain of TCRV that was isolated from bats is TRVL-11573 from *A lituratus*. Bats naturally infected with TCRV were described as showing similar symptoms as bats infected with rabies virus. Symptoms included perching in odd locations or solitarily, tremors, and unkempt coats [33]. It was recorded at the time that TCRV was also isolated from a mosquito pool. This isolation was the only one of its kind and the authors have considered the possibility that the samples may have been contaminated. TCRV has not been isolated from other mosquito pools. In an attempt to identify other mammals that might be infected with TCRV, over 2300 native and migratory birds and 2000 native mammals (mainly rodents) were tested for TCRV antibody. None were seropositive, and virus isolation was not accomplished [33].

Downs et al. performed experimental infections [33] using intramuscular injections of TCRV strain 11573 to examine the susceptibility of *Artibeus* bats to TCRV. Weanling mouse intracranial LD_{50} was found to be 10^{-2} per 0.03 mL. One group of *A. jamaicensis* and *A. lituratus* were experimentally infected with 828 weanling mouse LD_{50} and a second group received a virus inoculum of 10,000 weanling mouse LD_{50} [33]. These experimental infections led to inconclusive results. A total of 33 wild-caught Jamaican fruit bats were inoculated, and only one bat in the experiment showed post-inoculation immunity to TCRV. From the organs collected from that bat, virus was recovered from the salivary glands, spleen and liver. One weakness of this experiment was that the authors did not control the experiment by pre-infection bleeds. This could mean that the one bat that showed viral circulation could have already been infected in the wild before it was captured. Due to this error, there is no way to determine if the experimental infections were successful, or if the low incidence of infection was due to the low dose that was administered through the muscle.

Between 1972 and 1974, serum samples were collected from 39 different species of bats in Trinidad to identify viruses potentially carried by bats [176]. Antibodies specific for TCRV were detected in Jamaican fruit bats using mouse neutralization tests. Unfortunately, virus was not isolated and thus was not useful in determining current virus activity [34].

Experimental infections with Tacaribe virus have been performed with mice and guinea pigs, but no further bat studies had been performed since the initial isolation [42, 177]. It has been found that TCRV is lethal to newborn mice causing paralysis and necrosis in the central nervous system. In adult mice, TCRV replication was only observed in small amounts and was avirulent causing no notable pathogenesis [42].

TCRV has also been examined as a possible vaccine for Argentine hemorrhagic fever [166]. TCRV is not known to cause serious disease in adult laboratory animals,

and is thought to be unable to cause disease due to the inability of TCRV to antagonize the type I IFN pathway [74]. Due to TCRV having less virulent qualities in many laboratory animals and the fact that it is closely related to Junín virus (the etiologic agent of Argentine hemorrhagic fever), TCRV was chosen as a possible vaccine candidate [166, 178]. In this study, guinea pigs were experimentally infected with TCRV, and subsequently challenged with Junín virus. The authors examined the rodents for viral presence and tissue damage and found that no virus or antigen from Junín virus could be detected using immunofluorescence at any stage of infection. No damage was detected in the tissues that were collected. TCRV viral titers were found to peak between 7 to 10 days post infection and become undetectable around day 30-post infection. Neutralizing antibodies that were cross protective to Junín virus were produced. These results indicated that TCRV could possibly be used as a vaccine against Junín virus or other dangerous arenaviruses [166].

That TCRV can be manipulated at BSL-2 and the ease with which it can be propagated in Vero E6 cell culture makes it an attractive candidate as a New World arenavirus model [41, 77]. TCRV has been found to be nonpathogenic in many mammals, possibly due to its inability to antagonize the type I interferon response [74]. However, TCRV is closely related to viruses that cause South American hemorrhagic fevers [38, 41]. It shares 70% amino acid identity to Junín virus [178] and while it is not known to infect humans, or cause disease in many laboratory animals, more recent findings indicate that it may offer the possibility for the development of a disease model and the chance to identify a potential treatment for SAHF [41].

TCRV is also interesting because it is the only arenavirus that has been suggested to have a non-rodent reservoir. This raises the question of whether TCRV is truly a bat virus or if there is some unknown rodent-reservoir. In any case, TCRV is important as a representative of a group of viruses that cause serious human

hemorrhagic illnesses, and as a model which could possibly be used to study virus-host interactions of persistently infected mammals [2, 171]. Much more research about the virus and its interactions with potential host organisms is needed before we can fully understand it.

Detection of arenavirus infection

Even though Tacaribe virus is not known to cause serious human illness, it is closely related to other viruses that do [178]. The serious nature of SAHFs makes early detection of arenavirus infection important in saving human lives. Tests for detection of arenaviruses can help in the screening process of diseased humans as well as the screening of rodent populations. In the past, diagnosis of an arenavirus infection depended on the geographic location of the case, and the type of symptoms that were experienced by the individuals [171]. Other, more specific diagnostic tools have been developed to detect these infections.

There is currently a high-throughput real-time PCR assay that can be used for detection and quantitation of TCRV [171]. Although this assay is specific for TCRV, the authors believe that it can be used as a diagnostic model for other arenaviruses. This is more sensitive than the $TCID_{50}$ method of detection that is commonly used for arenaviruses. The RT-PCR assay can be performed in approximately 2 hours which gives another advantage of possibly diagnosing the patient before symptoms start to manifest [171]. One problem with this assay is that it detects any viral RNA, whether the virus is infectious or inactive.

Other diagnostic tools, such as sandwich ELISAs, have been developed for many arenaviruses including TCRV [179]. These assays are used to detect viral antigen in a sample rather than antibody to specific viruses. Indirect ELISAs, which screen for the presence of virus-specific antibodies in serum samples, have been developed for several arenaviruses, but many require the use of whole virus as an antigen and the

production of species specific secondary antibodies [180, 181]. Immunoassays can provide a faster way to monitor past and current infections in mammalian populations and diagnose infection in humans. However, many of these assays may still require 3 to 5 hours to complete and the transport of serum samples back to the laboratory [16, 182]. The amount of time required to perform these tests and the need for species-specific secondary antibodies are typically not conducive to rapid screening projects in the field. Modification of enzyme immunoassays to decrease the amount of time required to complete the assay has been shown to be effective [16, 110]. The development of a rapid immunoassay for TCRV might be advantageous in screening wild and experimentally infected animals.

While there has been and currently is progress in our understanding of bat immunology, there is still a great need for advancements of screening techniques and methods that can be used to characterize the immune systems and immune responses of bats. Many microorganisms that have been detected in or isolated from bats have been discussed in this review. However, it is likely that many other microorganisms, less well known, or even unknown, have not been discussed. The research project presented in the following chapters of this dissertation aims to address several aspects of bat immunology and virus-host interactions.

CHAPTER III

MOLECULAR AND PHYLOGENETIC CHARACTERIZATION OF CYTOKINE GENES FROM SEBA'S SHORT-TAILED FRUIT BAT (*CAROLLIA PERSPICILLATA*)

Abstract

Bats (order Chiroptera) represent nearly one fifth of all known species of mammals, yet many aspects of bat biology are poorly understood. Bats of certain species are well known reservoirs of rabies virus but within the past few years bats also have been identified as reservoirs or potential reservoirs of several other important human and livestock pathogens. Among these are severe acute respiratory syndrome coronavirus, Nipah and Hendra paramyxoviruses, several lyssaviruses, and both Marburg virus and ebolaviruses (filoviruses). In some instances, filoviruses may cause persistent infection without recognized pathology in the bat host. Even though these are medically important viruses, little is known as to how bat immune systems engage them or how they might evade a sterilizing host immune response. Recently, a project was initiated to research the immune systems of Seba's short-tailed fruit bats, which may be a reservoir for a newly discovered coronavirus. cDNAs of four cytokine genes; tumor necrosis factor (TNF), interleukin-10 (IL-10), interleukin-23a, (IL-23a), and granulocyte macrophage stimulating factor (GM-CSF) were cloned and sequenced. Sequence analysis shows these genes are highly conserved with regard to orthologous sequences and they provide some value for resolving phylogenetic relationships between mammals.

This work represents a first step in developing an infection and immunology model for a New World arenavirus in bats.

Introduction

In recent years, several zoonotic viral disease agents have been isolated from or detected in bats [2]. For some viruses, bats are known reservoir hosts, and for others they are suspected reservoirs or incidental hosts. Hendra virus, hosted by pteropid bats [57], was first identified from cases of fatal equine and human respiratory disease in Australia [183]. Nipah virus, also hosted by pteropid bats [184], has been associated with multiple outbreaks of human disease in Cambodia, Malaysia, India and Bangladesh, with case-fatality rates as high as 70% [100]. Lyssaviruses, including rabies virus, have been detected in bats throughout the world, except in Antarctica [131]. Severe acute respiratory syndrome coronavirus (SARS-CoV)-like viruses have been detected in Chinese horseshoe bats [7], and it is thought SARS-CoV is a direct descendent of these bat-borne viruses. Recent serological and molecular evidence suggests that ebolaviruses and Marburg virus are hosted by fruit bats in Africa [14]. While some infected reservoir host bats may show pathology, others appear to be asymptomatic [6]. Currently, more than 90 viruses have been identified from or detected in bat tissues, suggesting that bats are significantly underappreciated as hosts and vectors for human pathogens. Studies evaluating immune competency of bats are only now beginning to be conducted [118, 119, 121, 185, 186] and the emergence of cost-effective transcriptome sequencing should permit rapid advances in developing tools for assessing bat immune responses.

Three viruses have been detected in Seba's short-tailed fruit bats; an unspecified betaretrovirus, Venezuelan equine encephalitis virus (VEEV) and a group 1 coronavirus [141, 154, 155]. It is unknown whether bats of this species are reservoirs of these viruses or whether infections were incidental. Because these bats range from central

Mexico to southern Brazil [187], it is important to determine their possible role in maintenance and dissemination of these and other viruses. For this reason, we have begun a project to determine susceptibility of Seba's short tailed fruits bats to certain viruses.

Since the role of the host response is important in resistance and susceptibility, it will be necessary to develop methods for evaluating immune responses in the species. It is likely that some bats may become persistently infected without disease and that the viruses have evolved immune evasion strategies. Cloning of partial sequences of four cytokine genes from Seba's short-tailed fruit bat: tumor necrosis factor (TNF), interleukin-10 (IL-10), IL-23a, and granulocyte-macrophage colony stimulating factor (GM-CSF) has been accomplished. Molecular and phylogenetic analyses of the sequences are presented here.

Materials And Methods

Bats

The University of Northern Colorado (UNC) bat colony comprises neotropical fruit bats of two species, Seba's short-tailed fruit bats and Jamaican fruit bats. These are maintained together in a 36 m^3 room that allows unrestrained free flight of all individuals. The colony has been closed for 16 years and is rabies virus-free; PCR screening of rectal swab samples from 20 of these bats has failed to detect coronaviruses (K. Holmes, pers. comm.). The bats have access to roosting areas in the form of ceiling-hung baskets and various cloth drapes and are maintained under a light-cycle of 12L:12D. Ambient temperature is maintained between 20 and 25°C and humidity between 50% and 70% by a computer-controlled HVAC system. Bats are fed daily at midday with grueled apples, monkey chow (Harlan Teklad, Denver, CO), molasses, nonfat dry milk, cherry gelatin with raisins, and fresh bananas. In addition, a variety of other fruits, including mango, papaya, cantaloupe, banana, grapes, and watermelon, are

provided in food trays and also arrayed on skewers throughout the room to stimulate foraging behavior. For additional enrichment, artificial trees and vines are provided.

All procedures for the present work were approved by the University of Northern Colorado Institutional Animal Care and Use Committee and were in compliance with the USA Animal Welfare Act. Euthanasia was performed by respiratory hyperanesthesia with isoflurane followed by thoracotomy.

Ribonucleic acid extraction

A single cell suspension of splenocytes was prepared by gentle disruption of spleens between the ends of sterile frosted glass microscope slides. Red blood cells were lysed with ACK Lysis Buffer (Cambrex Biosciences, East Rutherford, NJ) for 5 min at room temperature followed by 3x wash in PBS (pH 7.4). Splenocytes were cultured in 5% fetal bovine serum (FBS) RPMI-1640 and activated in 2 µg/ml concanavalin A and 2 µg/ml and *E. coli* LPS (Sigma, St. Louis, MO) overnight at 37°C under 7% CO_2. RNA was extracted according to manufacturer's instructions using the Serious RNA Purification™ RNA Cell kit (Gentra Systems, Minneapolis, MN) and aliquots were stored at -70°C until used.

Reverse transcription-polymerase chain reaction

RNA was reverse-transcribed using qScript ™ cDNA synthesis kit (BioRad, Hercules, CA, USA) using a mixture of random hexamers and oligo-dT primers. PCR was performed using degenerate primers (Table 1, see page 64) with a PCR Core kit (Qiagen, Valencia, CA). Amplification was initially performed using 35 cycles of 95°C for 30 seconds, annealing at 58°C for 30 seconds, and extension at 72°C for 1 minute. After 35 cycles, samples were incubated at 72°C for 10 minutes and then held at 4°C. For genes that failed to amplify with 58°C annealing, the annealing temperature was

lowered to 50°C for the first five cycles, then at 58°C for 30 cycles. PCR products were then resolved on a 1% agarose gel to verify amplification.

Cloning of polymerase chain reaction products

Amplicons were cloned into TOPO-TA Sequencing (Invitrogen, Carlsbad, CA) vector according to the manufacturer's directions. Vectors were then used to transform competent DH5a *E. coli* cells that were then plated on LB/Amp. Several colonies were chosen from each gene and screened by PCR to verify the correct plasmid insert. Colony screening was performed by PCR (Promega 2x PCR Master Mix, Madison, WI) with primers used for the original amplifications. Selected colonies were then incubated overnight at 37°C in 4 mL of LB broth containing 50 μg/ml ampicillin. Plasmids were purified using a Qiagen QIAprep spin Miniprep kit (Valencia, CA), and recovery was verified using a 1% agarose gel.

Rapid amplification of complimentary deoxyribose nucleic acid ends

Rapid amplification of cDNA ends (RACE) PCR was used to obtain additional 5' and 3' sequences of Seba's short-tailed fruit bat IL-23a, IL-10, and GM-CSF genes. 5' regions were extended for all three genes, but 3' RACE fragments were not obtained. RACE was performed using a SMART™ RACE cDNA Amplification kit (Clontech, Mountain View, CA). Primers for RACE PCR were designed from conserved regions in previously obtained sequences described in section 2.4. RACE primers are included in Table 2 (see page 64).

Sequence analysis

Sequencing reactions were performed using T7 and T3 primers with Big Dye Terminator (Applied Biosystems, Foster City, CA). Sequences were edited and contigs built using Sequencher (GeneCodes, Ann Arbor, MI) and basic local alignment search tool (BLAST) was used to identify genes. Amino acid alignments were made using

orthologous cytokine sequences from human (*Homo sapiens)*, chimpanzee (*Pan troglodytes),* rhesus monkey (*Macaca mulatta)*, domestic dog (*Canis lupus familiaris*), domestic cat (*Felis catus*), and house mouse (*Mus musculus*). Accession numbers are listed for each protein in Table 3 (see page 65). The CLUSTAL algorithm in MacVector sequence analysis software (Cary, NC) was used to perform the alignments. Consensus sequences are listed at the bottom of each alignment. Creation of a consensus sequence required greater than 51% amino acid identity in a single position. Bat sequences were deposited into GenBank (TNF, EF653909; GM-CSF, EF653911; IL-23a, EU223817; IL-10, EF653910).

Phylogenetic analysis

Phylogenetic analyses were conducted using amino acid sequences collected from Seba's short-tailed fruit bat and orthologous sequences obtained from GenBank. Orthologous sequences were obtained from a single representative of as many taxonomic groups as possible. The following orthologous sequences were used (see Table 3 on page 65 for GenBank accession numbers); TNF from human, chimpanzee*,* rhesus monkey, domestic dog, domestic cat, house mouse, horse (*Equus caballus*), cattle (*Bos taurus*), pig (*Sus scrofa*), and chicken (*Gallus gallus*); IL-10 from human, chimpanzee*,* rhesus monkey, domestic dog, domestic cat, house mouse, horse, cattle, pig, and chicken; IL-23a from human, chimpanzee*,* rhesus monkey, domestic dog, domestic cat, house mouse, horse, cattle, pig, and platypus (*Ornithorhynchus anatinus*); and GM-CSF from human, chimpanzee*,* rhesus monkey, domestic dog, domestic cat, house mouse, horse, cattle, pig, and chicken.

All sequences were aligned using the CLUSTAL algorithm in MacVector sequence analysis software. PAUP* 4.0b10 [188] was used to perform maximum parsimony analyses for each region. All characters were weighted equally and the exhaustive search procedure was used to determine the most parsimonious tree. Trees

were rooted using chicken as an outgroup for GM-CSF, TNF, and IL-10, and platypus for IL-23a. Chicken sequence was chosen as an outgroup for GM-CSF, TNF, and IL-10 because it was the only non-mammal sequence available for all of the genes. Platypus was chosen as the outgroup sequence for IL-23a as the chicken sequence was not available in GenBank and the platypus could serve as an unrelated outgroup. Support for branching patterns were analyzed using a parsimony bootstrap procedure with 1000 replicates to. Parsimony bootstrapping is a statistical procedure that examines the repeatability of the placement of sequences into specific clades that are created during analysis.

Results

Strategy for cloning bat cytokine genes

A degenerate PCR cloning strategy was adapted from methods previously used with wild rodents [19, 80]. Orthologous sequences of several mammalian cytokine genes were aligned and degenerate primer sets were designed (Table 1, see page 64). Total RNA was extracted from concanavalin A and LPS-stimulated splenocytes to enhance transcription of cytokine genes. PCR was then performed and amplicons were cloned and sequenced. Translated bat sequences and orthologous sequences were aligned to identify conserved amino acid regions and analyze phylogenetic relationships. The numbering above each sequence alignment refers to the numbering of the bat sequence.

Tumor necrosis factor

The cloned cDNA of Seba's short-tailed fruit bat TNF was 675 nt and encodes approximately 96% of the TNF coding region. The nucleotide sequence was translated using MacVector's default translation table and then aligned with orthologous mammalian sequences. The nucleotide sequence encodes nearly all of the TNF

polypeptide (226 residues) and exhibits 82% identity and 84% similarity to human TNF (Figure 1, see page 59). The gene is highly conserved between species and shares a nearly identical transmembrane domain with orthologous TNF which was predicted based on amino acid alignments (residues 32-58) [189]. Serine residues at positions 83 and 84, the putative cleavage point for the release of soluble TNF homotrimer, appear to be similar to those of human, chimpanzee, rhesus monkey, dog, cat, and mouse TNF. The cysteine residues at positions 148 and 180 for intrachain disulfide bond formation are conserved in this bat [190]. A conserved histidine is present in the bat sequence at position 194, and is necessary for biological activity of TNF [191]. Residues L115, S165, and E225 are involved in receptor interaction of TNF [192], and are conserved in Seba's short-tailed fruit bat polypeptide.

Interleukin-10

The cloned cDNA fragment of Seba's short-tailed fruit bat IL-10 was 453 nt in length and encodes approximately 80% of the IL-10 polypeptide (151 residues) (Figure 2, see page 60). The polypeptide has 78% identity and 84% similarity to the human IL-10 sequence [193]. The amino acid residues for the first four helices are highly conserved in the Seba's short-tailed fruit bat sequence (helix A, residues 42-65; B, 73-82; C, 84-106; D, 111-134) [194]. The sequence is incomplete for the fifth helix. The four conserved cysteine residues that are responsible for interchain disulfide bonds [195] are also present in the bat sequence.

Interleukin-23a

The cloned fragment of IL-23a was 594 nt and encodes nearly 95% of IL-23a gene (Figure 3, see page 61). The nucleotide sequence encodes nearly all of the IL-10 polypeptide (197 residues) and exhibits approximately 74% identity and 81% similarity to human IL-23a. The bat sequence contains all four cysteine residues that are involved in

intrachain disulfide bonding (residues 48, 84, 88, and 100) and also includes all four helices (helix A, residues 39-58; B, 98-121; C, 127-147; D, 166-196) [31].

Granulocyte macrophage-colony stimulating factor

The nucleotide sequence for Seba's short-tailed fruit bat GM-CSF gene is 290 nt and encodes the first 96 residues (~69%) of the bat polypeptide. This region is highly conserved with a 25-residue signal peptide present (L, residues 1-25, Figure 4, see page 62) and shares 71% identity and 78% similarity to human GM-CSF. The bat polypeptide contains a partially conserved helix A that is involved in binding to the GM-CSF receptor (A, residues 29-44) [19, 196, 197], which differs by 6 amino acids compared to the mouse, and by 8 amino acids compared to the human sequences. The ß1 strand that is involved in receptor binding in other species is also present in this sequence (residues 59-61). Helix B is highly conserved (B, residues 72-84), and a partial helix C is encoded in the polypeptide (C, residues 91-97) [197]. The bat sequence also encodes a conserved cysteine residue (residue 71) that is used for the intrachain disulfide bond [196]. The paired cysteine is located near the C-terminus of the polypeptide in other species and would be found in a region of the bat sequence that has not been cloned.

Phylogenetic analysis

The alignment of TNF sequences included a total of 246 characters, of which 190 were variable and 45 were parsimony informative. The alignment of IL-10 sequences included a total of 188 characters, of which 128 were variable and 51 were parsimony informative. The alignment of IL-23a sequences included a total of 235 characters, of which 174 were variable and 39 were parsimony informative. The alignment of GM-CSF sequences included a total of 156 characters, of which 124 were variable and 56 were parsimony informative.

Analysis of the TNF data resulted in 5 equally parsimonious trees (C.I. = 0.9054; R.I. = 0.5272; length = 349). Consistency index (C.I.) values and retention index (R.I.) values measure relative amounts of homoplasy in a tree [198]. Length values indicate the number of character state changes that were necessary to arrive at the cladistic grouping shown on a tree. Fewer character state changes indicate that a tree is a better fit for the data [198]. The consensus tree placed Seba's short-tailed fruit bat as unresolved at the base of tree with house mouse, horse, and chicken (Figure 5A, see page 63). The bootstrap analysis strongly supported a clade with human, chimpanzee, and rhesus monkey (99% bootstrap), with human and chimpanzee exhibiting a sister relationship within that clade (71% bootstrap).

Analysis of the IL-10 data resulted in 2 equally parsimonious trees (C.I. = 0.8732; R.I. = 0.6500; length = 276). The consensus tree placed Seba's short-tailed fruit bat as basal to a clade containing domestic dog and domestic cat (Figure 5B, see page 63). However, bootstrap analysis did not support the placement of Seba's short-tailed fruit bat. The only clade that was strongly supported contains human, chimpanzee, and rhesus monkey (96% bootstrap), with human and chimpanzee exhibiting a sister relationship within that clade (96% bootstrap).

Analysis of the IL-23a data resulted in single most parsimonious tree (C.I. = 0.9313; R.I. = 0.6901; length = 320). Within the tree Seba's short-tailed fruit bat was placed basal to a clade containing all of the placental mammals except house mouse (Figure 5C, see page 63). Bootstrap analysis strongly supported the clade grouping Seba's short-tailed fruit bat with all of the placental mammals except house mouse (92% bootstrap) and the clade containing human, chimpanzee, and rhesus monkey (100% bootstrap), with human and chimpanzee exhibiting a sister relationship within that clade (91% bootstrap).

Analysis of the GM-CSF data resulted in 2 equally parsimonious trees (C.I. = 0.8859; R.I. = 0.6264; length = 298). The consensus tree placed Seba's short-tailed fruit bat as unresolved relative to all of the mammals except for mouse, which was unresolved at the base of the tree with chicken (Figure 5D, see page 63). The bootstrap analysis strongly supported a clade with human, chimpanzee, and rhesus monkey (100% bootstrap), with human and chimpanzee exhibiting a sister relationship within that clade (71% bootstrap).

Discussion

Until recently, bats have been underappreciated as reservoir hosts for pathogenic viruses. The relationships between bats and their viruses are poorly understood. Many zoonotic viruses hosted by other mammals often establish persistent infections without significant pathological consequences. Identification of the underlying mechanisms governing non-pathogenic infections of reservoirs may provide clues to virus ecology as well as pathogenesis of human diseases caused by many viruses [2, 87].

Laboratory models that could be used to understand the immunological relationships between viruses and their bat hosts are being developed. Several cytokine genes from bats have been successfully cloned and sequences show that they are substantially conserved among known mammalian orthologs.

TNF is thought to play a prominent role in pathogenesis of many infectious diseases, including hantavirus cardiopulmonary syndrome, dengue hemorrhagic fever and dengue shock syndrome [199]. It is a chemotactic inflammatory cytokine secreted by various immune cells and is expressed as a membrane-bound homotrimer that is cleaved by a cellular protease imbedded in the plasma membrane; its release induces inflammation by causing vascular leakage [28]. The sequence of TNF from Seba's short-

tailed fruit bat is highly similar to TNF of other species and, other than a three amino acid deletion at positions 64-66, contains no unusual differences from those sequences.

IL-10 is a noncovalently-linked homodimer secreted by many cells, including some regulatory T cell subsets, and is capable of suppressing inflammatory responses [32, 200, 201]. Each monomer is composed of six alpha helices and four conserved cysteine residues that are responsible for intrachain disulfide bond formation [194]. The bat polypeptide also is highly similar to IL-10 from vertebrates of other species, especially within the functional domains.

IL-23a is in the interleukin-12 family and is co-expressed with IL-12p40 to form a disulfide-linked heterodimer [31]. It is secreted from human and mouse dendritic cells generated in the presence of GM-CSF and IL-4, can induce IFNγ responses and promote proliferation of memory T cells in mice [202]. It is important for initiation of the adaptive immune response to some infections. The bat polypeptide is highly similar within domains to IL-23a of other mammalian species; however, it possesses a three amino acid insertion (GEK) at positions 68-70, between helices A and B, a region with unrecognized biological significance.

GM-CSF is a glycoprotein that can stimulate the growth and differentiation of many immune cells, including bone marrow stem cells [203, 204]. It is secreted by a variety of cells, including Th1 cells, controls the activities of a variety of phagocytic cells, is an important stimulator for the growth and development of immune cells, and is routinely used for *in vitro* propagation of dendritic cells from bone marrow progenitors [205]. Although the sequence of Seba's short-tailed fruit bat GM-CSF is highly similar to orthologous sequences, it has several differences in helix A, a receptor-binding domain.

Phylogenetic reconstructions did not provide clear resolution of the relationship between Seba's short-tailed fruit bat and other placental mammals. Phylogenetic trees from three cytokine genes (TNF, IL-23a, and GM-CSF) placed Seba's short-tailed fruit

bat in an unresolved position at or near the base of the tree. IL-10 provided the best phylogenetic resolution among taxa, placing Seba's short-tailed fruit bat in a clade containing domestic dog and domestic cat. This placement corresponds to recent mammalian phylogenetic analyses, which group bats, dogs, and cats into the Superorder Laurasiatheria [206]. However, Superorder Laurasiatheria also contains cattle, horse, and pig, which are dispersed throughout the IL-10 phylogenetic tree. The general lack of phylogenetic resolution indicates that additional taxonomic sampling will be required to accurately document the evolutionary divergence of these cytokine genes.

Parsimony is commonly used in phylogenetic analyses because it is relatively simple and generates phylogenetic trees using as few steps as possible. This method implies an evolutionary relationship and that the most simple explanation is likely the best [198]. It should be noted that other methods of phylogenetic analysis such as neighbor-joining or un-weighted pair group method with arithmetic mean (UPGMA), could lead to the generation of phylogenetic trees with different cladistic groupings. This is because each method uses different criteria for placing sequences into clades. Additional phylogenetic analyses using different methods of tree construction could be useful in resolving the evolutionary divergence of these cytokine genes.

Cloning and sequence analysis of additional cytokine genes and other immune related transcription factors of Seba's short-tailed fruit bat will allow the continued assessment of immune responses. It is believed these primer sets could be used for amplifying and cloning orthologous sequences from bats of many other species. In addition, the little brown bat (*Myotis lucifugus*) genome has been sequenced (7x, Broad Institute, Cambridge, MA), which should provide an important genomic resource for research efforts regarding reservoir bat host responses to viral infections.

Figure 1. Amino acid alignment of TNF sequences. Seba's short-tailed fruit bat cDNA was translated using the default translation table in MacVector DNA analysis software. The human, chimp, rhesus monkey, dog, cat and mouse TNF sequences were imported into MacVector from NCBI (accession numbers in table 2) and aligned using the default parameters in the CLUSTAL algorithm. Regions of sequence outlined in dark gray represent either consensus amino acid identities or similarities and white areas indicate non-similar amino acids. The consensus sequence is found at the bottom of the alignment. The bar that is denoted TM above the alignment represents the region of TNF that is involved in the transmembrane domain. Residues marked with asterisks represent conserved residues that are involved in biological function of TNF.

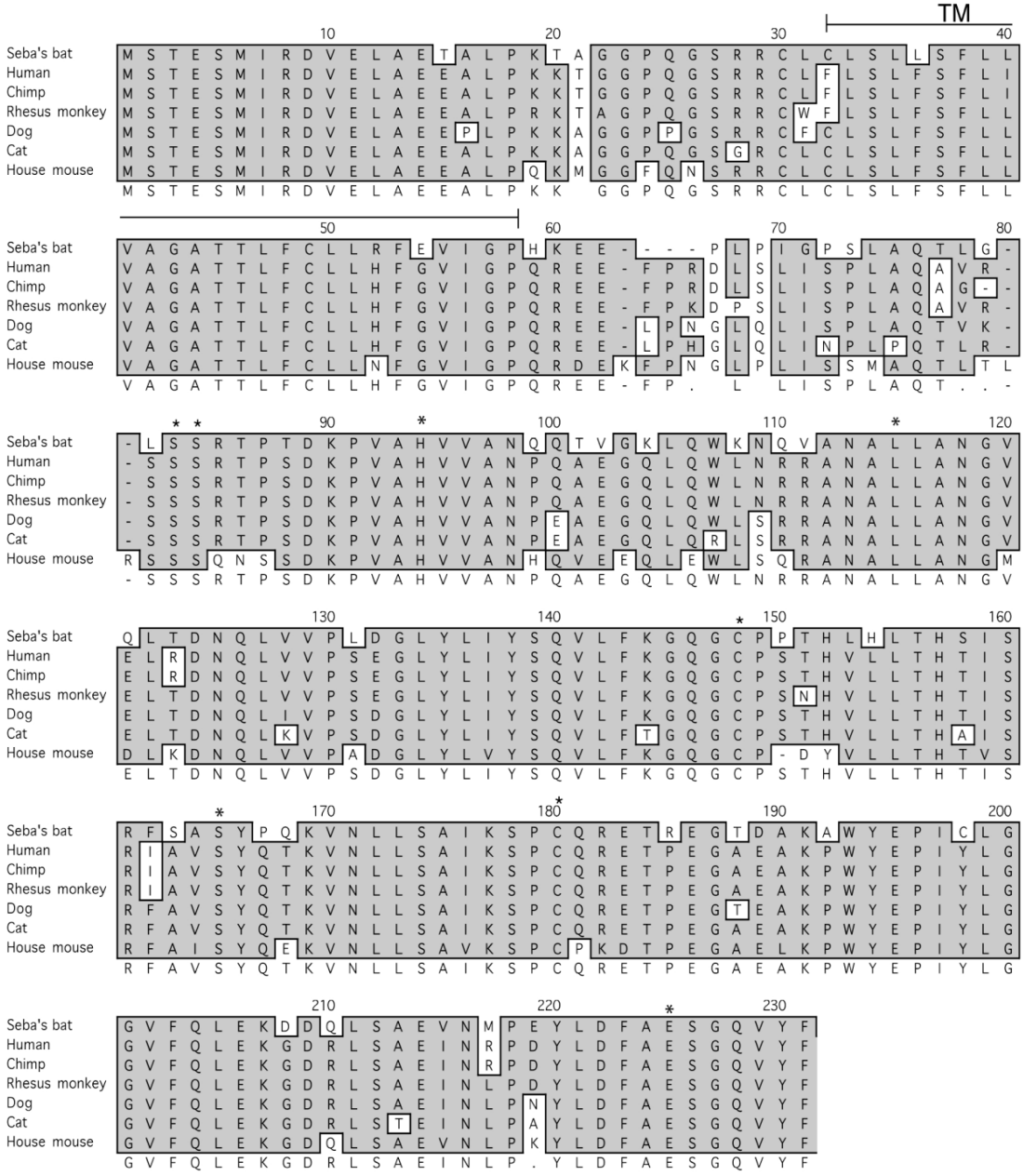


Figure 2. Amino acid alignment of IL-10. Seba's short-tailed fruit bat IL-10 amino acid sequence was aligned with other orthologous mammalian IL-10 sequences as described in Figure 1. Lettered bars (A, B, C, D, E) above the alignment indicate helix regions and the four asterisks denote conserved cysteines that form intrachain disulfide bonds.

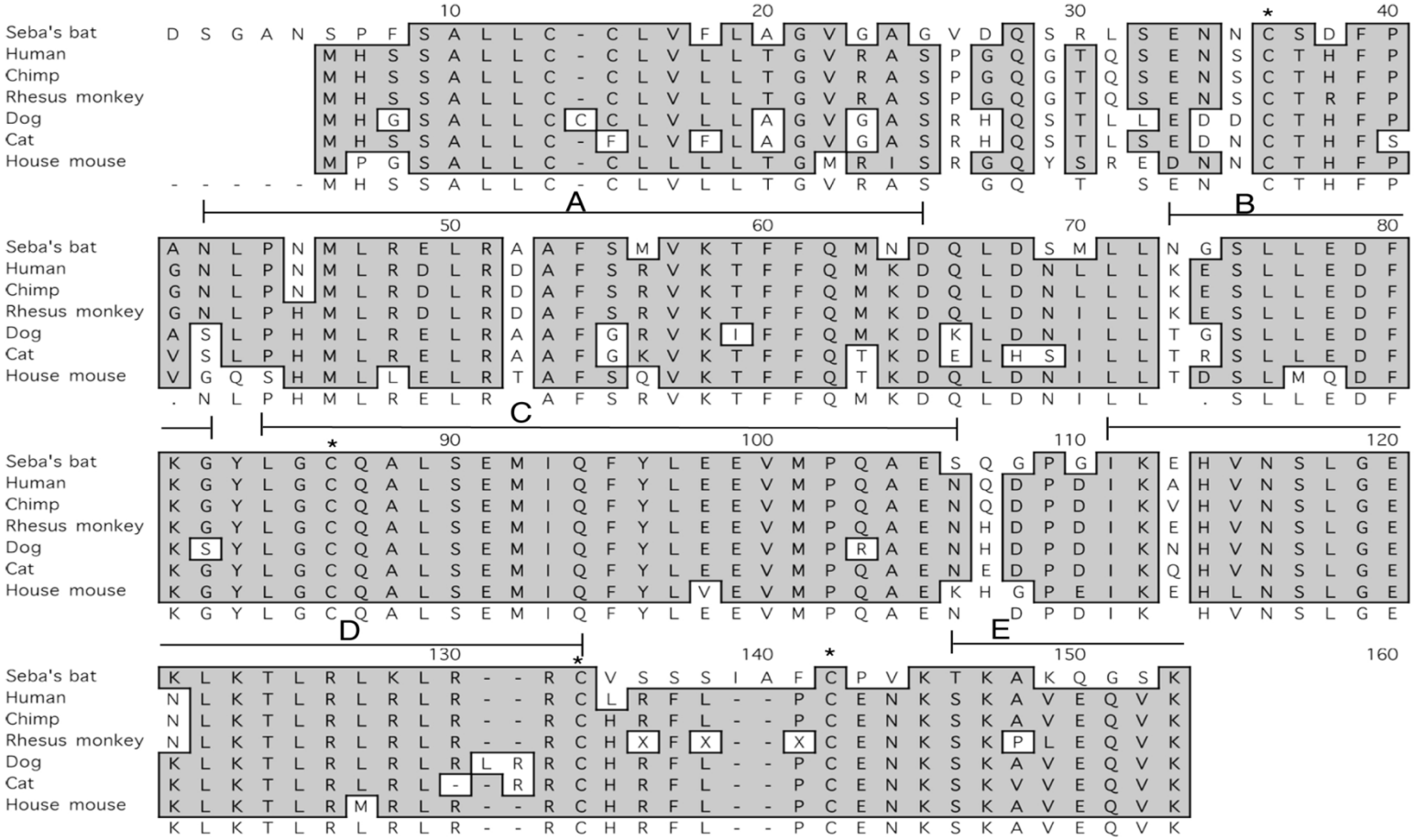


Figure 3. Amino acid alignment of IL-23a. Seba's short-tailed fruit bat IL-23a amino acid sequence was aligned with other IL-23a sequences as described in figure 1. Lettered bars (A, B, C, D) above the sequences denote four helix regions and asterisks indicate conserved cysteine residues.

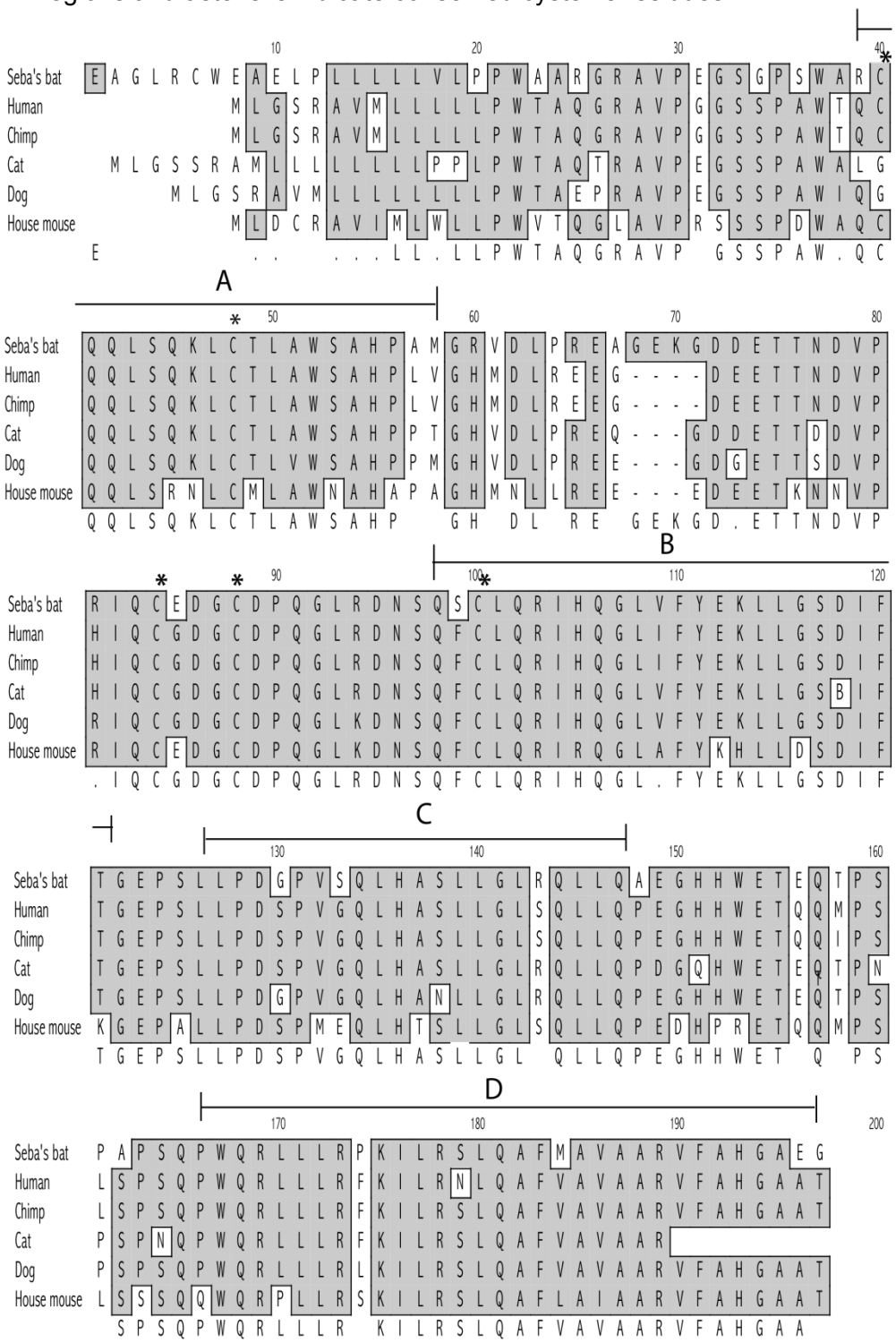


Figure 4. Amino acid alignment of GM-CSF. Seba's short-tailed fruit bat GM-CSF amino acid sequence was aligned with other mammalian GM-CSF sequences as described in figure 1. The region denoted with L indicates a 25 amino acid signal peptide, and region A indicates a partially conserved helix A. A highly conserved β1 strand is marked with a bar above the alignment. A highly conserved Helix B and an incomplete Helix C are

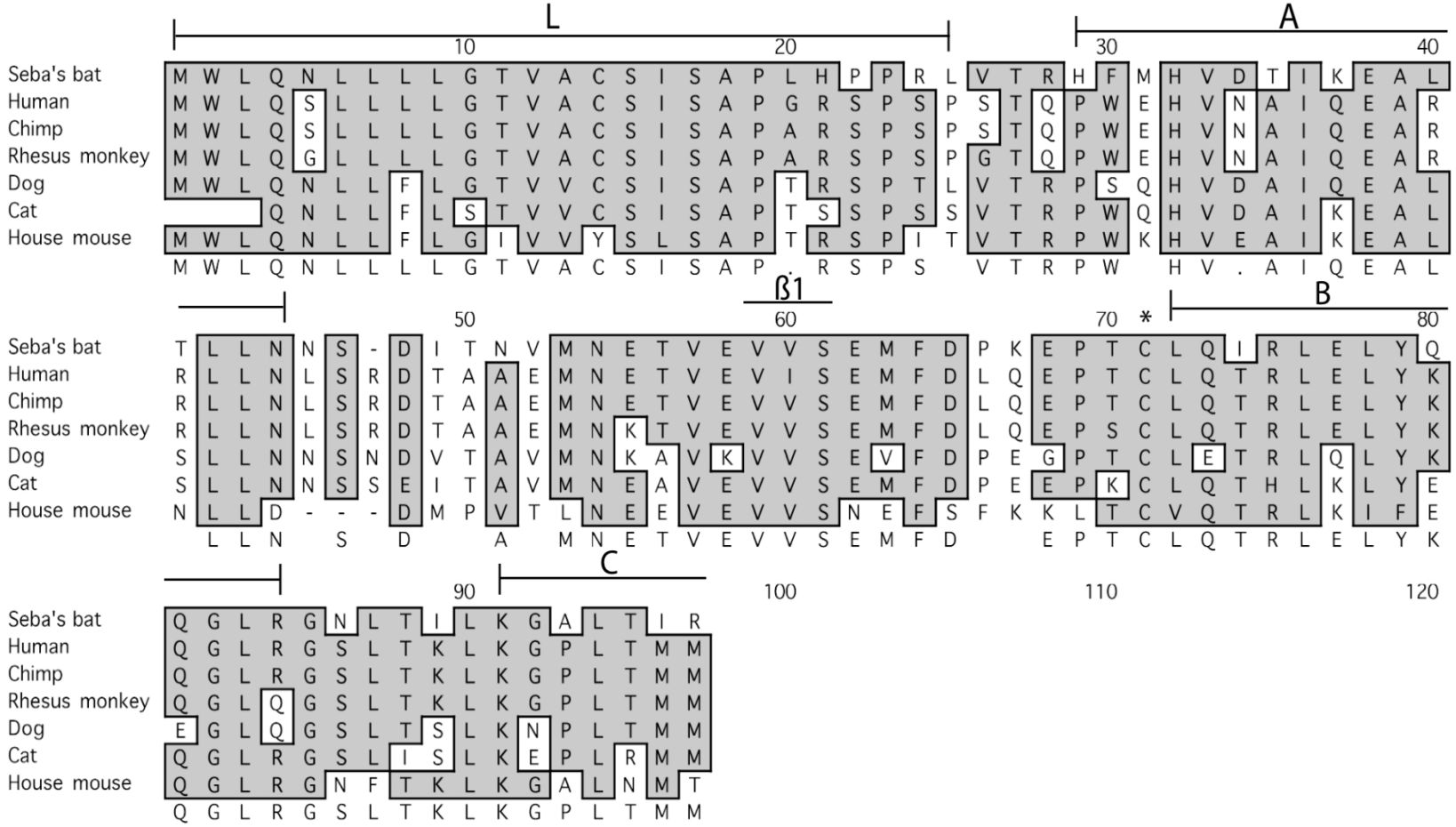
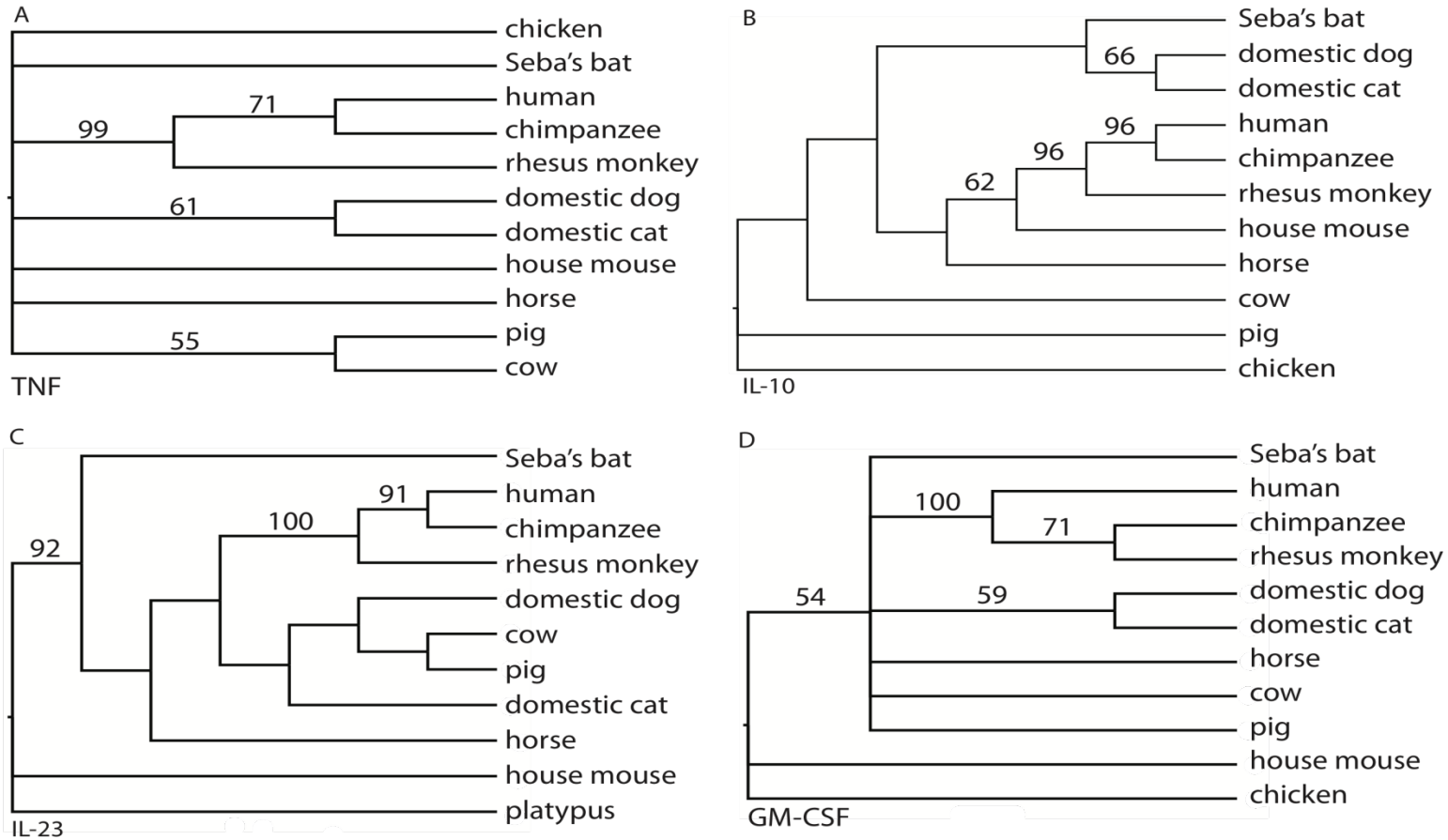


Figure 5. Most parsimonious phylogenetic tree reconstructions based on cytokine gene amino acid sequences. Genes are as follows: A, TNF; B, IL-10; C, IL-23a; D, GM-CSF. Bootstrap values greater than 50% are shown above the branches.



Tables

Table 1. Primer sequences used to clone partial cytokine cDNAs from Seba's short-tailed fruit bat. Sequences listed 5' to 3'.

Gene	Forward	Reverse	Size (bp)
TNF	GCGACGTAGAACTGGCAGAGG	CTGCGAAGTCTAGGTATTTGG	648
IL-10	CTCAGCACTGCTCTGTTGCC	1 GATGTCAAACTCAGTCATGG 2 GATGTCAAACTCATTCATGG	454
IL-23a	1 AGCCAGATCTGAGAAGAAGG 2 AGCCAGATCTGAGAAGCAGG	CTGCTCCGTGGGCAAAGACC	311
GM-CSF	1 CTGGAGGATGTGGCTGCAG 2 CTGGAGGATGTGGCTGAAG 3 CTGGAGGATGTGGCTTCAG 4 CTGGAGGATGTGGCTTAAG	1 ATGGTCAAGGGGCCCTTGAG 2 ATGGTCAAGGCGCCCTTGAG	293

Table 2. RACE primer sequences used to amplify 5' cDNA regions of Seba's short-tailed fruit bat cytokine genes. Sequences listed 5' to 3'.

Gene	Forward	Reverse
IL-10	TCGCCCTTCTCAGCACTGCTCTGTTGCC	GTTTGGGCAGGTTGGCTGGGAAGTCACTG
IL-23a	TGCCTGAAGGCAGCGGCCCCTCTTG	TCCACACGTCCATGGCTGGATGTGC
GM-CSF	CCTGCTTCTTCTGGGCACAGTGGCTTGC	GCGCCCTTGAGTATGGTGAGGTTGCCCC

Table 3. Polypeptide accession numbers of sequences used from NCBI

Polypeptide	TNF	GM-CSF	IL-10	IL-23a
Human	NP_000585.2	AAU21240.1	CAG46825	AAH66267.1
Chimp	NP_001038976.1	XP_527005.2	XP_525040.1	XP_522436.1
Rhesus monkey	NP_001040614.1	NP_001028121.1	ABI63892.1	NA
Dog	NP_001003244.4	NP_001003245.1	NP_001003077.1	XP_538231.2
Cat	NP_001009835.1	AAN11308.1	AAC64708.1	ABB01676.1
Mouse	NP_038721.1	NP_034099.2	NP_034678.1	NP_112542.1
Horse	NP_001075288.1	AAL41017.2	Q28374.1	ABZ91982.1
Cattle	DAA16338.1	P11052.1	NP_776513.1	ACC77208.1
Pig	NP_999187.1	NP_999283.1	NP_999206.1	NP_001123708.1
Platypus	NA	NA	NA	XP_001517448.1
Chicken	NP_989598.1	NP_001007079.1	NP_001004414.2	NA

CHAPTER IV

EXPERIMENTAL INFECTION SUGGESTS JAMAICAN FRUIT BATS (*ARTIBEUS JAMAICENSIS*) ARE NOT RESERVOIRS OF TACARIBE VIRUS

Abstract

Tacaribe virus (TCRV) was first isolated from nine *Artibeus* sp. bats captured in Trinidad in the early 1960s during a rabies virus surveillance program. Despite significant effort, no evidence of infection of other mammals, mostly rodents, was found that suggested other species harbored TCRV. For this reason, it was hypothesized that Artibeus bats naturally hosted TCRV. This is in stark contrast to other arenaviruses with known hosts in which all are rodents. Experimental infections of Jamaican fruit bats (*A. jamaicensis*) with TCRV were performed to examine this hypothesis. It was predicted that bats would become persistently infected without substantial pathology. Jamaican fruit bats were infected with TCRV strain TRVL-11573 and it was found that low-dose inoculations resulted in apathogenic infection and virus clearance, while high-dose inoculations caused substantial morbidity and mortality as early as 10 days post infection. In addition, transmission failed to occur in four experimental trials. Together, these data suggest that Jamaican fruit bats are not a reservoir host species for Tacaribe virus.

Introduction

Bats (order Chiroptera) represent approximately one fifth of the 5,400 known species of mammals [2]. In recent years, several bat species have been identified as hosts for human and veterinary viruses and are likely reservoirs of other important pathogens. Examples of viruses for which bats are reservoirs include rabies virus and related lyssaviruses, Hendra and Nipah viruses, ebolaviruses and Marburg virus, and SARS-like coronaviruses [9, 10, 14, 57, 59, 132]. Very little is known about virus-host interactions in bats since bats have not been extensively studied and little is known about their physiology, including immunology and host responses during infections. Research suggests that some bats may become persistently infected with many viruses without pathology [2, 114] and, because of a general lack of knowledge of bat biology it is unknown how they may become persistently infected.

Tacaribe virus (TCRV) is an arenavirus, and like all other arenaviruses, it is spherical, enveloped, and has a segmented single strand, ambisense RNA genome [38, 167]. While TCRV is not known to naturally cause human disease, at least two instances of nonfatal laboratory infections have occurred. The Tacaribe virus complex (New World) of arenaviruses includes Junín, Sabiá, Guanarito, Machupo and Chapare viruses, which cause Argentine hemorrhagic fever (HF), Brazilian HF, Venezuelan HF, Bolivian HF, and an as yet unnamed hemorrhagic fever, respectively [36, 38, 166, 207].

Previously, Tacaribe virus was isolated from Jamaican fruit bats and great fruit-eating bats (*A. lituratus)* near Port of Spain, Trinidad during a rabies virus surveillance program [33, 34]. Examination of more than 2,000 mammals, principally small rodents, in Trinidad failed to provide serological evidence of infection and led to the suggestion that *Artibeus* bats were reservoirs of TCRV [33]. This is unusual because all other arenaviruses with known hosts are rodent-borne [71, 208]. When TCRV was isolated from Jamaican fruit bats, it was observed that some bats appeared healthy while others

exhibited symptoms of rabies and were mistaken as rabid bats [33]. Previous experimental infections in rodents found that TCRV was lethal to newborn mice, causing paralysis and necrosis in the central nervous system. In adult mice, limited replication was observed and infection was avirulent [42, 166]. Experimental infection of Jamaican fruit bats with TCRV were attempted when the virus was first isolated; however, bats used in the experiment were wild-caught and not pre-bled prior to the experiment to determine serostatus. Results from these infections were inconclusive and may have been influenced by previous natural infections [33]. TCRV can cause persistent infection of Vero E6 cells; however, it is unknown if it can cause persistent infections in Jamaican fruit bats [77, 174].

Considering these ambiguous results that suggested *Artibeus* bats as reservoirs of TCRV, and the fact that other arenaviruses have rodent reservoirs, this question was reexamined under controlled laboratory conditions. Experimental infections of Jamaican fruit bats with TCRV were conducted to examine pathology, virus tropism, viral shedding, and whether the bats can become persistently infected. Bats inoculated with a high-dose of TCRV exhibited clinical symptoms of disease resembling those of the South American hemorrhagic fevers and either died or were euthanized for humane reasons. Low-dose inoculations resulted in asymptomatic infections and virus clearance as determined by PCR and virus isolation in cell culture. These findings suggest that Jamaican fruit bats are not reservoir hosts of Tacaribe virus.

Materials And Methods

Bats

The University of Northern Colorado (UNC) bat colony has two species of neotropical fruit bats, Seba's short-tailed fruit bats and Jamaican fruit bats. These are maintained together in a 36 m^3 room that allows unrestrained free flight of all individuals. The colony has been closed for 16 years and is rabies virus-free; PCR screening of

rectal swab samples from 20 of these bats has failed to detect coronaviruses (K. Holmes, pers. comm.) and serology samples for TCRV infections have been negative by neutralization testing. The bats have access to roosting areas in the form of ceiling-hung baskets and various cloth drapes and are maintained under a light-cycle of 12L:12D. Ambient temperature is maintained between 20°C and 25°C and humidity between 50% and 70% by a computer-controlled HVAC system. Bats are fed daily at midday with grueled apples, monkey chow (Harlan Teklad, Denver, CO), molasses, nonfat dry milk, cherry gelatin with raisins, and fresh bananas. In addition, a variety of other fruits, including mango, papaya, cantaloupe, banana, grapes, and watermelon, are provided in food trays and also arrayed on skewers throughout the room to stimulate foraging behavior. For additional enrichment, artificial trees and vines are provided.

Jamaican fruit bats were obtained from the University of Northern Colorado bat colony. Prior to infection, bats were transported to Colorado State University for acclimation and infection under ABSL-3 conditions. All procedures were approved by the University of Northern Colorado and Colorado State University Institutional Animal Care and Use Committees and were in compliance with the USA Animal Welfare Act.

During each infection experiment, bats were placed three to a cage for a total of four cages. Bats were fed a daily diet of fruit and water was made available in bowls inside each cage. Individual bats were identified using numbered wing tags.

Experimental infections

Two sets of experimental infections were conducted over the course of two years. Tacaribe virus strain TRVL-11573 propagated in Vero E6 cells was used for these studies. The titer of virus stock was 10^7 virions per ml of BA1 media containing 5% FBS.

For the initial experiment, twelve male bats were each inoculated with 100 μl at 10^6 subcutaneously (SC) on the abdomen, and 25 μl at 10^5 intranasally (IN). Bats were

monitored over the course of 18 days for development of pathological symptoms. As part of the protocol, some bats were routinely euthanized to examine disease progression. Two bats each were euthanized on day 4 PI, and day 8 PI. The remaining eight bats were euthanized as they began to show pathological symptoms of disease. In this initial experiment, one bat that was not housed with the twelve infected bats was included as a negative control.

The second experiment included three male and nine female Jamaican fruit bats that were randomly divided into four experimental groups receiving different doses and routes of virus inoculation. Group one consisted of two bats that received 10^6 virus IN and one bat that received PBS IN. Group two consisted of two bats that received 10^4 virus IN, and one bat that received PBS IN. Group three consisted of two bats that received 10^6 virus SC in the abdomen and one bat that received PBS SC in the abdomen. The fourth group consisted of two bats that received 10^4 virus SC in the abdomen, and one bat that received PBS SC in the abdomen. Bats were monitored for development of pathological symptoms over the course of 45 days. Any moribund bats were immediately euthanized.

In both experiments, intranasal inoculations were performed using a micropipettor and a sterile tip to administer the correct dose. Subcutaneous injections were performed using a sterile hypodermic syringe and needle. Inoculations were performed in a biosafety cabinet without anesthesia.

Weight records

Bat weights were recorded only on day zero during the initial experiment and every two days during the second experiment. During the second experiment, weights were recorded to examine the effect of viral infection on feeding behavior and weight gain or loss. Bats were carefully rolled up in a piece of cloth and placed on a balance to obtain weight in grams.

Swab collection

Oral and rectal swabs were routinely collected from each bat every two days to examine virus shedding. Oral and rectal swabs were collected from each bat using sterile cotton-tipped swabs. All oral and rectal swabs were collected and expressed in 500 μl of BA1 media containing 5% FBS and were frozen at -80^{o}C until processed.

Serum sample collection

Serum samples were routinely collected from each bat every two days. Bats were bled from the wing using a sterile 22-gauge needle and capillary collection tubes. Approximately 100 μl of blood was collected from each bat on collection days and placed into 200 μl of BA1 media containing 5% FBS. Following euthanasia final serum samples were collected by cardiac puncture. Serum samples were used in virus isolation and neutralizing antibody tests.

Organ sample collection

All bats were euthanized by an intracardiac injection of sodium pentobarbital. Necropsies were performed directly following euthanasia. Organ samples were collected for examination using conventional PCR, and virus isolation on Vero E6 cells followed by immunofluorescent antibody tests. All organ samples were frozen at -80^{o} C until processed. In the first experiment, testes, kidney, heart, lung, liver, spleen, large intestine, small intestine, and brain samples were collected. In the second experiment, organ sampling was the same except for the collection of the uterus from female bats, and the addition of collecting salivary glands.

Ribonucleic acid extraction

TCRV viral RNA was extracted from BA1 media containing 5% FBS that had been used to collect oral and rectal swabs using QIAamp (Qiagen, Valencia, CA) Viral RNA mini kits according to the manufacturer's instructions. All RNA samples were frozen at -80^{o} C until used for reverse transcription.

RNA was extracted from bat organ samples collected in RNAlater stabilization reagent (Qiagen, Valencia, CA). Organ samples were placed in 1.5 mL screw cap tubes and approximately twelve 2.3 mm zirconia/silica beads (BioSpec Products Inc., Bartlesville, OK) were added to each tube. A mini bead-beater (BioSpec Products Inc., Bartlesville, OK) was used to homogenize the organ samples. RNA extraction was performed using QiaShredder columns (Qiagen, Valencia, CA), and RNeasy mini kits (Qiagen, Valencia, CA) according to manufacturer's instructions. All RNA samples were immediately frozen at -80^{o}C until used for reverse transcription.

Reverse transcription and Polymerase chain reaction

RNA from oral swabs, rectal swabs, and organ samples was reverse-transcribed using qScript ^{TM} cDNA synthesis kits (Quanta Biosciences, Gaithersburg, MD) according to manufacturer's instructions. PCR was performed using Promega (Madison, WI) 2X PCR master mix kit and appropriate TCRV primers (Table 8, see page 91). Amplification was performed using 35 cycles of 94°C for 30 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for 1 minute. After 35 cycles, the reactions were held at 72°C for 10 minutes and finally held a 4°C until used. Amplified PCR samples were then resolved and visualized using a 1% agarose gel.

Cloning and sequence analysis

Several PCR products from organ samples were cloned and sequenced to verify amplification of TCRV. PCR products were cloned into TOPO-TA (Invitrogen, Carlsbad, CA) cloning vector according to the manufacturer's directions. Clones were then used to transform One Shot Chemically competent *E. coli* cells (Invitrogen, Carlsbad, CA), which were subsequently plated and grown on LB/ampicillin plates. Bacterial colony screening was performed using Promega (Madison, WI) 2X PCR master mix kit and TCRV primers to verify plasmid insert. Colonies with the TCRV insert were then incubated at 37°C

overnight in 4 mL of LB broth with 50 μg/ml ampicillin. Plasmids were then purified using a QIAprep spin Miniprep kit (Qiagen, Valencia, CA) according to manufacturer's directions. Plasmid recovery was confirmed using a 1% agarose gel. Sequencing reactions were performed using T7 and T3 primers with Big Dye Terminator (Applied Biosystems, Foster City, CA). Sequencing reactions were sent to CSU Macromolecular Services for sequencing. Sequencing files were edited using Sequencher (GeneCodes, Ann Arbor, MI) and BLAST (basic local alignment search tool) was used for gene identification.

Virus isolation

Organs that had previously been frozen at -80 C° were thawed in 500 µL of BA1 media containing 5% FBS. Approximately twelve 2.3 mm zirconia/silica beads (BioSpec Products Inc., Bartlesville, OK) were added to each tube and tissues were homogenized using a mini bead-beater (BioSpec Products Inc., Bartlesville, OK). Tubes were centrifuged at 13,000 rpm for 5 minutes to separate virus-containing supernatant from remaining solid tissue. Supernatants were then diluted into 96-well tissue culture plates in a log 10 dilution series beginning with neat supernatant and leaving the last row blank as a negative control. Supernatants were then added to a corresponding plate containing confluent Vero E6 cells. Cells were incubated with supernatants for 24 hours, at which point fresh media was added. Cells were kept at 37 °C, 5% CO_2 for 7 days. Cells were evaluated for presence of virus using immunofluorescent antibody staining. Media was removed and cells were fixed by incubating with acetone for 120 minutes. They were then incubated with mouse anti-TCRV ascites fluid diluted 1:200 in PBS for 30 minutes at 37° C. Cells were washed with 1X PBS and incubated with a 1:1600 dilution of DyLight goat anti-mouse IgG (Jackson Immunoresearch Cat.# 115-485-003), and incubated 30 minutes at 37° C. Cells were again washed and stored in 1X PBS protected from light until evaluation with a fluorescent microscope. The same protocol

for virus isolation was used on terminal serum samples from each experiment. Wells were evaluated as positive for virus isolation if greater than 25% of Vero E6 cells in each well were fluorescing.

Neutralizing antibody tests

Terminal serum samples from each bat were screened for anti-TCRV neutralizing antibody by neutralizing antibody tests and immunofluorescence. Fifty microliters of each serum sample was diluted with 75 μl of BA1 cell culture media and heat inactivated in a water bath for ten minutes. Heat-inactivated serum samples were diluted 1:2 with BA1 cell culture medium and placed in the first well of a 96-well tissue culture plate. Log 2 dilution series were made for each sample. A dilution series was performed with mouse anti-TCRV ascites fluid in BA1 cell culture medium, without the addition of serum to serve as an anti-TCRV positive control. A negative control sample was included by performing a dilution series with TCRV in BA1 cell culture medium, without the addition of serum. After all dilutions were made, 50 μl of TCRV (diluted 1:1 with BA1 cell culture media) was added to each well. Plates were then incubated at 37° C for 60 minutes. Fifty microliters of Vero E6 cells were then added to each well at a concentration of 400,000 cells per milliliter. Plates were kept at 37 °C, 5% CO_2 for 7 days. Cells were then screened for presence of TCRV infection using immunofluorescence, as was previously described for organ sample virus isolations.

Histopathology

Tissues were removed from bats at necropsy and collected in buffered formalin. Specimens were prepared and sectioned by Colorado Histprep, Inc. for evaluation by a veterinary pathologist (DG).

Results

Experimental infections

In the first experiment, twelve bats were inoculated with 10^6 $TCID_{50}$ (100 µl) subcutaneously (SC) and $2.5x10^5$ $TCID_{50}$ (25 µl) intranasally (IN) to determine TCRV susceptibility. During the initial experiment, it was planned to euthanize two bats on days 4, 8, 12, 16, and 28 post-infection (PI). Two bats were euthanized on day 4 and day 8 PI, but on day 10, bats began to show pathological symptoms of disease and some exhibited tremors and were unable to fly. One bat was found dead on day 10 PI and another was moribund on day 11 PI and was euthanized (Figure 6, see page 84). One bat was found dead on day 13 PI and another on day 16 PI. Two more bats became moribund on day 17 PI and were immediately euthanized. On day 18 PI, a bat was found dead and the last two bats were moribund and were euthanized. Symptoms of moribund bats included being poorly responsive to mechanical stimuli, abnormal wing, ear and head tremors, inability to coordinate movement, and inability to fly when released.

In the second experiment, two bats each were inoculated with high-dose IN (10^6 $TCID_{50}$), low-dose IN (10^4 $TCID_{50}$), high-dose SC (10^6 $TCID_{50}$) or low-dose SC (10^4 $TCID_{50}$) and were euthanized for humane reasons only if they showed pathological signs of disease. Included in each of these four cages was one uninfected bat to assess transmission. On day 21 PI, a bat that had received a 10^6 IN inoculation became moribund and was euthanized, and on day 23 PI, a bat that had received a 10^6 SC inoculation became moribund and was euthanized. On day 25 PI, a second bat from the 10^6 IN group became moribund and was euthanized. The remaining nine bats did not exhibit pathological symptoms during the 45 day experiment. The three bats that became moribund experienced the same symptoms as described in the initial experiment.

Bat weights

Bat weights were only recorded on day 0 in the initial experiment. In the second experiment, bat weights were recorded every two days as well as days that bats became moribund or were found dead. Bat weights appeared to fluctuate somewhat throughout the experiment with a notable decrease in weight as bats became moribund (Figure 7, see page 85).

Oral swab polymerase chain reaction

In the initial experiment, all oral swabs collected on days 0, 2 and 4 PI, were negative for virus by PCR (Table 4, see page 87). On day 6 PI, oral swabs from several bats were positive for virus and continued to show positive PCR results throughout the duration of the experiment.

In the second experiment, oral swabs collected on day 0 were all negative for viral RNA by PCR (Table 5, see page 88). On day 2 PI, the oral swab collected for one bat was positive. All of the day 4 PI oral swabs were negative, but by day 6 PI, oral swabs for five of the bats were positive for virus. Most of the bats continued to show positive oral swab PCR results throughout the experiment.

Rectal swab polymerase chain reaction

In the initial experiment, rectal swabs collected on days 0, 2, and 4 PI were negative for virus by PCR. By day 6 PI, and continuing through the entire experiment, many bats show rectal swabs that are positive for virus (Table 4, see page 87).

In the second experiment, rectal swabs collected on days 0, 2, 4, and 6 PI were negative for virus. From day 8 PI, continuing through day 45 PI, rectal swabs for many of the bats show positive results for virus by PCR (Table 5, see page 88).

Organ sample polymerase chain reaction

Organ samples collected during the initial experiment show that 11 of the 12 bats were positive for virus by PCR in several organ samples. One bat was not PCR positive for virus in any organ sample, and two other bats only showed positives in one organ sample each. The remaining nine bats were PCR positive for virus in multiple organs (Table 6, see page 89).

Organ samples collected during the second experiment show that only three of the twelve bats were positive for virus by PCR in various organ samples. One bat was positive in the brain only, while the other two bats were positive for virus by PCR in multiple organ samples (Table 7, see page 90).

Virus isolation

Virus isolation was attempted from organ homogenates from the initial experiment using Vero E6 cells followed by IFA (Table 6, see page 89) and were congruent with PCR results. Virus isolation from the second experiment also supported the findings from the organ PCR results with only two bats positive for virus in the organs (Table 7, see page 90). The same two bats were also positive for virus in the organ samples by PCR. Virus was not isolated from terminal serum samples in either of the infection experiments.

Neutralizing antibody test

Neutralizing antibody tests on terminal serum samples from the first experiment, in which bats died or were euthanized between 10 and 18 days PI, were negative. Only three bats from the second experiment had neutralizing antibody. All three of these bats were in subcutaneous inoculation groups and were negative for virus by PCR (Table 7, see page 90).

Histopathology

Tissues from the initial infection experiment were submitted for pathological examination. Eleven bats had neutrophilic interstitial pneumonia that ranged from mild to

moderate in severity, and with multifocal to diffuse distribution, while bat 738 exhibited no remarkable lesions. Six of the bats (bats 718, 747, 714, 720, 729, 738) exhibited myocardial lesions without inflammation, fibrosis or degenerate myofiber loss. Three bats (747, 714, 748) exhibited mild to moderate multifocal adrenocortical necrosis, and mild to moderate multifocal hepatocellular necrosis and vacuolar hepatocellular degeneration was observed in several bats (645, 647, 705, 747, 712). Most bats exhibited mild to moderate neutrophilic enteritis with crypt necrosis and epithelial degeneration.

Spleens from bats were grouped in two categories based upon histopathology: acute neutrophilic splenitis and white pulp hyperplasia/plasmacytic and histiocytic splenitis. Neutrophilic infiltration of the white pulp with fibrin tagging of the capsule occurred in many bats (645, 647, 705,747, 714, 712), which may be related to changes in the peritoneum; however, no conspicuous peritonitis was observed. The white pulp changes appear to be the result of proliferating lymphocytes instead of inflammation as plasma cells were often abundant.

Two bats (712 and 729) had lymphocytic leptomeningitis and mild to moderate multifocal gliosis in the brainstem and prosencephalon. Bat 712 also had mild neutrophilic encephalitis of the prosencephalon (Figure 8, see page 87).

Both of these bats, as well as bat 738, were PCR positive for viral RNA and exhibited tremors prior to euthanasia. Bat 714 was also PCR positive, but had died on day 11 PI and no tremors had been observed on day 10 PI. No remarkable lesions were observed in kidneys, ureters, bladder, testicles, thymus, esophagus, salivary gland, pancreas or stomach samples.

Discussion

Arenaviruses, for which the reservoirs are known, are rodent-borne. The one exception has been Tacaribe virus, which was thought to be hosted by *Artibeus* bats

based upon natural infections and virus isolation from bats, and a lack of evidence of a rodent host. This possibility has been addressed by infecting Jamaican fruit bats with TCRV and the results suggest that Jamaican fruit bats are not a likely reservoir of TCRV.

Conducting experimental infections with bat models are challenging because of the unique attributes of bats. The establishment of breeding colonies usually requires free-flight enclosures, appropriate foods, enrichment, including those that stimulate foraging behavior, and appropriate climate control to facilitate the highly social development of the colony. In addition, reproductive female bats typically produce one to three offspring per year and invest heavily in maternal care [175]. For *Artibeus* bats, reproductive females typically produce two pups per year in the wild, a number consistent with females in the UNC colony. This presents difficulty in conducting experiments with large sample sizes and, thus, can be a limiting factor in research with bats. Despite these limitations, efforts to infect Jamaican fruit bats with Tacaribe virus were successful.

Different doses and routes of TCRV inoculation were examined in the first and second infection experiments. Initial experiments were performed with coinoculation of high dose and two routes to determine if Jamaican fruit bats could be infected with TCRV strain TRVL-11573. TCRV was isolated in the early 1960s from the brain of a great fruit-eating bats (*A. lituratus*) and has an unknown passage history [33]. Initially, it was propagated in newborn mice and at some point the virus was subsequently propagated in Vero E6 cells.

It was found that this method of inoculation resulted in infection of Jamaican fruit bats with pathological symptoms and deaths. With the exception of the first four bats that were euthanized on schedule (two each on days 4 and 8), all remaining bats either died or became moribund and were euthanized for humane reasons. None of the bats survived past day 18 of the experiment.

In the second experiment, dose and route were partitioned between four groups (10^4 virus SC or IN, or 10^6 virus SC or IN) to determine the effect of dose and route. Three of the eight inoculated bats became moribund during the experiment and each was from high-dose groups.

In both experiments symptoms of moribund bats included poor response to mechanical stimuli, abnormal wing, ear and head tremors, inability to coordinate movement, weight loss and inability to fly when released. These symptoms are consistent with previous findings from Downs et al. [33].

Results from oral and rectal swabs suggest that some infected bats shed virus from excrement, which typically occurs in rodent reservoirs of other arenaviruses [71, 73]. In the first experiment, not all bats shed virus; however the two that had negative oral or rectal swabs, were euthanized on day 4 PI and were not likely to be shedding virus that early in the infection. The remaining 10 bats all showed positive oral or rectal swabs at some point during the experiment (Table 4, see page 87). Many bats appeared to be shedding virus either orally or rectally on specific days during the experiment, and not on other days. For example, in Table 4 (see page 87), bat number 720 appeared to be shedding virus rectally on days 6, 10, 12, and 16 PI, but not on day 8 PI. It is likely they were shedding virus, although it may not have been enough to be detected by PCR. This pattern was observed for many of the oral and rectal swabs collected for each of the bats in both experiments (Tables 4 and 5, see pages 87 and 88). During the first experiment, all remaining bats appeared to be shedding virus by day 10 PI. A similar pattern was observed in the second experiment, as all bats appeared to be shedding virus either orally or rectally by day 18 PI. The longer period of time between inoculation and virus shedding in the second experiment could have been due to bats receiving lower doses of virus, or the single route of inoculation. While three bats became moribund and were euthanized during the second experiment, the other

nine remained healthy and appeared to still be shedding virus (by PCR) on day 45 PI without pathological symptoms. However, it is uncertain if infectious virus was still present in the intestines of the surviving bats.

Organ sample PCR results from the first experiment showed all but one bat had viral RNA in various organs. The one negative bat was euthanized on day 4 likely before virus could be detected in the organs. There appeared to be no discernable pattern of virus distribution in the organs except for first detection in the spleen, and last in the heart and brain (Tables 6 and 7, see pages 89 and 90). It is possible that once virus reaches the brain death ensues rapidly. However, not all bats that became moribund or died in the first experiment had detectable virus in the brain. In the second experiment, only three bats were positive for virus in the organs, and there was no pattern of virus progression through the organs over time. For each experiment, virus isolation results were congruent with PCR results (Tables 6 and 7, see pages 89 and 90).

Neutralizing antibodies were found in the terminal serum samples of three bats from second experiment. These results suggest the bats mounted an immune response that cleared the virus.

Virus transmission was examined during the second experiment by including one uninfected bat in each experimental group. All uninfected bats were negative for virus by PCR on collected organ samples; however, all uninfected bats showed positive oral or rectal swabs for virus by PCR at some point in the experiment. None of these bats seroconverted nor had virus detected in organs by PCR or virus isolation. With no virus being detected in the organs of uninfected bats by PCR or virus isolation, it is likely that oral and rectal swab PCR results represent detection of contaminating virus RNA from the infected bats. This is suggested because these bats huddle closely together and typically engage in grooming activities of one another. Grooming and close contact

could lead to viral RNA detection in uninfected bat swabs. Thus, this experiment provides no support for transmission.

Histopathology from the first experiment revealed multiple organ involvement in TCRV disease. Pneumonia was noted in all but one bat and the livers and spleens had pathological changes as well. Three bats exhibited brain lesions and each exhibited tremors and were PCR positive for TCRV RNA, while a fourth brain PCR positive bat died early in the experiment on day 11 also exhibited tremors but had no remarkable brain lesions by histopathology. Tremors are also a feature of some cases of South American hemorrhagic fevers [209], suggesting bats as a potential model for human disease.

These experimental infections show that Jamaican fruit bats are susceptible to TCRV and that a high dose results in significant and fatal disease. Low-dose infections appeared to result in no disease, seroconversion in some and no detection of virus in organs. It was not possible to demonstrate transmission between bats despite the close contact of infected and uninfected bats for 45 days. Together, these data are not supportive of the hypothesis that Jamaican fruit bats are reservoirs of TCRV.

The distributional range of Jamaican fruit bats is throughout tropical Central and South America, the West Indies, including the Greater and Lesser Antilles, Trinidad and Tobago, the Yucatan and the Florida Keys [175]. Jamaican fruit bats are capable of flying long distances to forage [49, 175, 210, 211] and genetic studies indicate bidirectional movement of Jamaican fruit bats between the Antilles islands and mainland Mexico and South American countries [211, 212]. Trinidad, where TCRV TRVL-11573 was isolated, is 10 km from Venezuela and within the flight range of *Artibeus* bats [211]. It is possible that Jamaican fruit bats from which TCRV was first isolated may have been infected with TCRV in Venezuela or elsewhere and introduced the virus to the Antillean islands before experiencing pathological symptoms (which took 11 days to occur in the

work presented here). This could account for the lack of serological evidence in other mammals surveyed in Trinidad as reported by Downs et al [33].

The 19 isolates reported by Downs et al. (from 6 *A. jamaicensis* and 5 from *A. lituratus)* have apparently been lost, with the exception of TRVL-11573; although they were indistinguishable by complement fixation testing [33]. It is also possible that passage of TCRV TRVL-11573 in mice and cell culture has led to the selection of mutations that have altered the virus' infectious characteristics that are reported here, such that wild-type viruses may indeed be hosted by *Artibeus* bats but with different infectious outcomes. It is also possible that the routes of infections used (SC or IN; uninfected cage mates) do not replicate natural transmission, which could alter infectious outcome. Without having any of the original 19 isolates it is impossible to determine, thus additional fieldwork would be required in an attempt to reisolate virus. Finally, it is important to note that TRVL-11573 was isolated from a greater fruit-eating bat and thus its characteristics may differ in Jamaican fruit bats.

With other arenavirus infections, such as Guanarito virus infection of its natural reservoir, the cane mouse (*Zygodontomys brevicauda*) [71], and hantavirus infections of their hosts [68] interactions indicate the viruses cause persistent infections but without pathology, which was not observed in experimental bat infections. Thus, the weight of evidence suggests that Jamaican fruit bats are not reservoirs of TCRV.

Figure 6. Tacaribe virus is lethal to Jamaican fruit bats. Two bats were euthanized on days 4 and 8 PI and were asymptomatic. However, on day 10 one bat was found dead and during the next 8 days remaining bats died or were euthanized for humane reasons.

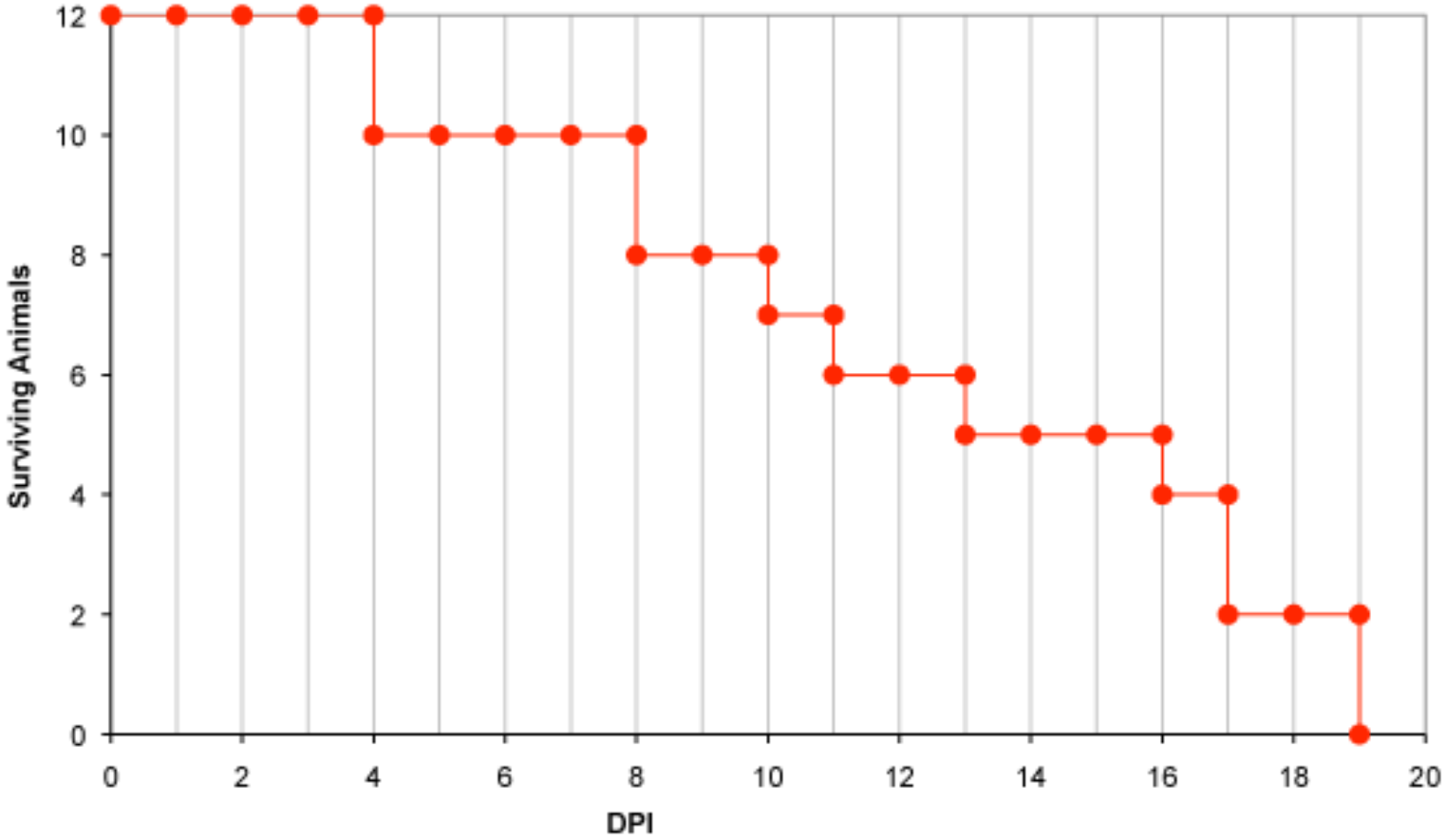


Figure 7. Weight changes after inoculation with Tacaribe virus. Weight loss was recorded for the 10^6 SC (A), 10^6 IN (B), 10^4 SC (C) and 10^4 IN (D) groups. Bats that survived had minimal weight fluctuations during the 45 day experiment. However, bats that became moribund (674, 676 and 677) each had weight decline prior to euthanasia. Each number represents an individual bat.

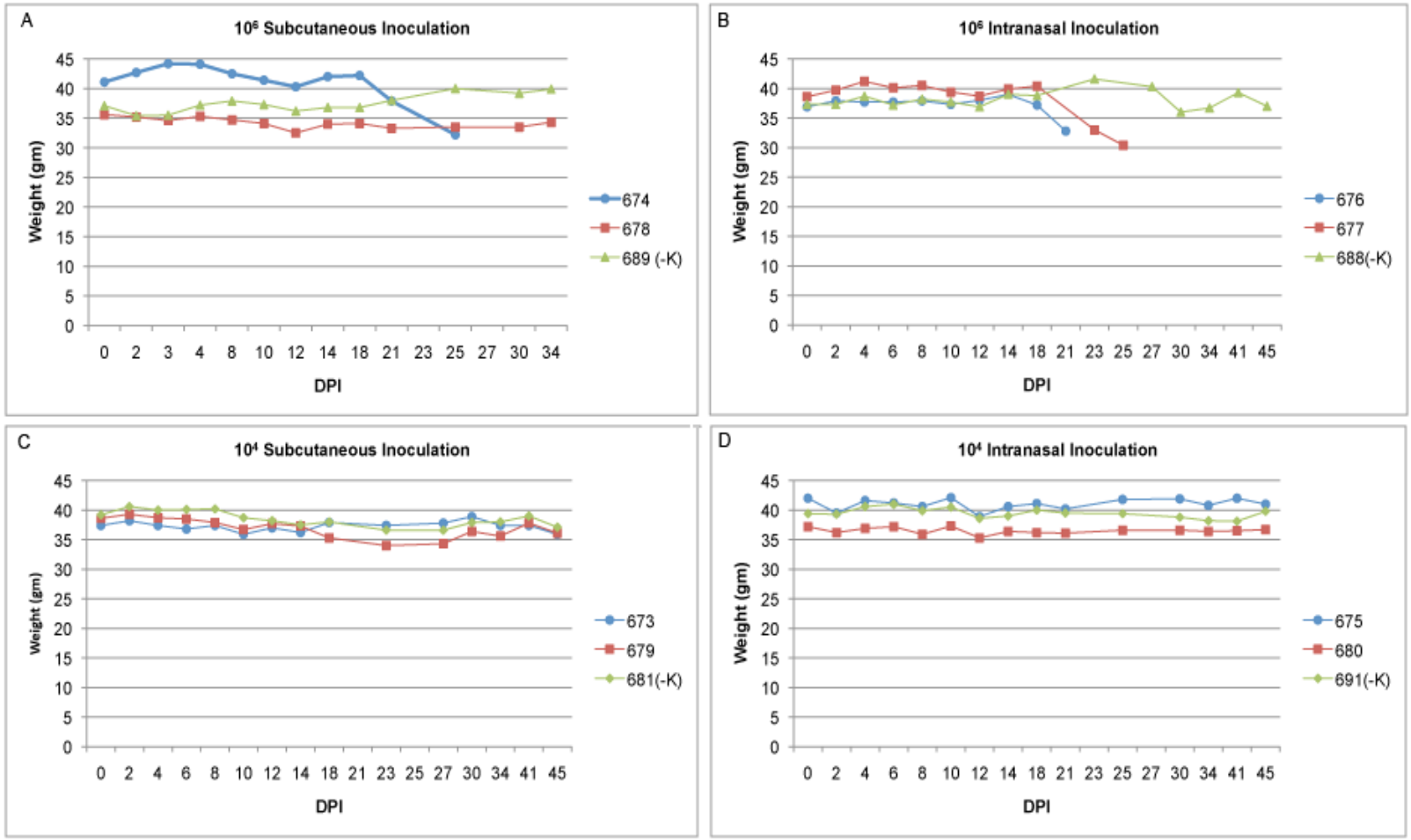


Figure 8. Histology of tissues from TCRV-infected bats in initial experiment. Bat 712 with encephalitis and multifocal gliosis (A). Bat 712 with lymphocytic meningitis (B). Bat 712 with interstitial pneumonia (C). Bat 718 with multifocal myocardial degeneration (D). Bat 718 with focal hepatic necrosis (E).

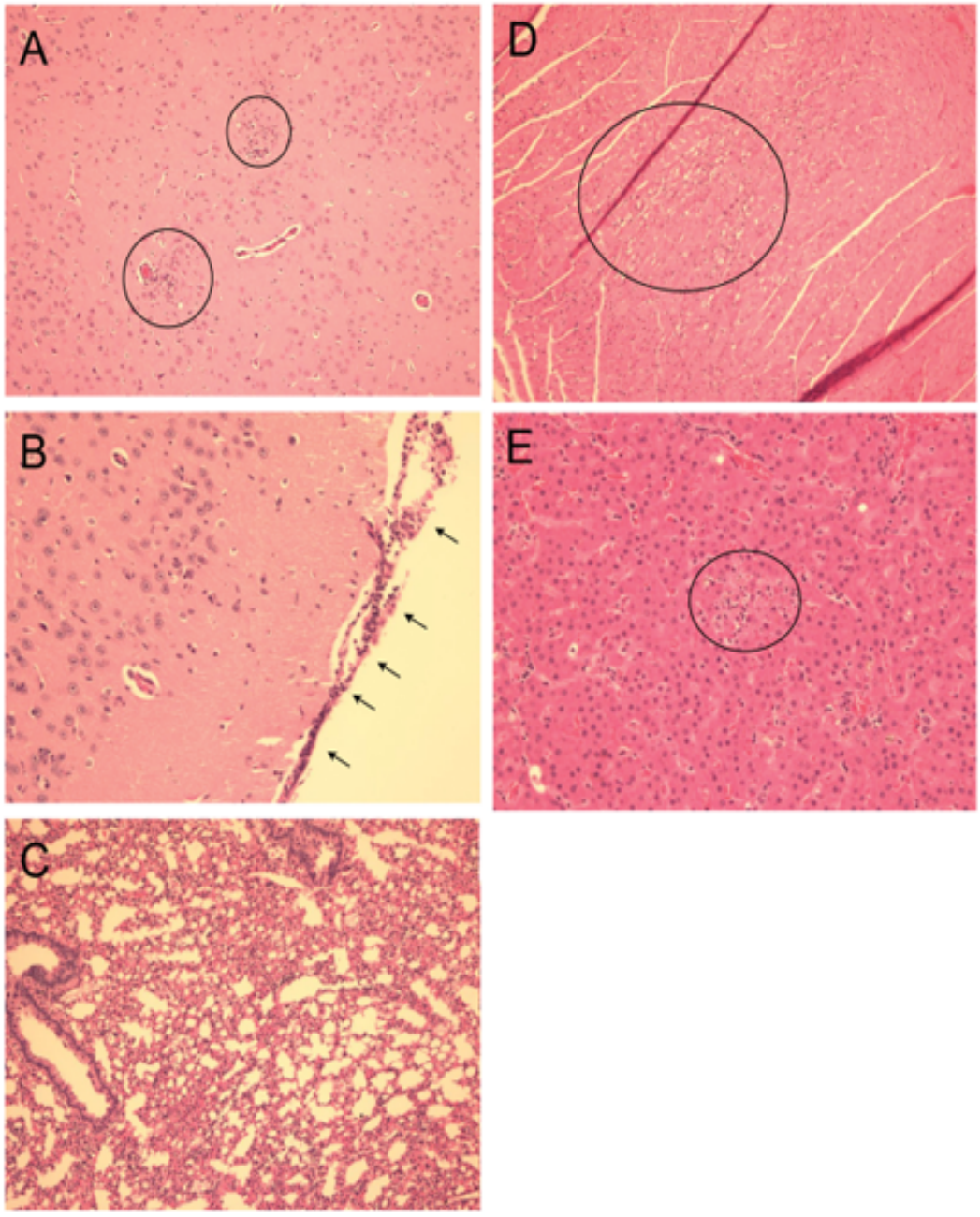


Table 4. Oral and rectal swab PCR results from the first experiment. An X indicates a PCR positive swab. Shaded in boxes indicate days that swabs were not collected from bats that died or were euthanized.

Bat	DPI Euth/Died	DPI	0	2	4	6	8	10	12	16
		Swabs								
645	4	Oral								
		Rectal								
647	4	Oral								
		Rectal								
747	8	Oral				X	X			
		Rectal								
705	8	Oral								
		Rectal				X	X			
714	11	Oral								
		Rectal						X		
703	12	Oral								
		Rectal					X			
718	13	Oral				X			X	
		Rectal						X	X	
748	16	Oral							X	
		Rectal						X	X	
712	17	Oral								
		Rectal						X		
720	17	Oral								X
		Rectal				X		X	X	X
729	18	Oral								X
		Rectal				X			X	
738	18	Oral						X		X
		Rectal						X		

Table 5. Oral and rectal swab PCR results from the second experiment. An X indicates a PCR positive swab. Shaded in boxes indicate days that swabs were not collected from bats that died or were euthanized.

Bat	Dose/Route	DPI	Days:	0	2	4	6	8	10	12	14	18	23	27	30	34	41	45
			Swabs															
691	UI 10^4 IN	45	Oral									X	X		X	X		
			Rectal						X					X		X		
680	10^4 IN	45	Oral				X					X	X		X	X		
			Rectal					X										
675	10^4 IN	45	Oral				X	X							X	X		
			Rectal											X			X	X
688	UI 10^6 IN	45	Oral				X					X		X		X		
			Rectal									X		X		X		
677	10^6 IN	25	Oral						X	X		X						
			Rectal							X	X	X	X					
676	10^6 IN	21	Oral							X		X						
			Rectal							X								
681	UI 10^4 SC	45	Oral									X				X		X
			Rectal											X		X		
679	10^4 SC	45	Oral					X				X			X	X		X
			Rectal					X				X		X	X	X		X
673	10^4 SC	45	Oral				X					X			X	X	X	
			Rectal					X	X			X				X		X
689	UI 10^6 SC	45	Oral					X						X		X		
			Rectal						X			X		X			X	
678	10^6 SC	45	Oral				X					X		X	X	X		
			Rectal							X		X	X	X	X	X	X	
674	10^6 SC	23	Oral		X				X			X						
			Rectal					X	X			X	X					

Table 6. Organ sample PCR and virus isolation results from the first experiment. An X indicates PCR positive organ samples. An O indicates PCR positive as well as virus isolation positive organ samples. Bat numbers marked with an asterisk indicate bats that experienced neurologic symptoms. DPI Euth/Died numbers shown in bold indicate bats that were found dead.

Bat	**645**	**647**	**747**	**705**	**703**	**714***	**718**	**748**	**712***	**720***	**729***	**738**
DPI Euth/Died	4	4	8	8	**10**	11	**13**	**16**	17	17	18	**18**
Organ												
Testes						X	X	X		X	X	
Kidney			X	X		X	X	X		X	X	
Heart						X	X				X	
Lung			X	X	X	**O**	**O**	X		X	X	
Liver			X	X		X	**O**	X		X	X	
Spleen	X		**O**	**O**	X	X	X	X		X	X	X
Lg. Intestine			X	X	X	X	**O**	**O**		X	X	X
Sm. Intestine				X		X	X	**O**		**O**	**O**	X
Brain									X		**O**	X

Table 7. Organ sample PCR, virus isolation, and neutralizing antibody test results from the second experiment. An X indicates PCR positive organ samples. An O indicates PCR positive as well as virus isolation positive organ samples. Bat numbers marked with an asterisk indicate bats that experienced neurologic symptoms. Boxes marked with an asterisk * denote bats positive for neutralizing antibody.

Bat	**691**	**680**	**657**	**688**	**677***	**676***	**681**	**679**	**673**	**689**	**678**	**674***
Dose/Route	**UI** 10^4 IN	10^4 IN	10^4 IN	**UI** 10^6 IN	10^6 IN	10^6 IN	**UI** 10^4 SC	10^4 SC	10^4 SC	**UI** 10^6 SC	10^6 SC	10^6 SC
DPI Euth/Died	45	45	45	45	25	21	45	45	45	45	45	23
Organ												
Reproductive												
Kidney					X							
Heart												
Lung					X							X
Liver												
Spleen					**O**							X
Lg. Intestine					X							
Sm. Intestine					**O**							X
Brain					X	X						**O**
Salivary												
Neut. Ab								*	*		*	

Table 8. TCRV primer sequences listed 5' to 3'.

Forward TCRV PCR primer	Reverse TCRV PCR primer	Size (bp)
AATTTGCGATCGAGAGCCTA	AGCTCATCCCAAACCATGAG	316

CHAPTER V

WORK TOWARD THE DEVELOPMENT OF A RAPID FIELD IMMUNOASSAY FOR DETECTING ANTIBODY TO TACARIBE VIRUS IN MAMMALS

Abstract

Work to develop a rapid field immunoassay that could be used for detection of antibody to Tacaribe virus in mammals has been initiated. Tacaribe virus is an arenavirus that was isolated from Jamaican fruit bats in Port of Spain Trinidad during a rabies virus surveillance program in the early 1960s. The natural reservoir of TCRV is uncertain, but due to the original isolation from bats, it was hypothesized that species of *Artibeus* bats were the natural reservoir. If it were found that Tacaribe virus is hosted by *Artibeus* bats it would be unusual because arenaviruses, for which the hosts are known, are rodent-borne viruses. By way of experimental infections, it has recently been identified that Jamaican fruit bats are not likely the reservoir of Tacaribe virus. These findings reveal that the reservoir host to this arenavirus is still unknown. Development of a rapid field immunoassay could be advantageous in discovering the natural reservoir of Tacaribe virus by allowing the rapid screening of many mammalian species in the field. A project to develop a rapid immunoassay is proposed by cloning and expressing a large fragment of the Tacaribe virus nucleocapsid for use as an antigen in an immunoassay using protein A/G-HRP conjugate.

Introduction

Arenaviruses belong to the family *Arenaviridae* and the genus *Arenavirus.* They are enveloped and have a single-strand, ambisense RNA genomes [167]. Arenaviruses have two gene segments, the large (L) segment and the small (s) segment. The large gene segment encodes an RNA-dependent RNA polymerase and a zinc binding protein called RING finger protein Z. The small segment encodes the nucleocapsid protein and a glycoprotein precursor. The structure of the arenavirus nucleocapsid is coiled and circular in arrangement [168, 169, 213].

Arenaviruses are assigned to two categories; the Lassa virus complex (Old World), and the Tacaribe virus complex (New World). The Lassa virus complex includes Lassa virus, found in Africa, which is the etiologic agent of Lassa fever in humans, Lymphocytic Choriomeningitis virus, which can cause aseptic meningitis in humans, and Lujo virus, which causes hemorrhagic fever in humans [35, 37, 75, 84, 170]. The Tacaribe virus complex includes Junín, Machupo, Sabia, Guanarito, and Chapare viruses, which cause Argentine hemorrhagic fever (HF), Bolivian HF, Brazilian HF, Venezuelan HF, and an as yet unnamed hemorrhagic fever respectively [35-38]. Arenaviruses, for which the hosts are known, are rodent-borne viruses. Many arenaviruses can establish persistent infections in the rodent hosts without pathology [40, 71, 172]. Phylogenetic studies have found that a tight coevolutionary relationship between arenaviruses and their rodent hosts may exist, which could account for the ability of the viruses to establish persistent infections without causing pathology [38, 67, 214].

Tacaribe virus (TCRV) is included in the Tacaribe virus complex, but it is not known to cause natural human infection and does not have a known rodent reservoir. TCRV was isolated from two species of *Artibeus* bats (*A. jamaicensis* and *A. lituratus*), during a rabies surveillance program in Port of Spain Trinidad in the early 1960s [33].

The isolation of TCRV from species of bats and the inability to identify TCRV in any other species has led researchers to hypothesize that *Artibeus* bats are the natural reservoir [33].

Experimental infections of Jamaican fruit bats with TCRV were performed to examine the virus-host interactions. It was hypothesized bats would become persistently infected with TCRV without developing pathology if they were reservoirs. Failure to develop persistent infections, and the development of pathogenic symptoms indicated that Jamaican fruit bats are not the reservoir of Tacaribe virus (Unpublished results- Chapter IV). These findings reveal that the natural reservoir of TCRV remains unknown. Serologic surveys of mammalian species in regions where *Artibeus* bats are distributed are necessary to identify the natural reservoir. The aim of the study presented here is to develop a rapid immunoassay for the detection of TCRV-specific antibody.

Immunoassays, such as sandwich ELISAs have been developed for the detection of Tacaribe virus and other arenavirus antigens, but these are not useful for the rapid detection of virus-specific antibody in the field [179-181]. Sandwich ELISAs can be used to detect current viral infections since they are designed to bind viral antigen, but they are not useful for detecting antibody that has been produced in response to a current or past infection. Indirect ELISAs are used to detect virus-specific antibody in serum samples of mammals. Many of these immunoassays include the use of whole virus as the antigen and species-specific secondary detection antibodies [110, 215].

Due to these requirements, many immunoassays may require 3 to 5 hours to complete, and thus are not conducive to rapid screening projects in the field. There is currently no indirect ELISA that may be used for the rapid detection of TCRV-specific antibody in the field. A rapid field immunoassay was recently developed for screening

populations of deer mice for SNV-specific antibody [16, 182, 216]. Development of a rapid indirect immunoassay would be advantageous for the detection of TCRV-specific antibodies in serum samples of bats captured in the field. Development of such an assay has required the identification of immunodominant epitopes in arenaviruses, and the production of an antigen that would be bound by TCRV-specific antibodies.

Studies using monoclonal antibodies (Mabs) have identified that arenaviruses have several different antigenic sites. There are 4 antigenic sites on the nucleoprotein, 2 on glycoprotein 1 and 6 on glycoprotein 2 [217, 218]. Another study used sequencing technology to examine antigenic characteristics of different types of arenaviruses. It was found that several shared amino acids (NP 118-126) within the nucleoprotein of arenaviruses make up the immunodominate cytotoxic T-lymphocyte (CTL) epitope in mice. The authors used amino acid alterations in the area that encodes this epitope to confirm that it is involved in binding CTLs and majorhistocompatability complex molecules (MHC) [219]. It is likely that an immunodominant epitope specific for binding TCRV-specific antibody can also be found on the nucleocapsid of TCRV. For this reason, it has been an aim of this project to clone and express a large fragment of the TCRV nucleocapsid.

Here, work that has been performed to develop a rapid field immunoassay to detect Tacaribe virus-specific antibody is described. A protocol that had previously been described for the development of a rapid protein-A/G horseradish peroxidase enzyme-linked immunosorbent assay (PAGEIA) that detects antibody to SNV was followed [16, 110].

Materials And Methods

RNA extraction

TCRV viral RNA was extracted from supernatant of Vero E6 cells that had been used to propagate Tacaribe virus strain TRVL-11573 using QIAamp (Qiagen, Valencia,

CA) Viral RNA mini kits according to the manufacturer's instructions. RNA samples were immediately used in reverse transcription PCR and were then frozen at -80° C.

Reverse transcription and PCR

Reverse transcription (RT) and gene amplification were both performed using TCRV specific primers (Table 9, see page 102) and a SuperScript III One-Step RT-PCR System with Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA). The reverse primer sequence used for this amplification was a primer that had been designed in previous studies [38]. cDNA synthesis and PCR amplification were performed using 1 cycle of 55°C for 30 minutes, 1 cycle of 94°C for 2 minutes, 40 cycles of 94°C for 30 seconds, 56°C for 30 seconds, 72°C for 2 minutes. After 40 cycles, reactions were held at 68°C for 5 minutes followed by a hold at 4°C until used. PCR cycle parameters detailed in the SuperScript III One-Step RT-PCR system were used. PCR products were resolved and visualized using a 1% agarose gel.

Cloning and sequence analysis

PCR products were cloned into TOPO-TA (Invitrogen, Carlsbad, CA) cloning vector according to the manufacturer's directions. Clones were then used to transform One Shot Chemically competent *E. coli* cells (Invitrogen, Carlsbad, CA) that were subsequently plated and grown on LB/ampicillin plates. Bacterial colony screening was performed using a Promega (Madison, WI) 2X PCR master mix kit and TCRV primers to verify plasmid insert. Amplification was performed using 35 cycles of 94°C for 30 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for 2 minutes. After 35 cycles, the reactions were held at 72°C for 10 minutes and finally held a 4°C until used. Amplified PCR samples were then resolved and visualized using a 1% agarose gel. Colonies with the TCRV insert were then incubated at 37°C overnight in 4 mL of LB broth with 50 μg/ml ampicillin. Plasmids were then purified using a QIAprep spin

Miniprep kit (Qiagen, Valencia, CA) according to manufacturer's directions. Plasmid recovery was confirmed using a 1% agarose gel. Plasmids were sent to SeqWright Inc. (Houston, TX) for sequencing using T7, T4, and internal TCRV primers (Table 10, see page 102). Sequencing files were edited using Sequencher (GeneCodes, Ann Arbor, MI) and BLAST was used for gene identification.

Addition of restriction sites

Following sequence identification of the plasmid insert as TCRV nucleocapsid, virus specific primers were designed with restriction enzyme sites flanking each primer (Table 11, see page 102). The forward primer was designed with an EcoRI site and the reverse primer was designed with a XhoI site. Primer design was carefully performed to ensure that the amplified portion of the nucleocapsid could be inserted into the expression vector in the correct reading frame for protein expression. PCR was performed on TOPO plasmids that contained the TCRV nucleocapsid. PCR products containing the EcoRI and XhoI overhangs were cloned back into TOPO cloning vector and used to transform One Shot Chemically competent *E. coli* cells (Invitrogen, Carlsbad, CA). Plasmid purifications were performed as described earlier, and plasmids were sent out for sequencing to confirm the presence of the restriction sites on either side of the TCRV nucleocapsid fragment.

Sub-cloning Into pET-21b

TOPO plasmids containing the TCRV nucleocapsid gene insert and pET-21b plasmids (Novagen, Sandiego, CA) were digested at 37°C for 4 hours using EcoRI and XhoI restriction enzymes in Buffer H (Promega, Madison, WI). Digests were resolved on a 1% agarose gel and gel extraction was performed using Wizard SV Gel and PCR Cleanup System (Promega, Madison, WI). Gel extracted digests were then ligated at 4°C overnight using T4 ligase (Promega, Madison, WI). Ligations were then used to transform competent NovaBlue *E. coli* cells (Novagen, San Diego, CA). Bacterial colony

screens and plasmid minipreps were performed as previously described. Plasmids were sent to SeqWright Inc. (Houston, TX) for DNA sequencing using T7, T7 terminator, and internal TCRV primers.

Results

A 1588 bp fragment of the TCRV nucleocapsid gene (nt 1716-3303) was successfully cloned into TOPO TA cloning vector with EcoRI and XhoI restriction sites flanking it. Sequence analysis showed that EcoRI and XhoI restriction sites are flanking the TCRV gene, and BLAST searches have confirmed that the gene insert that is present in TOPO TA is TCRV. It is believed that the TCRV nucleocapsid fragment has been successfully cloned into the pET-21b expression vector in the correct reading frame needed for protein expression with an attached his-tag, but confirmation by sequence analysis has not been successful. Plasmid purification from NovaBlue *E. coli* cells transformed with pET-21b vector yield low concentrations of plasmid. The concentration of the plasmid purification preparations has been so low that subsequent sequencing reactions have been unsuccessful.

Discussion

pET-21b

The expression vector, pET-21b, is ideal for expressing and purifying a portion of the TCRV nucleocapsid protein for several reasons. First, it has ampicillin and kanamyacin resistance genes, which allow for selection of bacterial colonies that have been transformed with the vector. This helps to ensure that bacterial colonies have been transformed with the expression vector and the TCRV gene insert. Also, pET-21b has a Lac operon encoded in its sequence, which allows for stimulation of protein expression using isopropyl β-D-1-thiogalactopyranoside (IPTG). IPTG is molecularly similar to allalactose. Treatment of bacterial cultures with IPTG causes stimulation of the Lac operon and thus the expression of gene fragments that have been cloned into

pET-21b. The pET-21b plasmid also encodes a 6x histidine tag (His-tag) that will be added to the C-terminus of the TCRV polypeptide during protein expression. Correct expression of the protein with the attached His-tag requires that the gene fragment be inserted into the vector in the correct reading frame. After protein expression, the TCRV nucleocapsid protein will have a C-terminal His-tag attached. This His-tag will allow for the purification of the TCRV antigen using column chromatography. These characteristics make the pET-21b vector ideal for expression of the TCRV antigen.

Problems that could possibly occur while trying to express the TCRV antigen include bacterial cell culture complications yielding low concentration of the expressed antigen, or that the insert is not in the correct reading frame for expression with an attached His-tag. One possible limitation to this approach include that the TCRV insert may be too large and could be problematic when expressing the protein. If this were the case, it might be necessary to identify the specific (smaller) fragment of the TCRV nucleocapsid that is the immunodominate epitope and only express that portion of the protein. A second limitation to this approach is that the expressed TCRV nucleocapsid antigen could possibly cross-react with antibodies that are specific for other closely related arenaviruses. Analysis of antibody binding capability would be necessary to determine if this is a problem.

Current status of the project

Sequence analysis has confirmed the successful cloning of a 1588 bp fragment of the TCRV nucleocapsid gene into TOPO TA cloning vector with EcoRI and XhoI restriction sites flanking it. It is believed that this fragment has been successfully cloned into pET-21b expression vector, but confirmation has not been obtained. A concentration of 200 ng/μl of DNA is required for quality DNA sequencing and we believe that previous attempts to sequence pET-21b vector have not been successful because it is a low-copy vector. Plasmid purifications using NovaBlue cells and pET-21b

do not yield sufficient concentrations of plasmid to allow DNA sequencing. It is likely that Maxiprep plasmid purification or a precipitation reaction of Miniprep plasmid purification yields may allow the acquisition of high enough concentrations to perform DNA sequencing.

Strategies for producing and purifying the antigen

Once sequence analysis confirms that the entire TCRV nucleocapsid insert is present in the expression vector, in the correct reading frame, without any deletions, it will be used to transform BL21 expression *E. coli* cells (Novagen, San Diego, CA). BL21 cells will be used for expression of the protein, and the antigen will be purified using column chromatography. These methods will be adapted from previously described methods [16, 110, 220]. Briefly, BL21 cultures will be treated with IPTG to stimulate protein expression from the Lac operon. Cells will then be lysed and extractions will be collected. Extractions will then be filtered by column chromatography using a cobalt column. This is also known as metal chelate-affinity chromatography [220]. Purified fractions will then be subjected to Western Blot analysis using anti-TCRV mouse ascites fluid as the primary antibody, and an anti-mouse alkaline phosphatase-conjugated antibody as the secondary antibody.

The purified fragment of the TCRV nucleocapsid will then be used as the antigen that is coated on 96-well polyvinyl chloride plates and used for the detection of TCRV-specific antibodies in mammalian serum samples. A rapid field test similar to the one described by Schountz et al. [16], will be developed. This will include 96 well polyvinyl chloride plates coated with the TCRV antigen and blocked using porcine gelatin prior to performing work in the field. Serum samples from wild-caught mammals will be added to the wells, and staphylococcal protein-A and streptococcal protein-G horseradish peroxidase (protein A/G-HRP) conjugate will be used to detect the presence of anti-

TCRV specific antibodies. Results will be visualized with the addition of activated ABTS substrate. Washes will be performed using PBS with 0.5% Tween-20 [16].

The immunoassay proposed here will likely take an hour to complete in the field whereas other similar assays can take as long as 3 to 5 hours [16, 182, 216]. The use of protein A/G-HRP makes the use of an anti-bat or other species-specific antibody unnecessary. It has been found that protein-A and protein-G HRP conjugates have the ability to bind the Fc regions of antibodies of a number of different mammalian species and can detect immunoglobulin M (IgM), IgA, and high affinity IgG [110]. These characteristics make this immunoassay ideal for screening wild mammals for TCRV-specific antibody.

The cloning a large fragment of the TCRV nucleocapsid into TOPO-TA cloning vector has been accomplished and it is believed that it has now been ligated into pET-21b expression vector. It is also believed that DNA sequence analysis will soon reveal that the TCRV nucleocapsid is in correct reading frame for protein synthesis with an attached 6x His-tag on the C-terminus of the protein. It is hypothesized that protein expression and purification will be possible. Tacaribe virus has an unknown natural host and it is likely that this antigen can be used in a rapid field immunoassay to screen mammals for TCRV-specific antibody. Due to the broad species specificity of this assay and the ease with which it might be used in the field, it is believed that it could be instrumental in identifying the natural reservoir of Tacaribe virus.

Table 9. Primer sequences used to amplify a fragment of the TCRV Nucleocapsid. Sequences listed 5' to 3'. Reverse primer sequence was found in Bowen et al., 1996.

Forward TCRV PCR primer	Reverse TCRV PCR primer	Size (bp)
AGTCTTACAGCTCACAGTGC	CGCACAGTGGATCCTAGGC	1800

Table 10. Primer sequences used in DNA sequencing of internal fragments of the TCRV nucleocapsid. Sequences listed 5' to 3'.

Forward TCRV PCR primer	Reverse TCRV PCR primer	Size (bp)
AATTTGCGATCGAGAGCCTA	AGCTCATCCCAAACCATGAG	316

Table 11. **TCRV primers used for the addition of restriction sites.** Sequences listed 5' to 3'. Restriction sites are underlined in each primer.

Forward TCRV PCR primer	Reverse TCRV PCR primer	Size (bp)
CCGAATTCGGGATTGAGTCAATTCACC	TACTCGAGTAGTTCTCCACTCATGGC	1595

CHAPTER VI

CONCLUSONS

Bats comprise one of the largest groups of known species of mammals and they have tremendous ecological importance. The general biology and ecology of bats has been extensively studied, yet many aspects of bat biology are still poorly understood. In the past decade, bats have been recognized as reservoirs of potential reservoirs of important human and veterinary pathogens. However, virtually nothing is known about the immune systems or immune responses of bats. This is a significant gap in the knowledge of bat biology.

Only recently have researchers begun to investigate this aspect of bat biology. In many instances, it appears that bats may become persistently infected with viruses without developing disease. This type of interaction is commonly seen between viruses and the natural reservoir. Spillover infections with many of the same viruses into human other mammalian populations can cause serious disease, including the South American hemorrhagic fevers. Not only may research on bat immunology help to fill the gap in knowledge that exists in bat biology, it could also provide insights to the mechanisms by which viruses are able to evade sterilizing immune responses, and persist in bat hosts without causing pathology. If researchers could identify the underlying mechanisms by which this interaction is accomplished, it could offer medically important information about possible treatments for human infections.

Another important reason for the examination of immune systems and immune responses of bats is the possible benefit this information could provide for bat

populations. Some interactions between bats and microorganisms can cause serious and fatal disease, and in many cases, this can be devastating to bat populations. It is possible that an understanding of bat immune systems and bat-microbe interactions could offer potential for solutions to this problem as well.

The work presented in this dissertation represents a significant step toward characterizing bat immune systems and the interactions that occur between bats and viruses. The results from cloning and characterization of bat cytokine genes have indicated that the cytokine genes of Seba's short-tailed fruit bats are highly conserved with respect to orthologous mammalian sequences. The high level of identity and similarity that was observed is likely an indication that the genetics of bat immune systems are very similar to the genetics of other mammalian immune systems. This could indicate that the regulation of immune genes during infection could contribute to the ability of bats to become persistently infected without developing disease. It is suggested that further research focus on examining the regulation of bat immune genes during infection. *In vitro* and *in vivo* experimental infections could be used to examine the modulation of immune genes during infection.

The results from experimental infections of Jamaican fruit bats with Tacaribe virus have indicated that Jamaican fruit bats do not become persistently infected with TCRV. Instead, it was found that high dose inoculations resulted in acute infections with severe and fatal disease, while low dose inoculations led to no disease with eventual virus clearance. These findings have led the conclusion that Jamaican fruit bats are likely not the reservoir of TCRV

It is speculated that when Downs et al. first isolated TCRV from *Artibeus* bats [33], infections were incidental. Due to the failure of an extensive search to identify a rodent reservoir of TCRV on the islands of Trinidad and Tobago [33], it is suggested that *Artibeus* bats came in contact with the virus in another location. *Artibeus* bats are found

in tropical regions of South America, the Caribbean Islands, the Florida Keys, and Central America [175]. Trinidad, where TCRV was first isolated is approximately 10 km from Venezuela. This distance is within the flight range of *Artibeus* bats [211]. It is possible that Jamaican fruit bats from which TCRV was first isolated may have been infected with TCRV in Venezuela and introduced the virus to the Antillean islands before experiencing pathological symptoms.

It could also be speculated that the natural reservoir of TCRV is a rodent species. This would make sense in light of the fact that all arenaviruses, for which the hosts are known, are rodent-borne viruses. More specifically, the known reservoirs of Junín, Machupo, and Guanarito viruses, (*Calomys musculinus*, *Calomys callosus* and *Zygodontomys brevicauda* respectively), are all rodents in the subfamily Sigmodontinae [71, 125, 170]. These rodents are known as the New World cricetines [221]. There are several genera of these rodents located in the areas where *Artibeus* bats are also located. There are five genera of New World cricetines that inhabit the Republic of Trinidad and Tobago. These are *Oryzomys, Akodon, Zygodontomys, Rhipidomys, and Nectomys* [221]. There are also 20 genera New World cricetines that inhabit areas of Venezuela. These include *Oryzomys, Akodon, Zygodontomys, Rhipidomys Nectomys, Neacomys, Aepeomys, Thomasomys, Neusticomys, Ichthyomys, Calomys, Holochilus, Sigmodon, Chilomys, Melanomys, Microorozomys, Oligorizomys, Sigmodontomys,* and *Oecomys* [221]. It is suggested that *Artibeus* bats came in contact with rodents or infectious secretions from rodents in Venezuela.

When considering the question of how bats might have come in contact with rodents or their secretions, it is suggested that a possible source could be rodents in the genus *Oecomys*. These rodents are found in Venezuela and are called the arboreal rice rats, and as the name implies, they live in trees [221]. While some *Artibeus* bats roost in caves, others utilize foliage or cavities in trees as a roost sites. It is possible that bats

could possibly be exposed to TCRV when roosting in close proximity to arboreal rice rats. While this is one possible route of exposure, it is feasible that bats could be coming in contact with other infected rodents.

Due to the lack of evidence of a reservoir of TCRV in Trinidad and Tobago, it is suspected that the natural reservoir of TCRV could be found in Venezuela. Venezuela is the closest country to Trinidad and Tobago and has a large number of mammalian species that inhabit it [221]. It is suggested that a serologic survey of mammals (especially rodents) in areas of Venezuela where *Artibeus* bats could have come in contact with TCRV, should be conducted. It may also be advantageous to conduct another serologic survey of mammals in the Antillean islands to ensure that nothing was previously missed.

Although the interaction between TCRV and Jamaican fruit bats appears to not be that of a virus and a natural reservoir, this study has provided important information about the interaction between TCRV and Jamaican fruit bats. The findings of this research project represent an important development in the world of arenaviruses, as it was previously believed that TCRV was the only arenavirus, for which the host is known, to have a non-rodent host. Evidence thus far supports that development of a rapid field immunoassay that can be used to detect TCRV-specific antibody will soon be available. The rapid field immunoassay may be useful in the identification of the true reservoir of TCRV.

Other future work that is recommended is the exploration of TCRV infection in Jamaican fruit bats as a disease model for South American hemorrhagic fevers. Due to the fact that Jamaican fruit bats infected with TCRV developed symptoms such as tremors, that are very similar to the symptoms experienced by humans infected with Junín virus, it is possible that Jamaican fruit bats could serve as an animal model for SAHF [172]. An animal model of SAHF could provide the opportunity to evaluate how

disease progresses in human cases, and offer the potential to identify more effective treatment options.

While many researchers fear that the identification and examination of bats as reservoirs may lead to the persecution of bats, it seems irrational to ignore such a large portion of bat biology and ecology. This fear of persecution and eradication of bats may be founded on real concerns and care must be taken with such a controversial topic. A balance must be found between examining the immune systems and immune responses of bats with bat conservation. Possible solutions to this problem include closely integrating the fields of bat ecology, conservation, disease ecology, and microbiology, as well as increased education of the general public on the ecological and economic importance of bats. Bats engage in many important activities including seed dispersal, pollination, and insect control, and thus, it must not be forgotten that bats have an enormous amount of ecologic significance. It must be remembered that conservation of bat species is necessary and that eradication of bat species could be counterproductive. However, it is important for immunologic research in bats to continue, as it is important to the comprehensive understanding of bat biology, and could offer potentially advantageous information in relation to the management of infectious disease in humans and bats.

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