DISSERTATION

OF BATS AND BUGS: CHARACTERIZING ARBOVIRAL TRANSMISSION AT THE HUMAN-WILDLIFE INTERFACE

Submitted by

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ABSTRACT

OF BATS AND BUGS: CHARACTERIZING ARBOVIRAL TRANSMISSION AT THE HUMAN-WILDLIFE INTERFACE

Bats and the viruses they carry have been of increasing interest to the scientific community since the determination nearly 20 years ago that severe acute respiratory syndrome coronavirus (SARS-CoV) likely originated in Chinese bats. However, studies into the relationship between bats and the pathogens they carry date back to the mid-20th century, when researchers began analyzing the susceptibility of bats to rabies and other viruses – many of them transmitted by mosquitoes. Since these initial explorations into bats' ability to serve as hosts of arboviruses, scientific and technological advancement have permitted countless discoveries surrounding viral evolution, host range, and transmission dynamics of these viruses. The dawn of whole-genome sequencing has permitted the rapid discovery and phylogenetic characterization of viruses from man, animal, and arthropod, and highly sensitive detection methods have improved our ability to detect pathogens in free-ranging animals.

Despite these advances, questions remain surrounding the role that bats and other wildlife may plan in the ongoing transmission of arboviruses. As we seek to more accurately define the term "viral reservoir," it is important to synthesize existing information and identify knowledge gaps on which to focus our research efforts. Chapter 1 of this dissertation summarizes current knowledge and provides examples of characterizing these tripartite relationships between virus, vertebrate host, and arthropod vector.

Within family *Reoviridae*, genus *Orbivirus* is comprised of 22 species vectored by mosquitoes, ticks, sandflies, and biting midges (*Culicoides*). In 2013, a novel orbivirus was isolated from an Egyptian fruit bat in Uganda, which was subsequently named Bukakata virus for the region of Uganda in which it was isolated. We sought to characterize the genome of this orbivirus and three other orbiviruses – Fomede virus, Ife virus, and Japanaut virus - isolated from Old World bats in the 20th century. Using whole-genome sequencing, we conducted phylogenetic analyses to ascertain in which arthropod vector clade each fell. The results of our sequencing and genome comparison suggest that Bukakata and Fomede viruses are both tick-borne orbiviruses, and likely serotypes of the Chobar Gorge serogroup (named for Chobar Gorge virus, which was isolated from *Ornithodoros* spp. ticks in Nepal). Japanaut and Ife viruses are likely vectored by either *Culicoides* biting midges or sandflies. To investigate growth kinetics in mammalian cell lines, we performed multi-step growth curves with Bukakata orbivirus and determined it replicates to high titers on Vero cells, BHK-13 cells (derived from the Syrian golden hamster), and R06E cells (derived from the Egyptian fruit bat, the species from which Bukakata was initially isolated). To contextualize these results within an ecological context, archived Egyptian fruit bat and *Ornithodoros* spp. tick samples were screened for BUKV RNA. Though no field samples were positive, this chapter provides a comprehensive framework in which to characterize novel arboviral isolates from bats.

After characterizing the genome and *in vitro* growth kinetics of a newly discovered arbovirus, a logical next step is to assess vertebrate host susceptibility, including pathogenesis, viremia profiles, and immune kinetics. Ideally, these studies are performed in ecologically relevant vertebrate hosts – in this case, the Egyptian fruit bat would be an ideal animal model, though only one captive colony of Egyptian fruit bats exists in North America. Captive colonies

of any bat species are valuable and rare, and Colorado State University is fortunate to maintain a colony of Jamaican fruit bats (*Artibeus jamaicensis*). We characterized the susceptibility of Jamaican fruit bats, a New World fruit bat, to Bukakata orbivirus, isolated from the Old World Egyptian fruit bat. Three bats were initially inoculated in our pilot study and all developed respiratory distress 12 days post-infection (dpi). Upon euthanasia, we noted extensive pathology in the lungs and injected vasculature in the other organs. Low levels of viral nucleic acid were detected in two of the bats, so we opted to further characterize pathogenesis over a timecourse study carried out to 15 days. While none of these bats had detectable viremia or viral RNA in tissues, four bats from later timepoints (2 at 10 dpi and 2 at 15 dpi) mounted a neutralizing antibody response. Seroconversion in the absence of detectable virus may speak to the unique innate and adaptive immune features characterized in many bat species, and likely indicative of an immune response sufficiently robust to neutralize and clear virus.

Shifting focus to another group of arboviruses, the third chapter of my dissertation used droplet digital PCR, a highly sensitive molecular diagnostic platform, to detect subgenomic flavivirus RNA in archived Ugandan bat samples. This molecular target has been shown to persist in host tissues longer than genomic RNA owing to its resistance to degradation by host exoribonuclease activity. While the ddPCR platform presented unique challenges, we did confirm presence of ZIKV sfRNA in four bats (from three species, all in family Pteropodidae). These results echo the findings of mid-20th century studies characterizing susceptibility of Old World fruit bats to ZIKV and other flaviviruses. Interestingly, while the product we amplified and sequence was only 123 base pairs, all four samples were most closely related to Asian lineage ZIKV (PRVABC59) which caused a large outbreak in the Americas in 2015. These results indicate that sfRNA is a valuable and sensitive target for detection of residual flavivirus

nucleic acid in host tissues, and highlight the need to further characterize flavivirus circulation in East African bat populations.

In chapter 5, I discuss current limitations in diagnostic platforms and sample types used in pathogen surveillance studies at present. In balancing the interests of conservation and public health, it is imperative to focus efforts on validation of non-lethal and minimally invasive sampling strategies. As such, I describe a protocol used for detection of haemosporidian parasite DNA from blood spots on filter cards. Unfortunately, the nucleic acid was degraded in a sizeable proportion of them, but nevertheless this pipeline will be valuable for future surveillance efforts and should be further investigated for detection of RNA viruses in samples. The second diagnostic pipeline described in this section discusses the use of non-lethal sampling for detection of coronavirus RNA in bats in captivity. As SARS-CoV-2 almost undoubtedly has evolutionary origins in the order Chiroptera, many questions remain surrounding the potential for New World bats to become infected. Given frequent interaction between bats and humans during fieldwork, rehabilitation, and management of captive populations in zoos and sanctuaries, zooanthroponotic transmission is a viable risk and ongoing monitoring is warranted. We tested the feces of juvenile bats in rehabilitation facilities for SARS-CoV-2 RNA, and none were positive. This fecal testing pipeline, however, could be used for monitoring of cross-species transmission in environments where interspecies contact is high.

Lastly, I discuss future directions for research investigating the potential for bats to act as reservoirs for arboviruses. Sample prioritization of vertebrate and arthropod species should be informed by previous experimental results and genetic analyses, and validation of non-lethal and minimally invasive sampling strategies will be paramount to protecting the health of both bats and humans. Lastly, building off of previous ‘arbovirus vertebrate reservoir’ guidelines proposed

by Goro Kuno and colleagues in 2017, I highlight opportunities and challenges in fulfilling these criteria when studying bats.

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CHAPTER 1: INTRODUCTION[1]

Bats and the viruses they harbor have been of interest to the scientific community due to the unique association with some high consequence human pathogens in the absence of overt pathology. Virologic and serologic reports in the literature demonstrate the exposure of bats worldwide to arboviruses (arthropod-borne viruses) of medical and veterinary importance (Calisher et al., 2006). However, the epidemiological significance of these observations is unclear as to whether or not bats are contributing to the circulation of arboviruses.

Historically, a zoonotic virus reservoir has been considered a vertebrate species which develops a persistent infection in the absence of pathology or loss of function, while maintaining the ability to shed the virus (e.g., urine, feces, saliva) (Abdussalam, 1959; Mims, 1975; Rodhain, 1998). Haydon et al. (2002) extended this definition of a reservoir to include epidemiologically connected populations or environments in which the pathogen can be permanently maintained and from which infection is transmitted to the defined target population. The significance of the relative pathogenicity of the infectious agent to the purported reservoir host has been debated (Haydon et al., 2002). In the case of bats as a reservoir species, rigorous field and experimental evidence now exist to solidify the role of the Egyptian rousette bat (*Rousettus aegyptiacus*) as the reservoir for Marburg virus (Towner et al., 2009; Amman et al., 2015; Schuh et al., 2017). Considering arboviruses, additional criteria must be met in order to consider a particular vertebrate species a reservoir. Reviewed by Kuno et al., these criteria include the periodic isolation of the infectious agent from the vertebrate species in the absence of seasonal vector activity, and the coincidence of transmission with vector activity (Kuno et al., 2017). Further, the vertebrate reservoir must also develop viremia sufficient to allow the hematophagous arthropod to acquire an infectious bloodmeal (Kading et al., 2014) in order for vector-borne transmission to occur. Bats have

[1] This chapter includes the complete manuscript cited as "Fagre, A.C. & Kading, R.C. (2019). Can bats serve as reservoirs for arboviruses? *Viruses 11*(3), 215. doi: 10.3390/v11030215". This article is reproduced with permission and only minimal modifications were made to meet formatting requirements.

long been suspected as reservoirs for arboviruses (Kumm, 1932), but experimental data that would support a role of bats as reservoir hosts for certain arboviruses remain difficult to collect. Here we synthesize what information is currently known regarding the exposure history and permissiveness of bats to arbovirus infections and identify knowledge gaps regarding their designation as arbovirus reservoirs.

1.1: What is known about bats' ability to host arboviruses?

1.1.1: Members of the order *Bunyavirales*

The order Bunyavirales is divided into eight families, four of which pose threats to public health and veterinary medicine – families *Nairoviridae*, *Peribunyaviridae*, *Phenuiviridae*, and *Hantaviridae* (Maes et al., 2018). While bats have been demonstrated to host hantaviruses, these viruses do not rely on an arthropod in their transmission cycle and thus will not be discussed (Guo et al., 2013). Viruses in order Bunyavirales that have been experimentally examined in bats or described in field studies are described in Table 1.1.

1.1.1.1: Family *Nairoviridae*, Genus *Orthonairovirus*

Members of the genus *Orthonairovirus* of medical and veterinary significance include Crimean Congo hemorrhagic fever virus (CCHFV) and Nairobi sheep disease virus (NSDV) (Maes et al., 2018). CCHFV is transmitted by ticks in genera *Rhipicephalus* and *Hyalomma* (Tonbak et al., 2006). While neither live virus nor nucleic acid of CCHFV has been detected from bats, serologic evidence suggests past infection of populations of bats across a diverse geographic range (Tkachenko et al., 1969; Saidi et al., 1975; Müller et al., 2016). Further, bats are often parasitized by both soft and hard ticks, which occupy a diverse range of ecological niches in endemic countries (Hornok et al., 2016; Luz et al., 2016; Schuh et al., 2016). A 2016 seroprevalance study by Müller and colleagues examining 16 African bat species (n = 1,135) found that the prevalence of antibodies against CCHFV was much higher in cave-

dwelling bats (3.6%–42.9%, depending on species) than foliage-living bats (0.6%–7.1%). They also screened 1,067 serum samples by RT-PCR, but all were negative for CCHFV nucleic acid (Müller et al., 2016). Experimental studies to assess the ability of bats to support replication of CCHFV have not been published.

1.1.1.2: Family *Peribunyaviridae*, Genus *Orthobunyavirus*

Members of the genus *Orthobunyavirus* include many viruses of importance to human and veterinary medicine, including Bunyamwera virus, California encephalitis virus, Jamestown Canyon virus, Kaeng Khoi virus, and La Crosse encephalitis virus (Maes et al., 2018), but limited evidence exists regarding the exposure or potential involvement of bats in the circulation of viruses in this family. Kaeng Khoi virus (KKV) has been isolated from cimicid bugs (Order: Hemiptera, Family: *Cimicidae*) (*Stricticimex parvus* and *Cimex insuetus*) and from suckling wrinkle-lipped bats (*Tadarida plicata*) in caves in Thailand, but was not isolated from soft ticks tested in the same area (*Ornithodorus hermsi*) (Williams et al., 1976). Additionally, KKV has been implicated in the case of several mine workers who reported illness and were discovered to have seroconverted(Neill, 1985), demonstrating spillover of this virus to humans in association with the cave environment, and suggesting that cimicids may play a role in vectoring virus between bat and human hosts. To date, no experimental data have been generated to address this hypothesis.

Spence and colleagues attempted to experimentally infect Jamaican fruit bats (*Artibeus jamaicensis*) via intramuscular injection with Nepuyo virus (Group C serogroup), yet no infectious virus was subsequently recovered from the bats (Spence et al., 1966). This is interesting considering two strains of Nepuyo virus were isolated from Jamaican fruit bats (*Artibeus jamaicensis*) and great fruit-eating bats (*Artibeus literatus*) in Honduras, and protective sera were found in Jamaican fruit bats in Trinidad (Calisher et al., 1971; Price, 1978). Bats of undetermined species were involved in a large serosurvey in Brazil that examined antibodies in wildlife against the Gamboa serogroup orthobunyaviruses, though none were found to be positive (Chiang et al., 2018). Seven and twelve species of Trinidadian bats were

examined for antibodies by HI against Caraparu (Group C serogroup) and Maguari (Bunyamwera serogroup) viruses, respectively, and were all found to be negative (Price, 1978).

1.1.1.3: Family *Phenuiviridae*, Genus *Phlebovirus*

Viruses in the genus *Phlebovirus* (family *Phenuiviridae*) of importance to human and animal health include Rift Valley fever virus (RVFV) and severe fever with thrombocytopenia syndrome virus (SFTSV) (Maes et al., 2018). Bats of the species *Miniopterus schreibersii* (n = 1) and *Eptesicus capensis* (n = 2) were experimentally infected with RVFV and the *M. schreibersii* bat's urine and liver tested positive for antigen (Oelofsen and Van der Ryst, 1999). A more recent study by Balkema-Buschmann and colleagues experimentally infected Egyptian rousette bats (*Rousettus aegyptiacus*) with vaccine strain MP-12 and recovered infectious virus from spleen and liver of some animals (Balkema-Buschmann et al., 2018). Oelofsen & Van der Ryst (1999) examined 350 samples from 150 field-caught bats in Africa, yet none were positive for antigen by use of ELISA (Oelofsen and Van der Ryst, 1999). Kading et al (2018) detected neutralizing antibodies against RVFV in Egyptian rousette bats and little epauletted fruit bats (*Epomophorus labiatus*) in Uganda, a country that has recently experienced human cases of RVFV (Kading et al., 2018; Nyakarahuka et al., 2018). Whether or not bats serve as a reservoir of RVFV during interepidemic periods remains to be determined.

1.1.1.4: Unclassified Bunyaviruses

Bangui virus (BGIV) is an unclassified bunyavirus and was isolated from an unidentified bat in the Central African Republic (CAR) (Chippaux and Chippaux-Hyppolite, 1965). Mojuí dos Campos virus (MDCV) is another ungrouped bunyavirus isolated from an unidentified bat species (Karabatsos, 1985; Wanzeller et al., 2002)

Table 1.1: Bat species with published results describing virus isolation, molecular evidence of infection, or seroconversion as they concern family *Bunyavirales*

Family	Virus	Virus Isolation/ Molecular Evidence	Serologic Evidence	Ref(s)
Naidoviridae, genus *Orthonairovirus*	Ahun virus	*Myotis mystacinus, Pipistrellus pipstrellus*		(Dacheux et al., 2014)
	Crimean-Congo Hemorrhagic Fever Virus (CCHF)		*Rousettus aegyptiacus, Coleura afra, Hipposideros cf. caffer, Miniopterus inflatus, Hipposideros gigas, Eidolon helvum, Epomops franqueti, Hypsignathus monstrosus, Micropteropus pusillus, Myonycteris torquata, Myotis dasycneme, Myotis daubentonii, Myotis blythii, Nyctalus noctula* Unidentified species (France)	(Tkachenko et al., 1969; Saidi et al., 1975; Müller et al., 2016)
	Gossas (GOSV)	*Tadarida* sp.		(Walker et al., 2015)
	Issyk-Kul (IKV)	*Nyctalus noctula*, *Myotis blythii*, *Vespertilio serotinus*, and *Vespertilio pipstrellus*		
	Kasokero (KKOV)	*Rousettus aegyptiacus*		(Kalunda et al., 1986; Zeller et al., 1989; Walker et al., 2015)
	Keterah (KTRV)	Tick larvae (*Argus pusillus*) collected from *Scotophilus temmincki*		(Varma and Converse, 1976)
	Leopards Hill (LPHV)	*Hipposideros gigas*		(Ishii et al., 2014; Walker et al., 2015)
	Uzun Agach (UAV)	*Myotis blythii oxygnathus*		(Al'khovskii et al., 2014)
	Yogue (YOGV)	*Rousettus aegyptiacus*		(Kalunda et al., 1986; Zeller et al., 1989; Walker et al., 2015)
Peribunyaviridae, genus *Orthobunyavirus*	Bunyamwera virus	*Myotis lucifugus, Eidolon helvum, Rousettus aegyptiacus, Mops condylurus*	*Eidolon helvum, Rousettus aegyptiacus, Tadarida condylura*	(Reagan et al., 1954; Shepherd and Williams, 1964; Williams et al., 1964; Simpson Di Fau - O'Sullivan and O'Sullivan, 1968; Simpson et al., 1968)

	Bimiti		*Anoura geoffroyi, Carollia perspicillata, Phyllostomus hastatus, Pteronotus parnellii, Natalus tumidirostrus*	(Price, 1978)
	Catú virus	*Molossus obscurus*	*Anoura geoffroyi, Carollia perspicillata, Phyllostomus hastatus*	(WOODALL, 1967; Price, 1978)
	Guama virus (GMAV)	Unidentified bat	*Anoura geoffroyi, Phyllostomus hastatus, Artibeus literatus*	(Price, 1978; Karabatsos, 1985)
	Kaeng Khoi Virus (KKV)	*Chaerephon plicata* (*Tadarida plicata*)	*Taphazous theobaldi, Tadarida plicata*	(Williams et al., 1976; Osborne et al., 2003; Groseth et al., 2014)
	Manzanilla		*Molossus ater*	(Price, 1978)
	Mojuí dos Campos virus (MDCV)	Unidentified bat		(Karabatsos, 1985; Wanzeller et al., 2002)
	Nepuyo virus (NEPV)	*Artibeus jamaicensis*, *Artibeus literatus*	*Artibeus jamaicensis, Phyllostomus hastatus*	(Calisher et al., 1971; Price, 1978)
	Oriboca		*Artibeus literatus*	(Price, 1978)
	Restan		*Artibeus literatus, Artibeus jamaicensis, Carollia perspicillata*	
Phenuiviridae, genus *Phlebovirus*	Malsoor virus	*Rousettus leschenaultia*		(Mourya et al., 2014)
	Rift Valley fever virus (RVFV)	*Miniopterus schreibersii, Eptesicus capensis, Micropteropus pusillus, Hipposideros abae, Hipposideros caffer*	*Rousettus aegyptiacus, Epomophorus minor*	(Boiro et al., 1987; Oelofsen and Van der Ryst, 1999; Konstantinov et al., 2006; Olive et al., 2012; Kading et al., 2018)
	Toscana virus (TOSV)	*Pipistrellus kuhli*		(Verani et al., 1982, 1988)

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1.1.2: Members of the Family *Flaviviridae*

The family Flaviviridae includes many high-consequence emerging arboviruses, including Zika virus (ZIKAV), yellow fever virus (YFV), and Dengue virus (DENV). Flaviviruses associated with bats that do not appear to utilize an arthropod vector ("no-known vector flaviviruses") have been reviewed elsewhere (Blitvich and Firth, 2017). Viruses in family *Flaviviridae* that have been experimentally examined in bats or described in field studies are descried in Table 1.2.

1.1.2.1: Dengue Virus

Interestingly, despite DENV isolations from *Artibeus* spp. bats in the wild, experimental infections of great fruit-eating bats (*A. intermedius*) with DENV-2 and Jamaican fruit bats with DENV serotypes 1 and 4 resulted in low levels of viremia, low rates of seroconversion, and lack of detection of viral RNA in the organs via RT-PCR, indicating that bats may not act as a suitable reservoir host (Cabrera-Romo et al., 2014; Sotomayor-Bonilla et al., 2014; Perea-Martinez et al.). Experimental infection of the Indian flying fox (*Pteropus medius*) resulted in no viremia or clinical signs, but intracerebral inoculation of little brown bats (*Myotis lucifugus*) resulted in irritability, paralysis, and death (Reagan and Brueckner, 1952; Shah and Daniel, 1966). DENV nucleic acid and anti-DENV antibodies have been detected in Mexican bats on the Gulf and Pacific coast, and nucleic acid has been detected in the liver and/or sera of wild-caught bats in French Guiana (Aguilar-Setién et al., 2008; Thoisy et al., 2009). Anti-DENV antibodies have been detected in multiple bat species in Uganda (Kading et al., 2018). However, a survey in Central and Southern Mexico analyzing 240 individuals representing 19 bat species by RT-PCR resulted in no detection of viral nucleic acid (Cabrera-Romo et al., 2016). A 2017 study by Vicente-Santos and colleagues examined 12 bat species from Costa Rica and found a cumulative seroprevalence of 21.2% (51/241) by PRNT and a prevalence of 8.8% (28/318) in organs tested by RT- PCR (Vicente-Santos et al., 2017). No infectious virus was isolated and viral loads were considered too low for the bats to function as amplifying hosts.

Rather, Vicente-Santos and colleagues surmised a spillover event from humans to bats, with bats functioning as a dead-end host (Vicente-Santos et al., 2017). The serum of Jamaican fruit bats and great fruit-eating bats from Grenada (n = 50) were also tested for antibodies against DENV1, 2, 3, and 4, and none were seropositive (Stone et al., 2018). While field evidence supports the exposure of bats to DENV in multiple geographic areas, experimental infections conducted to date are consistent in that bats are not likely to support DENV replication and circulation to levels high enough to infect blood-feeding mosquitoes.

1.1.2.2: Japanese Encephalitis Virus

Multiple studies conducted experimental infections of insectivorous bats with Japanese encephalitis virus (JBEV) and found that bats were susceptible to infection with this virus. Three species of bats (big brown bats (*Eptesicus fuscus*), little brown bats (*Myotis lucifigus*) and Eastern pipistrelles (*Pipistrellus subflavus*)) were inoculated with JBEV in the laboratory and maintained infection while held under simulated hibernation conditions. Bats infected prior to hibernation were viremic upon arousing from hibernation, with circulating virus detectable as long as 112 days after the initial infection (La Motte, 1958).

Big brown bats also demonstrated recurrent viremia in the absence of clinical signs in a subsequent study (Sulkin, 1962). Importantly, researchers demonstrated a mosquito-bat-mosquito transmission cycle and postulated this may be an overwintering mechanism for JBEV since mosquitoes did successfully transmit JBEV to bats at low temperatures (La Motte, 1958). Eastern pipistrelles also became infected with JBEV after consumption of infected mosquitoes, demonstrating that bats could be infected orally as well as through a mosquito bite (La Motte, 1958). No demonstrable pathologic effects noted during infection of three bat species [big brown bats, little brown bats, and Mexican free-tailed bats (*Tadarida brasiliensis*) with various strains of JBEV or St. Louis encephalitis virus (SLEV) (Sulkin et al., 1963). No pathology nor viremia was appreciated when pipistrelles (*Pipistrellus abramus*) were infected with JBEV (Ito and Saito, 1952). While experimental data demonstrated that some bat species can sustain

JBEV infections and support mosquito-borne transmission of this virus, the epidemiological significance of these observations in the field remains unclear.

JBEV has been isolated from wild-caught bats in Taiwan (*Miniopterus fuliginosus* and *Hipposideros armiger terasensis*) (Cross, 1971; Karabatsos, 1985), China (*Rousettus leschenaultia* and *Murina aurata*) (Zhang et al., 2001; Wang et al., 2009), Japan (*Miniopterus schreibersi fuliginosus* and *Rhinolophus cornutus cornutus*) (Sulkin et al., 1970). Antibodies against JBEV have been detected in wild-caught bats in Indonesia (unspecified species) (Olson et al., 1983), China (*Rousettus leschenaultia, Cynopterus sphinx, Taphozous melanopogon, Miniopterus schreibersii, Pipistrellus abramus, Rhinolophus macrotis* and *Miniopterus fuliginosus*) (Cui et al., 2008; Jiang et al., 2015), Australia (*Pteropus scapulatus* and *Pteropus gouldi*) (Rowan and O'CONNOR, 1957), Taiwan (unspecified species)(Wang et al., 1962), India (*Pteropus giganteus*, *Hipposideros pomona, Hipposideros speoris, Hipposideros bicolor, Hipposideros cineraceus, Megaderma lyra, Cynopterus sphynx*, and *Rhinolophus rouxi*) (Carey et al., 1968; Kaul et al., 1976; Banerjee et al., 1988), and Japan (*Miniopterus schreibersi fuliginosus, Rhinolophus ferrum equinum Nippon, Vespertilio superans, Myotis macrodactylus, Rhinolophus cornutus cornutus, Pipistrellus abramus, Myotis mystacinus, Plecotus auritus sacrimontis*, and *Murina leucogaster hilgendorfi*) (Miura et al., 1970). Multiple isolations of JBEV from locations where the virus is endemic, in addition to the fact that genetic characterization of isolates has supported their similarity to strains identified from human and mosquito isolates, support the role of bats in ongoing circulation of JBEV (Liu et al., 2013).

1.1.2.3: St. Louis Encephalitis Virus

Another medically important flavivirus with both field-obtained information and *in vivo* experimental inoculation is SLEV. A 1983 study by Herbold and colleagues demonstrated that 9% of wild-caught *Eptesicus fuscus* and *Myotis lucifugus* (n = 390) in Ohio possessed neutralizing antibodies to SLEV (Herbold et al., 1983). Other serosurveillance efforts in North America and Grenada focused on detection of SLEV in free-ranging bat populations have resulted in largely negative findings (Sulkin et al.,

1963; Pilipski et al., 2004). Following experimental infection, viremia and transplacental transmission (albeit infrequent) was appreciated in Mexican free-tailed bats (*Tadarida brasiliensis*)(Sulkin et al., 1963, 1964). The viremia in these bats reached 4 log units, likely too low a titer to facilitate transmission to a blood-feeding mosquito (Kading et al., 2014). Upon inoculation, little brown bats (*Myotis lucifugus*) appear to be resistant or only slightly susceptible to SLEV(Sulkin et al., 1963). Herbold and colleagues (1983) demonstrated that inoculation of *Eptesicus fuscus* with SLEV results in infection and virus was maintained throughout hibernation (70 days), with viremia developing four days following arousal (105 days post-infection) (Herbold et al., 1983). Low levels of viremia upon experimental inoculation in conjunction with low seroprevalence data indicate this virus likely does not utilize bats as a reservoir host in nature.

1.1.2.4: West Nile Virus

To date, biosurveillance testing of bats in Central America for WNV have turned up negative results. Grenadian *Artibeus jamaicensis* and *Artibeus literatus* (n = 50) bats were negative for WNV neutralizing antibodies by PRNT(Stone et al., 2018), 14 Trinidadian bat species (n = 384) were negative by ELISA for WNV antibodies(Thompson et al.), and 16 Mexican bat species (n = 146) tested for WNV RNA by RT-PCR were negative (Sotomayor-Bonilla et al., 2017). In North America, results have been negative or indicative of low levels of circulation in bat populations tested. Tissues from 312 field-collected bats representing seven species in Illinois tested by RT-PCR were all negative for WNV, and the same study reported one big brown bat (*Eptesicus fuscus*) with neutralizing antibodies (n = 97) (Bunde et al., 2006). A field survey taking place in New Jersey and New York reported one big brown bat and one northern long-eared bat (*Myotis septentrionalis*) with neutralizing antibodies to WNV (n = 83) (Pilipski et al., 2004). In another field study, only two of 149 free-tailed bats (*Tadarida brasiliensis*) possessed neutralizing antibodies against WNV (Davis et al., 2005). In Uganda, Kading et al. (2018) detected neutralizing antibodies to WNV in 2/8 African straw-colored flying foxes (*Eidolon*

helvum), and 3/44 little epauletted fruit bats (*Epomophorus labiatus*) (Kading et al., 2018). Simpson and O'Sullivan (1968) demonstrated experimental inoculation of African straw-colored flying foxes did not result in viremia though two of three bats developed neutralizing antibody. In the same study, two of three Egyptian rousette bats were infected but only trace viremia was detected and seroconversion was not appreciated (Simpson Di Fau - O'Sullivan and O'Sullivan, 1968). Experimental inoculation of free-tailed bats (*Tadarida brasiliensis*) did not result in viremia, and infection of big brown bats resulted in low titers (10–180 PFU/mL) (Davis et al., 2005), not capable of supporting transmission to feeding mosquitoes (Kading et al., 2014).

1.1.2.5: Yellow Fever Virus

Attempts to experimentally infect vampire bats (*Desmodus rotundus*) and black mastiff bats (*Molossus rufus*) by mosquito bite (*Aedes aegypti*) were unsuccessful (Kumm, 1932). Experimental inoculation of multiple bat species (*Eumops perotis, Carollia perspicillata, Phyllostomus hastatus* and bats in the genus *Molossus*) were similarly unsuccessful (Hughes and Perlowagora, 1948). Still, Kading et al. detected a significant neutralizing antibody titer against YFV in one Egyptian rousette bat in Uganda in 2012, indicating bats are exposed to this virus in nature (*Kading et al., 2018*). Uganda has experienced outbreaks of YFV in recent years (Nyakarahuka et al., 2018).

1.1.2.6: Zika Virus

While multiple African bat species (*Eidolon helvum, Rousettus aegyptiacus*, and *Rousettus angolensis*) demonstrated viremia following inoculation with ZIKAV, *Mops condylurus* did not become viremic, although did contain low virus titers in the kidney (Shepherd and Williams, 1964; Simpson Di Fau - O'Sullivan and O'Sullivan, 1968). Experimentally-infected little brown bats (*M. lucifugus*) were susceptible to the ZIKAV by the intraperitoneal, intradermal, intracerebral and intrarectal routes of exposure, but not susceptible intranasally (Reagan et al., 1955). Following inoculation of Jamaican fruit

bats with the strain of ZIKV causing the outbreak in the Americas (PRVABC59), seroconversion was confirmed and viral RNA was detected in testes, brain, lung and salivary glands (Malmlov et al., 2019). However, it is unclear how or whether ZIKAV circulates in free-ranging bat populations. Kading et al. (2018) did not detect neutralizing antibodies to ZIKAV among 292 Ugandan bats screened (Kading et al., 2018). Flavivirus infections of bats with an emphasis on the potential role in Zika virus ecology has been reviewed elsewhere (Kading and Schountz, 2016).

1.1.2.7: Other Members in Family Flaviviridae and Pan-Flavivirus Surveillance

Flavivirus serology has been historically challenging due to the cross-reactivity of viral epitopes to circulating antibodies (Calisher et al., 1989). Therefore, the results of serologic surveillance studies must be interpreted cautiously (Machain-Williams et al., 2013; Kading et al., 2018). Further, multiple methods exist for antibody detection (e.g., HI, PRNT, ELISA), and the biological significance of neutralizing vs. non-neutralizing antibodies must be taken into account.

In 2010, the serum of 140 Mexican bats from three species (*Glossophaga soricina*, *Artibeus jamaicensis*, and *A. literatus*) was assayed by PRNT using WNV, SLEV, and DENV 1–4, and 26 were positive for flavivirus-specific antibodies (19%). None of the titers exceeded 80, and all samples were also negative when tested for flavivirus nucleic acid by RT-PCR (Machain-Williams et al., 2013). In a 2015 serosurvey, eight bats (2.6%) displayed non-specific hemagglutination-inhibition (HI) results indicating cross-reactivity or antibodies against an undetermined flavivirus (Thompson et al.). Kading and colleagues performed a serosurveillance study in Ugandan bats and identified 13.6% (85/626) had non-specific flavivirus antibodies by plaque reduction neutralization assay (*Chaerephon pumilus*, *Hipposideros ruber*, *Mops condylurus*, *Nycteris macrotus*, *Eidolon helvum*, *Epomophorus minor*, and *Rousettus aegyptiacus*) (Kading et al., 2018). Still, results generally supported the widespread exposure of bats in Uganda to flaviviruses (Kading et al., 2018).

In 2018, Sotomayor-Bonilla and colleagues reported that liver and spleen samples from 12 Mexican bat species tested negative using pan-flavivirus NS5 primers (Sotomayor-Bonilla et al., 2018). A

recent study in Brazil suggested a lack of arboviral circulation in bat populations, as 103 individuals from 9 species were tested for molecular and serologic evidence of alphavirus and flavivirus infection and all were negative (Bittar et al., 2018). Results of experimental infection of Egyptian rousette bats with WNV and of Angolan free-tailed bats (*Mops condylurus*) with Ntaya virus resulted in very low levels of viremia, while infection of African straw-colored fruit bats with Ntaya virus resulted in neither pathology nor detectable viremia (Simpson Di Fau - O'Sullivan and O'Sullivan, 1968).

Table 1.2. Bat species with published results describing virus isolation, molecular evidence of infection, or seroconversion for viral species in family *Flaviviridae.*

Virus	Virus Isolation/ Molecular Evidence	Serologic Evidence	Ref(s)
Banzi (SAH336)		*Eidolon helvum, Epomophorus anuras, Miniopterus schreibersii, Tadarida pumila, Tadarida condylura*	(Shepherd and Williams, 1964)
Bussuquara virus (BSQV)		*Artibeus jamaicensis*	(Stone et al., 2018)
Central European encephalitis virus	Unidentified bat		(Calisher et al., 2006)
Dengue virus (DENV)	*Desmodus rotundus, Artibeus jamaicensis, Carollia brevicauda, Myotis nigricans, Glossophaga soricina, Artibeus literatus, Artibeus planirostris, Carollia perspicillata, Myotis lucifugus*	*Myotis nigricans, Pteronotus parnellii, Natalus stramineus, Artibejus jamaicensis, Artibeus* spp., *Uroderma* spp., *Molossus* spp., *Chaerephon pumilus, Mops condylurus, Anoura geoffroyi, Artibeus cinereus, Artibeus jamaicensis, Artibeus literatus, Carollia perspicillata, Molossus ater, Molossus molossus, Phyllostomus hastatus, Pteronotus davyi, Pteronotus parnellii, Sturnira* spp., *Pteropus gouldii, Pteropus giganteus*	(Reagan and Brueckner, 1952; O'CONNOR et al., 1955; Rowan and O'CONNOR, 1957; Shah and Daniel, 1966; Price, 1978; Platt et al., 2000; Aguilar-Setién et al., 2008; Thoisy et al., 2009; Machain-Williams et al., 2013; Sotomayor-Bonilla et al., 2014; Vicente-Santos et al., 2017; Abundes-Gallegos et al., 2018; Kading et al., 2018; Perea-Martinez et al.)
Ilheus		*Anoura geoffroyi, Phyllostomus hastatus, Pteronotus davyi, Artibeus jamaicensis, Artibeus literatus, Desmodus rotundus, Molossus ater*	(Price, 1978)
Japanese encephalitis virus (JEV)	*Murina aurata*, *Rousettus leschenaultia, Eptesicus fuscus, Myotis lucifugus, Pipistrellus subflavus, Tadarida brasiliensis mexicana, Hipposideros armiger terasensis, Miniopterus schreibersii, Rhinolophus cornutus*	Undetermined species, *Rousettus leschenaultia*, *Taphozous melanopogon*, *Miniopterus fuliginosus, Myotis macrodactylus*, *Miniopterus shreibersii, Eptesicus fuscus, Pteropus goldii, Pteropus scapulatus,* Genera *Hipposideros* and *Miniopterus, Pteropus giganteus, Rhinolophus cornutus, Murina leucogaster, Pipistrellus*	(Ito and Saito, 1952; Rowan and O'CONNOR, 1957; La Motte, 1958; Wang et al., 1962, 2009; Sulkin et al., 1963, 1964, 1966b, 1966a, 1970; Carey et al., 1968; Miura et al., 1970; Cross, 1971; Kaul et al., 1976; Olson et al.,

		abramus, Plecotus auritus, Rhinolophus ferrum-equinum, Vespertilio superans	1983; Banerjee et al., 1984, 1988; Karabatsos, 1985; Zhang et al., 2001; Cui et al., 2008; van den Hurk et al., 2009; Jiang et al., 2015)
Jugra virus	*Cynopterus brachyotis*		(Karabatsos, 1985)
Kyasanur forest disease virus (KFDV)	*Rhinolophus rouxi*, *Cynopterus sphinx*	*Rousettus leschenaultii, Cynopterus sphinx, Pteropus giganteus, Rhinolophus hipposideros, Rhinolophus rouxi*	(Pavri and Singh, 1965, 1968; Rajagopalan et al., 1969)
Murray Valley encephalitis		*Eptesicus pumilus, Pteropus gouldi, Pteropus scapulatus*	(O'CONNOR et al., 1955; Rowan and O'CONNOR, 1957; Stanley and Choo, 1964)
Ntaya virus	*Tadarida condylura*	*Eidolon helvum*	(Simpson Di Fau - O'Sullivan and O'Sullivan, 1968; Simpson et al., 1968)
St. Louis encephalitis virus (SLEV)	*Eptesicus fuscus, Myotis lucifugus, Tadarida brasiliensis mexicana*	*Artibeus intermedius, Artibeus jamaicensis, Artibeus literatus, Artibeus phaeotis*, *Glossophaga soricina*, *Molossus major*, *Phyllostomus hastatus, Sturnira lilium, Eptesicus fuscus, Myotis lucifugus, Molossus ater, Anoura geoffroyi, Carollia perspicillata, Molossus ater, Molossus molossus, Natalus tumidirostris, Pteronotus davyi, Pteronotus parnellii, Sturnira* spp.	(Sulkin et al., 1960, 1963, 1964, 1966b, 1966c; Rueger et al., 1966; Allen et al., 1970; Price, 1978; Herbold et al., 1983; Ubico and McLean, 1995; Machain-Williams et al., 2013; Thompson et al.)
Tick-borne encephalitis virus (TBEV)	*Myotis myotis, Barbastella barbastellus, Plecotus auritus*	*Barbastella barbastellus, Myotis myotis, Plecotus auritus, Rhinolophus hipposideros*	(Havlik and Kolman, 1957; Kolman et al., 1960; Nosek et al., 1961)
Turkey meningoencephalitis		*Rousettus aegyptiacus*	(Akov and Goldwasser, 1966)
Uganda S virus		Unspecified	(Andral et al., 1968)
Usutu virus	*Rousettus aegyptiacus*	*Eidolon helvum*, *Rousettus* sp.	(Simpson Di Fau - O'Sullivan and O'Sullivan, 1968; Simpson et al., 1968)
West Nile virus (WNV)	*Rousettus leschenaultia, Rousettus aegyptiacus*	*Eptesicus fuscus, Myotis lucifugas, Myotis septentrionalis, Eidolon helvum,*	(Rowan and O'CONNOR, 1957; Shepherd and Williams, 1964;

		Epomophorous minor, Pteropus scapulatus, Tadarida condylura, Tadarida pumila, Rousettus sp., *Rousettus aegyptiacus*, Undetermined species	Akov and Goldwasser, 1966, 1966; Andral et al., 1968, 1968; Simpson Di Fau - O'Sullivan and O'Sullivan, 1968; Simpson et al., 1968; Rajagopalan et al., 1969; Paul et al., 1970; Pilipski et al., 2004; Bunde et al., 2006; Machain-Williams et al., 2013; Kading et al., 2018)
Yellow fever virus (YFV)	*Eidolon helvum, Rousettus aegyptiacus, Tadarida (Mops) condylurus, Epomophorus* sp., *Eptesicus fuscus*	*Eidolon helvum, Rousettus aegyptiacus, Phyllostomus hastatus, Artibeus cinereus, Artibeus jamaicensus, Artibeus literatus, Carollia perspicillata, Glossophaga soricina, Molossus ater, Molossus molossus, Phyllostomus hastatus, Pteronotus davyi, Pteronotus parnellii, Vampyrops helleri, Tadarida condylura, Tadarida pumila, Epomophorus* sp., *Rousettus* sp.	(Laemmert et al., 1946; REAGAN et al., 1955; Sulkin, 1962; Haddow et al., 1964; Shepherd and Williams, 1964; Williams et al., 1964; Andral et al., 1968; Simpson Di Fau - O'Sullivan and O'Sullivan, 1968; Simpson et al., 1968; Sérié et al., 1968; Price, 1978; Kading et al., 2018)
Zika virus (ZIKV)	*Eidolon helvum, Rousettus aegyptiacus*	Undetermined species, *Eidolon helvum, Rousettus aegyptiacus*, *Tadarida condylura, Tadarida pumila, Eidolon helvum, Rousettus* sp., Unspecified	(Shepherd and Williams, 1964; Andral et al., 1968; Simpson Di Fau - O'Sullivan and O'Sullivan, 1968; Simpson et al., 1968; Olson et al., 1983)

1.1.3: Members of the Family Reoviridae

Few studies have examined the presence of viruses in genus Coltivirus in bat populations, and to date, a single isolation has been made (Table 1.3) (Weiss et al., 2017). A 1984 study by Chastel and colleagues failed to detect antibodies to Eyach Virus (*Reoviridae*, Colorado Tick Fever group) in the serum of two field-caught bats (Chastel et al., 1984). To date, five orbiviruses have been isolated from wild-caught bats and serologic evidence exists for exposure of Australian and South American bats to orbiviruses (Table 1.3). While no evidence of human exposure exists for these bat-associated orbiviruses, Bukakata (BUKV) and Fomede (FOMV) appear to be strains of the Chobar Gorge species (Fagre et al., 2019). CGV was isolated from *Ornithodoros* spp. ticks in Nepal, and serum from nearby humans and ruminants possessed anti-CGV antibodies, indicating past exposure (Centers for Disease Control and Prevention. Arbovirus Catalog: Chobar Gorge). Further investigation is warranted to determine the true virus-vector-host tripartite relationships of these viruses and their zoonotic potential. Viruses in family *Reoviridae* that have been experimentally examined in bats or described in field studies are described in Table 1.3.

Table 1.3: Bat species with published results describing virus isolation, molecular evidence of infection, or seroconversion for viral species in family *Reoviridae*

Genus	Virus	Virus Isolation/ Molecular Evidence	Serologic Evidence	Ref(s)
Coltivirus	Taï Forest reovirus (TFRV)	*Chaerephon aloysiisabaudiae*		(Weiss et al., 2017)
Orbivirus	Bukakata (BUKV)	*Rousettus aegyptiacus*		(Fagre et al., 2019)
	Elsey virus (PHSV)		*Pteropus* spp.	(Attoui et al., 2009)
	Fomede (FOMV)	*Nycteris nana, Nycteris gambiensis*		(Boiro et al., 1986; Butenko, 1996; Konstantinov et al., 2006; Centers for Disease Control and Prevention. Arbovirus Catalog: Fomede)
	Heramatsu	*Myotis macrodactylus*		(Miura et al., 1970; Zhao et al., 2013)
	Ife (IFEV)	*Eidolon helvum*		(Kemp et al., 1988; Centers for Disease Control and Prevention. Arbovirus Catalog: Ife)
	Japanaut (JAPV)	*Syconycteris crassa*		(Schnagl and Holmes, 1975; Centers for Disease Control and Prevention. Arbovirus Catalog: Japanaut)
	Matucare		Genera *Myotis* and *Noctilio*	(Justines and Kuns, 1970)

1.1.4: Members of the Genus Alphavirus (Family: *Togaviridae*)

Viruses in genus Alphavirus (family *Togaviridae*) that have been experimentally examined in bats or described in field studies are described in Table 1.4.

1.1.4.1: Chikungunya

Enzootic circulation of CHIKV is understood to occur among non-human primates and forest-dwelling mosquitoes (Higgs and Vanlandingham, 2015), but other vertebrates including rodents, bats, reptiles and amphibians have been shown to support CHIKV replication (Bosco-Lauth et al., 2016, 2018). The range of peak viremia developed by big brown bats was relatively low, but within the range of infectivity to blood feeding mosquitoes (Kading et al., 2014; Bosco-Lauth et al., 2016). When Indian flying foxes (*Pteropus giganteus*) and big brown bats were experimentally infected with CHIKV, bats developed viremia but no clinical signs of disease, indicating they could play a role in the natural transmission of this virus (Shah and Daniel, 1966; Bosco-Lauth et al., 2016). Experimental infection of African straw-colored flying foxes did not result in viremia or seroconversion to CHIKV, supporting a separate study which reported lack of viremia in experimentally infected Egyptian rousette bats and African straw-colored flying foxes (Shepherd and Williams, 1964; Simpson Di Fau - O'Sullivan and O'Sullivan, 1968)[43,44]. In 2015, the serum of 42 wild-caught Grenadian bats (genus *Artibeus*) were subjected to PRNT and 15 (36%) were found to possess neutralizing antibody to CHIKV (Stone et al., 2018). CHIKV has been circulating in Central and South America since 2013 (Higgs and Vanlandingham, 2015). Whether or not bats are contributing to the natural circulation of CHIKV in endemic areas or areas of introduction remains to be determined and will require the detection of CHIKV nucleic acid or live virus from free-ranging bats.

1.1.4.2: EEEV/VEEV/WEEV

Serological evidence exists supporting exposure of bats to encephalitic alphaviruses in the field,

and experimental data demonstrate the susceptibility of bats to infection with alphaviruses including VEEV. Four Mexican bat species were examined for molecular evidence of infection with Venezuelan equine encephalitis virus (VEEV), Western equine encephalitis virus (WEEV), and Eastern equine encephalitis virus (EEEV). No individual bats were positive for WEEV or EEEV, but 3% (5/150) representing all four species were positive for VEEV (Sotomayor-Bonilla et al., 2017). Field-caught Jamaican fruit bats (*Artibeus jamaicensis*) and great fruit-eating bats (*Artibeus literatus*) were negative by PRNT for EEEV and WEEV antibodies, but 2.6% (1/38) had neutralizing antibodies to VEEV (Stone et al., 2018). Similarly, the serum of 384 bats representing 14 species was subjected to ELISA, and 2.9% (11/384) contained VEEV-specific antibodies. ELISA and HI assays for EEEV and WEEV antibodies, respectively, were all negative (Thompson et al.). Four species of wild-caught bats from the northeastern United States were tested for neutralizing antibody against EEEV and WEEV. Samples were negative for antibodies against WEEV, but 1.3% of the 128 bats tested did possess EEEV-neutralizing antibody (MAIN, 1979b). Bats of the genera *Myotis* and *Eptesicus* were experimentally infected with EEEV, and developed viremia but failed to develop neutralizing antibodies. Infection of big brown bats with EEEV by bite of *Culiseta melanura* and *Aedes aegypti* mosquitoes was successful, and resulted in more non-hibernating than hibernating bats seroconverting (MAIN, 1979a).

1.1.4.3: Other Emerging Alphaviruses

Mayaro is an emerging mosquito-transmitted alphavirus circulating in South America. Antibodies against Mayaro virus were detected by HI in 37 Trinidadian bat species tested (Price, 1978). In a recent serosurveillance study, 2/432 bats were seropositive by plaque reduction neutralization assay to Babanki virus (BBKV) and 9/626 Egyptian rousette bats had non-specific alphavirus antibodies (Table 1.4) (Kading et al., 2018). Multiple isolates of BBKV were obtained from *Cx. perfuscus* mosquitoes collected from multiple locations in Uganda during this same sampling period as when bats were sampled (Mossel et al., 2017). Mosquito blood meals from bats comprised 7.5% of the total blood meals identified from the

species Cx. Perfuscus (Crabtree et al., 2013). It is unclear whether bats contribute to the transmission cycle of BBKV or are merely incidentally exposed through mosquito bites.

Ten *Pteropus poliocephalus* bats were experimentally infected with Ross River virus, and five developed low (log_{10} 2.2 $TCID_{50}$/100 L) detectable and short-lived (2 days) viremia. Still, 2% of the colonized mosquitoes (*Aedes vigilax*) that fed on the bats between days 1–4 post-infection became infected (Ryan et al., 1997). Kading et al. (2014) modeled that for viremias <log10 2.0/mL, the probability of a mosquito becoming infected was around 0.1 or less given the low circulating titer and the volumetric constraints of a small mosquito blood meal; therefore, at least 10 mosquitoes would need to feed on an animal with a low viremia in order for one mosquito to ingest virus (Kading et al., 2014). In the case of RRV, published data demonstrate that infection of mosquitoes fed on RRV-viremic bats is still possible despite a viremia and low titer (Ryan et al., 1997). Therefore, if bat and mosquito populations are in high numbers, 50% of bats develop a detectable viremia, and 2% of mosquitoes become infected, mosquito-borne transmission could take place even though experimentally determined efficiencies are low (Ryan et al., 1997).

Table 1.4. Bat species with published results describing virus isolation, molecular evidence of infection, or seroconversion for viral species in family Togaviridae, genus Alphavirus

Virus	Virus Isolation/ Molecular Evidence	Serologic Evidence	References
Babanki virus		*Epomophorus minor, Rousettus aegyptiacus*	(Kading et al., 2018)
Chikungunya (CHKV)	*Eptesicus fuscus, Rousettus leschenaultia, Pteropus giganteus, Scotophilus* sp., *Hipposideros cafer*	*Artibeus literatus*, *Artibeus jamaicensis,* unknown species, *Pteropus giganteus, Megaderma lyra*	(Bres et al., 1963; Brès and Chambon, 1964; Shah and Daniel, 1966; Olson et al., 1983; Zhang et al., 1989; Bosco-Lauth et al., 2018; Stone et al., 2018)
Eastern equine encephalitis virus (EEEV)	*Eptesicus fuscus*	*Artibeus intermedius, Artibeus jamaicensis, Artibeus literatus, Glossophaga soricina, Rhynchonycteris naso, Sturnira lilium, Carollia perspicillata, Phyllostomus hastatus, Vampyrops helleri, Eptesicus fuscus, Myotis lucifugus*	(Karstad and Hanson, 1958; La Motte, 1958; Daniels et al., 1960; Shepherd and Williams, 1964; Price, 1978; MAIN, 1979b, 1979a; Ubico and McLean, 1995)
Mucambo		*Molossus ater, Phyllostomus hastatus, Carollia perspicillata*	(WOODALL, 1967; Price, 1978)
O'Nyong Nyong			
Ross River virus (RRV)	*Pteropus poliocephalus*	*Pteropus poliocephalus*, *Pteropus scapulatus,* undetermined species	(Gard et al., 1973; Olson et al., 1983; Ryan et al., 1997)
Sindbis virus	*Rhinolophidae* sp., *Hipposiderae* sp., *Myotis lucifugus*, Eidolon helvum*		(Reagan et al., 1956; Simpson Di

			Fau - O'Sullivan and O'Sullivan, 1968)
Semliki Forest Virus	*Myotis lucifugus, Eidolon helvum, Rousettus aegyptiacus, Tadarida (Mops) Condylurus*	*Eidolon helvum, Rousettus aegyptiacus, Tadarida condylura*	(Reagan et al., 1954, 1956; Shepherd and Williams, 1964; Simpson Di Fau - O'Sullivan and O'Sullivan, 1968)
Venezuelan equine encephalitis virus (VEEV)	*Carollia perspicillata, Eptesicus fuscus, Artibeus planirostris, Sturnira lilium, Artibeus phaeotis, Desmodus rotundus, Artibeus literatus*	*Artibeus* sp., *Carollia brevicauda, Carollia subrufa, Carollia perspicillata*, *Desmodus rotundus*, *Glossophaga soricina*, *Noctilio leporinus, Sturnira lilium, Sturnira ludovici, Artibeus literatus, Carollia sowelli, Sturnira parvidens*	(Corristan et al., 1956, 1958; GRAYSON and Galindo, 1968; Constantine, 1971; Wong-Chia and Scherer, 1971; Price, 1978; Ubico and McLean, 1995; Sotomayor-Bonilla et al., 2017; Guzman et al., 2018; Stone et al., 2018; Thompson et al.)
Western equine encephalitis virus (WEEV)	*Tadarida brasiliensis*	*Artibeus jamaicensis*	(McLean et al., 1979)

1.2: Relationships between Arthropods and Bats

A number of hematophagous arthropods feed on bats, including bat flies (genera *Nycteribiidae* and *Streblidae*), bat bugs and bed bugs (family *Cimicidae*), and ticks (families *Argasidae* and *Ixodidae*) (Williams et al., 1976; Fritz, 1983; Bertola et al., 2005; Bartonička and Gaisler, 2007; Patterson et al., 2007; Balvín et al., 2012, 2013; Hornok et al., 2016; Luz et al., 2016). Viruses of medical and veterinary significance have also been isolated from these arthropods (Williams et al., 1976; Rush et al., 1980; Brown et al., 2009; Kurhade et al., 2018). However, the contribution that these ectoparasites play in the circulation of medically important viruses among bats and other hosts is unclear and necessitates further investigation.

1.2.1: Mosquitoes

Kading and Schountz (2016) reviewed instances in the literature where mosquito blood meals have been identified as originating from bats (Kading and Schountz, 2016). Information on primary mosquito vectors feeding on bats is very limited. Tiawsirisup et al. 2012 collected mosquitoes from five genera inside a bat cave in Thailand to investigate sylvatic circulation of JBEV. While these collections included arbovirus vectors *Cx. quinquefasciatus* and *Cx. tritaenhiorhynchus*, the only blood-fed mosquitoes collected from the cave were *Cx. quinquefasciatus*, at least 20 of which had fed on Leschnault's rousette (*Rousettus leschenaulti*) bats (Tiawsirisup et al., 2012). *Culiseta morsitans Theobald* mosquitoes (vector of Eastern equine encephalitis virus) were found to have fed on Eastern pipistrelle bats (*Pipistrellus subflavus*), but these blood meals comprised only 1% of the total blood meals identified from this mosquito species (Molaei et al.). No information was found on any blood meals from bats being detected in *Aedes (Stegomyia)* species, but it is unclear how much investigation has been done in this area. Sixteen of 20 field-collected *Ae. funereus* mosquitoes (vector of RRV) had fed on *Pteropus alecto* bats (Ryan et al., 1997). In Africa, mosquito species in which bat blood meals have been identified and are known to be associated with a number of medically-important arboviruses include: *Coquillettidia*

(Cq.) fuscopennata (Theobald) (YFV, Sindbis, chikungunya viruses), *Culex (Cx.) perfuscus Edwards* (WNV, Oropouche, Sindbis, Wesselsbron, Usutu, Babanki viruses), *Cx. (Cx.) neavei Theobald* (WNV, Babanki, Spondweni, Sindbis, Koutango viruses), and *Cx. (Cx.) decens* group (WNV, chikungunya, Babanki viruses(Mutebi et al., 2012; Crabtree et al., 2013). While mosquitoes in the subgenus *Cx. (Cx.)* are recognized as primary vectors of WNV, Sindbis, Babanki, and Usutu viruses, only for Babanki virus has additional field data been collected so far that support a potential role for bats in virus circulation (discussed above) (Kading et al., 2018).

1.2.2: Bat flies

Bat flies (Order: Diptera; Families: Nycteribiidae, Streblidae) are highly host-specific obligate hematophagus ectoparasites of bats(Dick and Patterson, 2006; Estrada-Villegas et al., 2018). A novel fusogenic orthoreovirus, tentatively named Mahlapitsi virus (MAHLV), was discovered in bat flies (*Eucampsipoda africana*) associated with Egyptian rousette bats (Jansen van Vuren et al., 2016). A novel orthobunyavirus, tentatively named Wolkberg virus (WBV), was isolated from the same species of bat flies in a similar geographic region (Jansen van Vuren et al., 2017). Dengue virus RNA was detected by RT-PCR in bat flies (*Strebla wiedemanni*, *Trichobius parasiticus*) associated with common vampire bats (*Desmodus rotundus*) (Abundes-Gallegos et al., 2018). *Bartonella* spp. bacteria have also been found infecting both fruit bats and the bat flies parasitizing them in Madagascar, providing a documented example of a pathogen-vector-bat association involving bat flies (Brook et al., 2015). More research in this area is needed to elucidate the role of bat ectoparasites in spreading pathogens among individual bats in a roost.

1.2.3: Bat bugs and other arthropods in family Cimicidae

In addition to parasitizing humans by infesting their dwellings, bed bugs and other arthropods in family Cimicidae are found in close association with bat populations (Balvín et al., 2012). Some cimicids

are known to play a role in alphavirus transmission. Cliff swallow bugs (*Oeciacus vicarious*) transmit Fort Morgan virus and Buggy Creek virus (Hayes et al., 1977; Rush et al., 1980; Hopla et al., 1993; Brown et al., 2009) among passerine birds. Tonate virus, which is closely related to VEEV, and also causes fatal encephalitis in man, has also been isolated from swallow bugs in the 1970's (Monath et al., 1980). As previously described, bat bugs (*Cimex insuetus* and *Strcticimex parvus*) host the orthobunyavirus KKV which likely causes disease in humans (Williams et al., 1976; Neill, 1985), but this also remains a largely understudied research area.

1.2.4: Acari (ticks and mites)

Ticks and mites are known to parasitize bats and they both fill similar ecological niches. Many studies have identified viruses in bats that cluster phylogenetically with other arboviruses transmitted by ticks. Other isolations are indicative of bats playing a role in viruses isolated from either hard-bodied or soft-bodied ticks. For example, Estero Real Virus was first isolated from ticks (*Ornithodoros tadaridae*) obtained from a palm tree colonized by Cuban bats (Málková et al., 1985). Due to the close ecological association between Egyptian rousette bats (*R. aegyptiacus*) and soft-bodied ticks in caves, the argasid tick *Ornithodoros faini* was screened for Marburgvirus RNA but all pools were found to be negative (Schuh et al., 2016). To date, evidence does not support the role of arthropods in the transmission of filoviruses in nature. Bats are also known to host mite species in families Macronyssidae, Dermanyssidae, and Spinturnicidae, though the significance of their role in virus transmission between bats and other vertebrates is largely unknown (Bradshaw and Ross, 1961; Moras et al., 2013; Colin-Martinez and Garcia-Estrada, 2016). Species in Macronyssidae and Dermanyssidae have also been demonstrated to efficiently transmit arboviruses (further reviewed in Moro et al 2005) (Moro et al., 2005).

1.2.5: Other bat-arthropod associations

A recent study explored viruses of potential arthropod-origin in the fruit bat *Hypsignathus monstrosus* and identified five viruses: one dicistrovirus (family *Dicistroviridae*, order Picornavirales), one nodavirus (family Nodaviridae), and two tombus-like viruses. They also detected a related tombus-like virus isolated from fig wasps and primitive crane flies co-habitating with bats, suggesting the ability of arthropods to host "bat-associated" viruses (Bennett et al., 2019). Tombus viruses typically infect plants, but tombus-like viruses infect a wide range of hosts, including arthropods and marine invertebrates (Stuart et al., 2004; Shi et al., 2016; Dolja and Koonin, 2018). Ingestion of arthropods by fruit bats has been documented (Barclay et al., 2006; Clulow and Blundell, 2011), and could be a potential mechanism explaining the association between viromes of arthropods and frugivorous bat species.

1.3: Considerations for Reservoir Competence and Characterizing the Relationship between Bats, Arthropods, and Pathogens

With the advent of next-generation sequencing and decrease in costs and labor associated with high-throughput sequencing options, metagenomic investigations into bats and their virome have flourished (Li et al., 2010; Wu et al., 2016b; Letko et al., 2020; Virome of Bat Guano from Nine Northern California Roosts). With this, the number of viruses associated with bat species in the order *Chiroptera* has also increased (Wu et al., 2016b). Global efforts into pathogen discovery from wildlife species (e.g. USAID PREDICT) have sequenced fragments of genome from ~1,100+viruses to date (949 of them novel), bolstering our understanding of viral diversity in bats and other free-ranging wildlife species (PREDICT Project). The goal of these efforts is to identify species of interest for targeted surveillance and specific points at the human-animal interface where interventions may be necessary to avoid spillover into human populations. However, a major limitation in these studies is the absence of *in vitro* or *in vivo* characterization of these pathogens, and in many instances, the absence of whole genome characterization of the pathogen itself.

Within the next two chapters of my dissertation work (Chapters 2 and 3), further viral characterization of a novel orbivirus is undertaken using both *in vitro* and *in vivo* approaches to define potential impact on human and animal populations with higher resolution. In Chapters 4 and 5, novel diagnostic methodologies and molecular targets are applied to improve our understanding of the tripartite relationships between bats, arthropods, and pathogens infecting one or both populations.

CHAPTER 2: GENOMIC AND IN VITRO CHARACTERIZATION OF BUKAKATA ORBIVIRUS, A NOVEL VIRUS ISOLATED FROM A UGANDAN BAT[2]

2.1: Introduction

Serological and virological evidence documents the exposure of various East African bat species to several arboviruses including Rift Valley fever, dengue, and yellow fever virus(Calisher et al., 2006; Kading et al., 2018), however, little is known about the potential role of bats as arbovirus reservoirs or potential amplifying hosts. Orbiviruses (*Reoviridae*:*Orbivirus*) are 10-segmented dsRNA vector-borne viruses that cluster phylogenetically by arthropod vector group (Attoui et al., 2011). Recent research has also reported a novel orbivirus that may represent the first recognized insect-specific virus in the genus Orbivirus (Harrison et al., 2016). While mostly recognized as veterinary pathogens (i.e., bluetongue virus, African horse sickness), several orbiviruses have been associated with neurologic disease in humans (Libikova et al., 1978; Malkova et al., 1980; MacLachlan and Guthrie, 2010; Mohd Jaafar et al., 2014).

Prior to this study, four orbiviruses had been isolated from wild bats. Japanaut virus (JAPV) was isolated from the blood of a southern blossom bat (*Syconycteris crassa*) and a pool of mixed culicine mosquitoes in the Sepik District, New Guinea in 1965 (Schnagl and Holmes, 1975; Centers for Disease Control and Prevention. Arbovirus Catalog: Japanaut). Heramatsu orbivirus was isolated from the blood of an eastern long-fingered bat (*Myotis macrodactylus*) trapped in a mine in Heramatsu, Kagoshima, Japan in 1965 (Miura et al., 1970). The genome of Heramatsu orbivirus (isolate KY-663) has been partially sequenced (Zhao et al., 2013). Eight isolates of Ife virus (IFEV) were isolated from the blood and organs of straw-colored fruit bats (*Eidolon helvum*) in Nigeria, Cameroon, and the Central African Republic in 1971 and 1974 (Kemp et al., 1988). Gambian pouched rats (*Cricetomys gambianus*), African grass rats (*Arvicanthis niloticus*), and domestic ruminants in Nigeria were also found to be seropositive for IFEV (Ezeifeka et al., 1987, 1989; Centers for Disease Control and Prevention. Arbovirus Catalog:

[2] This chapter includes the complete manuscript cited as "Fagre, A.C., Lee, J.S., Kityo, R.M., Bergren, N.A., Mossel, E.C., Nakayiki… & Kading, R.C. (2019). Discovery and Characterization of Bukakata orbivirus (Reoviridae:Orbivirus), a Novel Virus from a Ugandan Bat. *Viruses 11*(3), 209. doi: 10.3390/v11030209". This article is reproduced with permission and only minimal modifications were made to meet formatting requirements.

Ife). Fomede virus (FOMV) was isolated from the brain, liver, and spleen of a dwarf slit-faced bat (*Nycteris nana*) in Kindia, Guinea in 1978, and has been repeatedly isolated from Nycteridae bats in Guinea (Boiro et al., 1986; Butenko, 1996; Konstantinov et al., 2006). Additionally, serologic evidence exists for exposure of Bolivian bats (genera *Myotis* and *Noctilio*) to Matucare virus, an orbivirus isolated from Ornithodoros ticks in 1963 (Justines and Kuns, 1970). Australian fruit bats were found to be seropositive to Elsey virus, a serotype of the mosquito-borne Peruvian horse sickness virus (Attoui et al., 2009).

In 2013, a novel orbivirus, tentatively named Bukakata orbivirus (BUKV), was isolated from an Egyptian fruit bat (*Rousettus aegyptiacus leachii* (A. Smith, 1829)) (ERB) captured in Kasokero Cave, Uganda (Figure 2.1). This is the fifth orbivirus isolated from bats; however, aside from partial sequencing of Heramatsu, no bat-associated orbivirus has been genetically characterized. The specific aims of this project were to:

1. Sequence the genomes of the four bat-associated orbiviruses without published existing genetic information (JAPV, IFEV, FOMV, & BUKV) and conduct phylogenetic analyses to ascertain their potential arthropod associations, as orbiviruses cluster phylogenetically based on their arthropod vector
2. Determine the replication kinetics of BUKV in multiple vertebrate cell types
3. Assess the prevalence of BUKV RNA in additional archived field samples from Uganda

2.2: Materials and Methods

2.2.1: Viruses and Cells

JAPV (MK 6357), IFEV (IbAn 57245), and FOMV (DakAnK 654) viruses were all sourced from the Arbovirus Reference Collection at the Centers for Disease Control Arbovirus Diseases Branch in Fort Collins, CO. BUKV (UGA432), isolated during this study, was deposited in the Arbovirus Reference

Collection following isolation on Vero cells (African green monkey kidney epithelial cells) (ATCC CCL-81).

Vero cells and BHK-21 [C-13] cells (Syrian golden hamster kidney fibroblast) (ATCC CCL-10) were maintained in Dulbecco's minimal essential media supplemented with 5% fetal bovine serum (FBS) (F0500-A, Atlas Biologicals, Fort Collins, CO, USA) and 1% penicillin/streptomycin (P/S) (15140122, ThermoFisher Scientific, Rockford, IL, USA). R06E cells (BEI resources, NIAID, NIH: R06E, *R. aegyptiacus leachii* (Egyptian fruit bat), Immortalized Fetal Cell Line, NR-49168) (Jordan et al., 2009) were maintained in DMEM-F12 (11330032, ThermoFisher Scientific, Rockford, IL, USA) with 5% FBS and 1% P/S. R06E cells were tested and confirmed negative for contamination with Vero cells via DNAzol extraction and Sanger sequencing using Barcode of Life CO1 primers(Messing, 1983; Ivanova et al., 2007). All cell lines were tested for presence of *Mycoplasma* spp. using PCR and were found to be negative using the ATCC Universal Mycoplasma Detection Kit (Cat. no. 30-1012K) (ATCC, Manassas, VA, USA).

2.2.2: Bat capture and sampling

Bats were captured from multiple locations throughout Uganda during 2011–2013 (Kading et al., 2018). Seventy-one additional combined liver and spleen tissue RNA samples from ERBs captured previously at Maramagambo Forest in 2009 were provided for inclusion in this study (Table 2.1, Figure 2.1). All bat captures were conducted under the approval of IACUC protocols 1731AMMULX (samples from Maramagambo Forest) and 010-015 (all other samples). Bats were captured using harp traps or mist nets. Sampling locations, numbers and species captured, and blood collection and serological results from these samples are described elsewhere (Towner et al., 2009; Kading et al., 2018). All bats were euthanized according to approved IACUC protocols in accordance with AVMA guidelines to harvest tissues for virus isolations. Tissues collected included lung, intestine, liver, spleen, and oral and fecal swabs. Tissue sections were immediately placed in cryotubes and liquid nitrogen dry shippers. Duplicate

aliquots of serum and liver/spleen were analyzed first for filovirus RNA at the CDC Viral Special Pathogens laboratory in Atlanta, GA USA.

Filovirus-negative liver and spleen specimens were homogenized for virus isolation. Approximately 0.5 cm^3 sections of tissue were mechanically homogenized in a 2.0 mL snap cap tube containing 1 mL BA1 medium (Hanks M-199 salts, 0.05 M Tris pH 7.6, 1% bovine serum albumin, 0.35 g/L sodium bicarbonate, 100 U/mL streptomycin, 1 µg/mL Fungizone) and one or two stainless steel 5 mm beads in a Qiagen mixer mill (Qiagen, Valencia, CA, USA) at 25 cycles/sec for four minutes. Homogenates were clarified by centrifugation at approximately 12,800× g for 8 min at 4 °C and stored at −80 °C. A 100 µL aliquot of supernatant was inoculated directly onto Vero cell monolayers, with one sample per well on a 6-well plate for virus isolation by double-overlay plaque assay(Miller et al., 1989). A second overlay containing neutral red was added four days post infection, and plates were observed for plaques up to 10 days post infection.

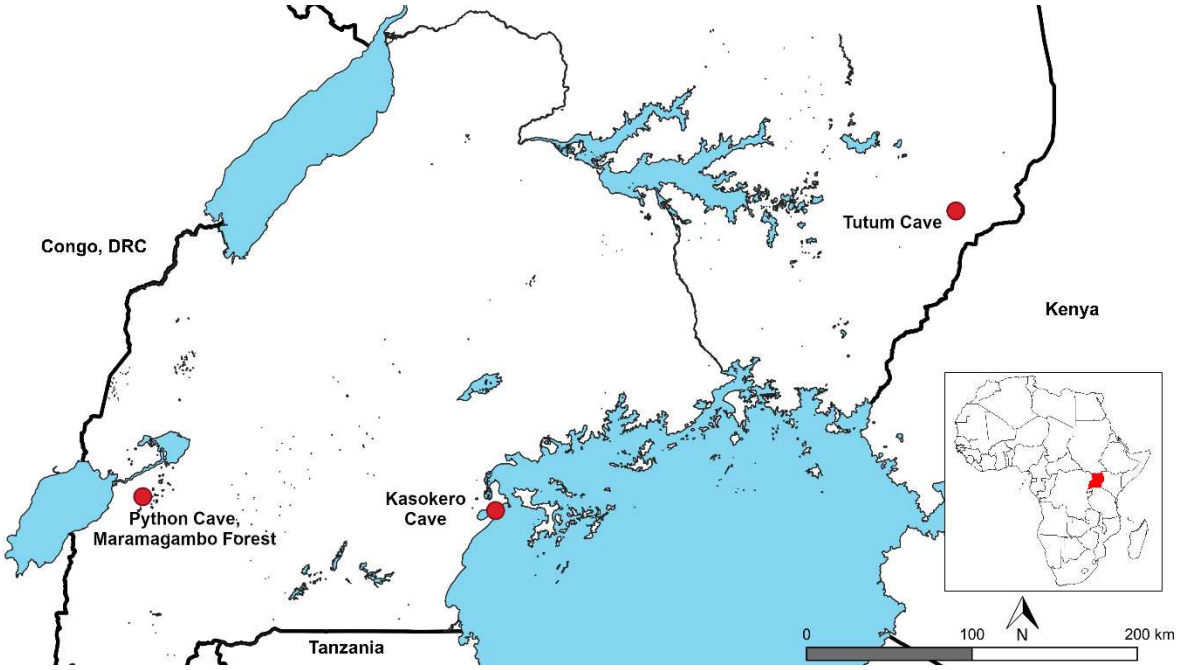


Figure 2.1 Sampling locations for bats within Uganda, 2009–2013. Capture locations for all bats tested in this study.

Table 2.1 Sampling location and dates for fruit bats (*R. aegyptiacus*) screened for BUKV RNA (Kading et al., 2018)

Location	Latitude	Longitude	N	Tissue Type	Date collected
Python Cave, Maramagambo Forest	-0.26667	30.05000	71	Liver and spleen	Nov 2009
Kasokero Cave	-0.34214	31.96627	56	Spleen	Jan 2013
Tutum Cave	1.28333	34.46667	45	Spleen	Feb 2012

Cells from plaque-positive wells were harvested into 1 mL DMEM + 10% fetal bovine serum and clarified by centrifugation and the infected supernatant was stored at −80 °C. The viral RNA from plaque-positive samples was extracted from 200 µL of the supernatant and eluted into a final volume of 140 µL AE buffer using the Qiagen BioRobot EZ1 Workstation using the EZ1 Virus Mini Kit v2.0. From the infected bat, additional sections of liver, lung, intestine, and oral and fecal swabs were also processed for virus isolation as above.

2.2.3: Sequencing and Bioinformatics Analysis

Initial sequencing of the novel virus isolate and bioinformatics analysis was performed following published methods (Kading et al., 2013b). RNA was extracted from FOMV, IFEV, and JAPV virus isolates using the MagMAX Viral RNA Isolation Kit (Life Technologies, Carlsbad, CA, USA) and DNAse treated using the TURBO DNA-free™ kit (Ambion, Austin, TX, USA). Library preparation was carried out using ScriptSeq™ v2 RNA-Seq Library Preparation Kit (Epicentre Biotechnologies, Madison, WI, USA) and ScriptSeq™ Index PCR Primers (Epicentre Biotechnologies, Madison, WI, USA). Libraries were pooled and sequenced on the Illumina MiSeq using the MiSeq Reagent Kit v2 (300 cycles) (Illumina, San Diego, CA, USA). RNA was Trizol extracted from Vero cell supernatant for the novel orbivirus isolate, BUKV (UGA432), and the library was prepared using the Kapa RNA HyperPrep kit (Kapa Biosystems, Wilmington, MA, USA). The library was sequenced on the Illumina MiSeq using the MiSeq Reagent Micro Kit v2 (300 cycles) (Illumina, San Diego, CA, USA).

Low-quality bases and barcoded sequencing adapters were removed using Cutadapt, duplicate reads were collapsed using cd-hit, and host reads were filtered using Bowtie2 (Martin, 2011; Fu et al.,

2012; Langmead and Salzberg, 2012). *De novo* assembly was performed using SPADES assembler and contigs were searched for homology against the NCBI Viral RefSeq database at the nucleotide (blastn) and amino acid (blastx) levels (Bankevich et al., 2012). Contigs representing orbivirus genomic segments were validated by remapping the quality- and host-filtered reads using Bowtie2 (Langmead and Salzberg, 2012). The resulting alignments were visually inspected to confirm that mapping depth and base-calls were sufficient for accurately determining the sequence of each open reading frame for each genomic segment. Putative open reading frames were characterized using open reading frame (ORF) prediction (Geneious v11.1.15, Biomatters, Auckland, New Zealand) and comparing the output (ORF start/stop/length etc.) to related orbiviruses. This workflow resulted in a coding-complete consensus sequences for all ten segments of JAPV, FOMV, and IFEV, and for nine of ten segments for BUKV.

2.2.4: Phylogenetic Analysis

Genome regions of interest included the highly conserved RNA-dependent RNA polymerase (VP1), sub-core shell protein (T2 (VP2/VP3)), and the major-core surface protein (T13 (VP7)) (Belhouchet et al., 2010; Belaganahalli et al., 2011). Multiple alignment and phylogenetic analysis were analyzed for these three genes using the newly-derived consensus sequences obtained for the four bat-borne orbiviruses in addition to previously genetically characterized orbivirus sequences obtained from GenBank (AHSV = African horse sickness, BTV = Bluetongue virus, CGLV = Changuinola virus, CGV = Chobar Gorge virus, CORV = Corriparta virus, EEV = Equine encephalosis virus, EHDV = Epizootic hemorrhagic disease virus, EUBV = Eubenangee virus, FENGV = Fengkai virus, GIV = Great Island virus, KEMV = Kemerovo virus, LEBV = Lebombo virus, LIPV = Lipovnik virus, ORUV = Orungo virus, PALV = Palyam virus, PATAV = Pata virus, PHSV = Peruvian horse sickness virus, SVIV = Sathuvachari virus, SCRV = St. Croix River virus, SLOV = Stretch Lagoon orbivirus, TIBV = Tibet orbivirus, TILV = Tilligerry virus, TRBV = Tribec virus, UMAV = Umatilla virus, WMV = Wad Medani virus, WALV = Wallal virus, WARV = Warrego virus, YUOV = Yunnan virus) (Table 2.2). Putative open reading frames were translated and aligned in SeaView version 4 (Gouy et al., 2010) using Clustal

Omega (Sievers et al., 2011), and then back-translated to nucleotide sequence. Nucleotide alignments were trimmed using TrimAl in order to remove poorly aligned regions (Capella-Gutiérrez et al., 2009).

Table 2.2: Sequences used in phylogenetic analyses of bat-associated orbiviruses.

Isolate	Abbrev.	VP1	T2	T13
AHSV-1 (HS29/62)	AHSV-1	FJ183364	FJ183366	FJ183371
AHSV-2 (strain 2/E.caballus-c/ZAF/2012/Mokopane-E120203)	AHSV-2	KT030360	KT030362	KT030366
AHSV-9	AHSV-9	NC_006021	GQ506549*	U90337*
BTV-12	BTV-12	GU390658.1	GU390660	GU390663
BTV-17	BTV-17	L20447	JX272451*	JX272455
BTV-1e (IND1992/01)	BTV-1	JQ282770	JQ282771	JQ282774
BTV-25	BTV-25	GQ982522	GQ982523	EU839843*
BTV-26 (KUW2010/02)	BTV-26	JN25516	HM590643	HM590644
BTV-8w (NET2006/04)	BTV-8	AM498051	AM498053	AM498057
Changuinola (BE AR 49042)	CGLV	JQ610655	JQ610657	JQ610661
Chenuda virus	CHUV	NC_027534	NC_027535	NC_027550
Chobar Gorge virus	CGV	NC_027553	NC_027554	NC_027559
Corriparta virus	CORV	KC853042	KC853043	KC853049
EEV (Cascara)	EEV	HQ630912	HQ630914	HQ630918
EHDV-1w (USA1955/01)	EHDV-1	AM744977	AM744979	AM744983
EHDV-2	EHDV-2	AM744987.1	AM744989	AM744993
Eubenangee virus (AUS1963/01)	EUBV	JQ070376	JQ070378	JQ070382
Fengkai (Tibet)	FENGV	NC_027803.1	NC_027812	NC_027805
Great Island Virus (CanAr 42)	GIV	HM543465	HM543466	HM543471
Kemerovo virus	KEMV	HQ266591	HQ266592	HQ266597
Lebombo virus	LEBV	JQ610665	JQ610666	JQ610671
Lipovnik (CzArLip 91)	LIPVh	HM543475	HM543476	X
Orungo virus (UGMP 359)	ORUV	JQ610675	JQ610677	JQ610681
Palyam virus	PALY	KT002588	KT002590	KT002594
Pata (CAF1968/01)	PATAV	JQ070386	JQ070388	JQ070393
Peruvian horse sickness virus	PHSV	DQ248057	DQ248058	DQ248063
Sathuvachari (IAn66411)	SVIV	KC432629	KC432631	KC432635
St Croix River virus	SCRV	NC_005997	NC_005998	NC_006004
Stretch lagoon orbivirus_K49460	SLOV	NC_012754	NC_012755	X
Tibet (XZ0906)	TIBV	KF746187	KF746189	KF746193
Tilligerry virus (AUS1978/03)	TILV	JQ070366	JQ070368	JQ070372
Tribec virus	TRBV	HM543478	HM543479	HQ266588
Umatilla virus (USA1969/01)	UMAV	HQ842619	HQ842620	HQ842626
Wad Medani virus	WMV	KP268804	KP268805	KP268811
Wallal (Ch 12048) - AUS1978/09	WALV	KJ495745	KJ495747	KJ495751
Warrego (Ch 9935) - AUS1969/01	WARV	KJ495755	KJ495757	KJ495761
Yunnan virus	YUOV	NC_007656.1	NC_007657	NC_007663

The best-fit substitution models for nucleotide alignment and protein alignment were determined using jModelTest 2.1.10 (Guindon and Gascuel, 2003; Darriba et al., 2012) and ProtTest 3.4.2 (Ronquist and Huelsenbeck, 2003; Darriba et al., 2011), respectively. Best-fit substitution models were selected based on lowest BIC and AIC scores. The best-fit substitution models selected for amino acid multiple alignment as determined by ProtTest was LG with gamma distribution including estimation of invariant sites (LG + G + I) for VP1, T2, and T13. The best-fit substitution models selected for nucleotide multiple alignment as determined by jModelTest were generalized-time reversible with gamma distribution including estimation of variant sites (GTR + G + I) for VP1 and T2, and generalized-time reversible with gamma distribution (GTR + G) for T13. Nucleotide trees were prepared using the Bayesian Markov Chain Monte Carlo method, as implemented in MrBayes 3.2.5 (Ronquist and Huelsenbeck, 2003). The analysis was performed for five million steps, with sampling every 1000 steps and discarding the first 10% as burn-in. Amino acid maximum-likelihood trees were prepared in MEGA7 with 1000 bootstrap replicates (Le and Gascuel, 2008; Kumar et al., 2016).

2.2.5: Multi-Step Growth Curves of BUKV

Confluent T-12.5 flasks of Vero cells, BHK-21 [C-13] cells, and R06E cells were inoculated with BUKV in triplicate at a MOI of 0.01. Triplicate mock-infected flasks served as negative controls. An additional flask for each cell line was prepared to count cells and ensure an accurate MOI calculation. Cells were allowed to incubate with virus for 60 min at 37 °C, and then washed with 1× phosphate buffered saline (PBS) three times prior to replacing maintenance media (DMEM with 2% FBS and 1% P/S for Vero cells and BHK-21 [C-13] cells, DMEM-F12 with 2% FBS and 1% P/S for R06E cells). Immediately, 100 µL was taken for the 0 h post-infection timepoint and proportion FBS was increased to 20% prior to freezing at −80 °C. Additional time points were collected at 12, 24, 48, 72, and 96 h post-infection. Back-titrations were performed in duplicate to confirm each inoculum was within two-fold of the desired titer. Viral quantification was assessed by plaque titration as previously described using Vero cell plaque assay (Beaty et al., 1989).

2.2.6: Screening of Archived Field Samples for BUKV RNA

In total, RNA extractions from 171 ERBs representing three populations in Uganda were available for screening (Kading et al., 2018) (Table 2.1, Figure 2.1). RNA was extracted using the MagMax 96 total RNA isolation kit (Applied Biosystems/Ambion, Austin, TX, USA). Eluted RNA from splenic samples was tested in a quantitative reverse-transcriptase PCR (qRT-PCR) assay using TaqMan Fast Virus 1-Step Master Mix (ThermoFisher Scientific, Foster City, CA, USA). Each sample was run in duplicate using primers and probe (5′-3′) (forward: GCA GAC TGT ATC GCG GAA AG, reverse: TAA GTT TCG CTT TCC TCC CGA, probe: CTG AAA CTC GAT CTC CGC AAC GTT CTT) targeting the VP1 gene (RdRp) of BUKV and as single reactions using primers and probe targeting the GAPDH gene (forward: GTC GCC ATC AAT GAC CCC TTC, reverse: TTC AAG TGA GCC CCA GCC, probe: CCA CCC ATG GCA AGT TCA AAG GCA CA) to ensure RNA integrity. Each reaction contained 5 µL splenic RNA, 5 µL TaqMan Fast Virus 1-Step Master Mix, 500 nM each primer, 250 nM probe, and 7.5 µL H2O. All samples were run on a QuantStudio 3 thermocycler using the following cycling parameters: 50 °C for 5 min; 95 °C for 20 s; 95 °C for 3 s, 60 °C for 30 s (40×).

As a positive control, supernatant from BUKV-infected Vero cells was 10-fold serially diluted and from each dilution, RNA was extracted using the Mag-Bind Viral DNA/RNA 96 kit (Omega Bio-Tek, Norcross, GA, USA) on the Kingfisher® extraction system (Thermo Scientific, Rockford, IL, USA). Additionally, plaque assays of the dilution series were performed in duplicate on Vero cells to quantify viral titer (Beaty et al., 1989). A linear regression was performed to correlate viral titer to cutoff threshold value determined by QuantStudio™ Design & Analysis Software run on the QuantStudio™ 3 polymerase chain reaction system (Thermo Scientific, Rockford, IL, USA). The same parameters were used to screen the tick samples. Archived RNA extracted from pooled Ornithodoros faini soft ticks collected in Python Cave, Maramagambo forest, Uganda, were screened for BUKV RNA following the same protocol as for archived bat sample testing (Schuh et al., 2016).

Samples with questionable or suspect-positive results (cycle threshold value of 35–40 or nearing the cycle threshold cutoff) using qRT-PCR were subjected to a nested PCR protocol using the Phusion

HiFi Master Mix (New England BioLabs, Beverly, MA, USA) and run on a T100™ Thermal Cycler (Bio-Rad, Hercules, CA, USA). As a positive control, a synthetic oligonucleotide control was generated targeting a 406 bp region of VP1 to facilitate recognition of a false positive result by Sanger sequencing (Integrated DNA Technologies, Inc., Coralville, IA, USA) (Figure 2.2). Cycling parameters for both Round 1 (forward: 5′-CGC TCC GTC ATT GGT TTG CA-3′, reverse: 5′-ATA GCT TCG CTT ACG CCG GT-3′) and Round 2 (forward: 5′-TCC TTG TCT CAA TCG CGC GT-3′, reverse: 5′-AGC GAC AAA GCC TCC ACA GA-3′) were as follows: 98 °C for 30 s; 98 °C for 7 s, 68.5 °C for 15 s, 72 °C for 20 s (30×); and 72 °C for 6 min.

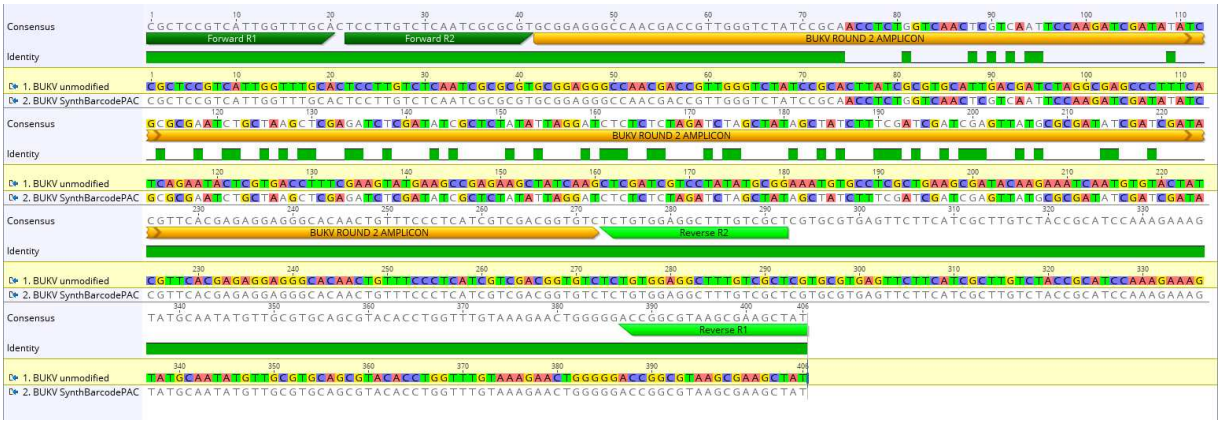


Figure 2.2. Schematic diagram of synthetic barcoded positive control for detection of BUKV in archived tissue samples using nested PCR (with primers for both rounds annotated). Within the 231 bp amplicon, disagreements to the actual BUKV VP1 sequence are highlighted to show differences appreciated upon Sanger sequencing.

2.3: Results

2.3.1: Virus Isolation

Four days post-inoculation on Vero cells, plaques were visible for the spleen of bat #432 (UGA432, JCK8050, FMNH 223820) (Figure 2.3), a young male ERB captured in Kasokero cave in January 2013 (Table 2.1, Figure 2.1). Of the additional tissues harvested from the infected bat and tested for BUKV, only liver and spleen were positive for infectious virus by plaque assay; lung, oral and fecal swabs, and intestine were negative.

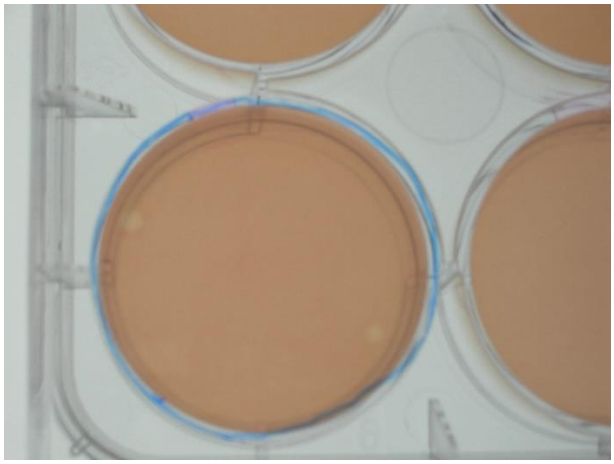


Figure 2.3. Plaque assay demonstrating cytopathic effect caused by BUKV on Vero cells derived from splenic homogenate from Bat UGA432.

2.3.2: Genome Sequencing

Complete coding sequences were obtained for all ten segments of FOMV, IFEV, and JAPV, and for all segments besides segment 3 of BUKV. Each segment possessed one gene encoding one protein, with the exception of segments 9 and 10, which each contained a second shorter ORF similar to other orbiviruses (Van Dijk and Huismans, 1988; Belhouchet et al., 2011). The size and GC% content for each ORF, in addition to its closest match to available data on GenBank using BLASTX (predicted translation product from six reading frames) and its GenBank accession number are listed in Tables 2.3-2.6.

2.3.3: Phylogenetic Analysis

Amino-acid maximum-likelihood trees constructed in MEGA7 for VP1, T2, and T13 are provided (Figure 2.4-2.6), as are nucleotide phylogenetic trees estimated using the MCMC method in MrBayes (Figures 2.7-2.9). Phylogenetic tree topology consistently places BUKV and FOMV viruses with the tick-borne orbiviruses and JAPV and IFEV with the Culicoides/sandfly-borne orbiviruses. Pairwise distances generated in Geneious using MAFFT for both nucleotide and amino acids are listed for each of the four newly sequenced orbiviruses in addition to the other previously-sequenced orbiviruses obtained from Genbank in Figure 2.10 (T2), Figure 2.11 (VP1), and Figure 2.12 (T13).

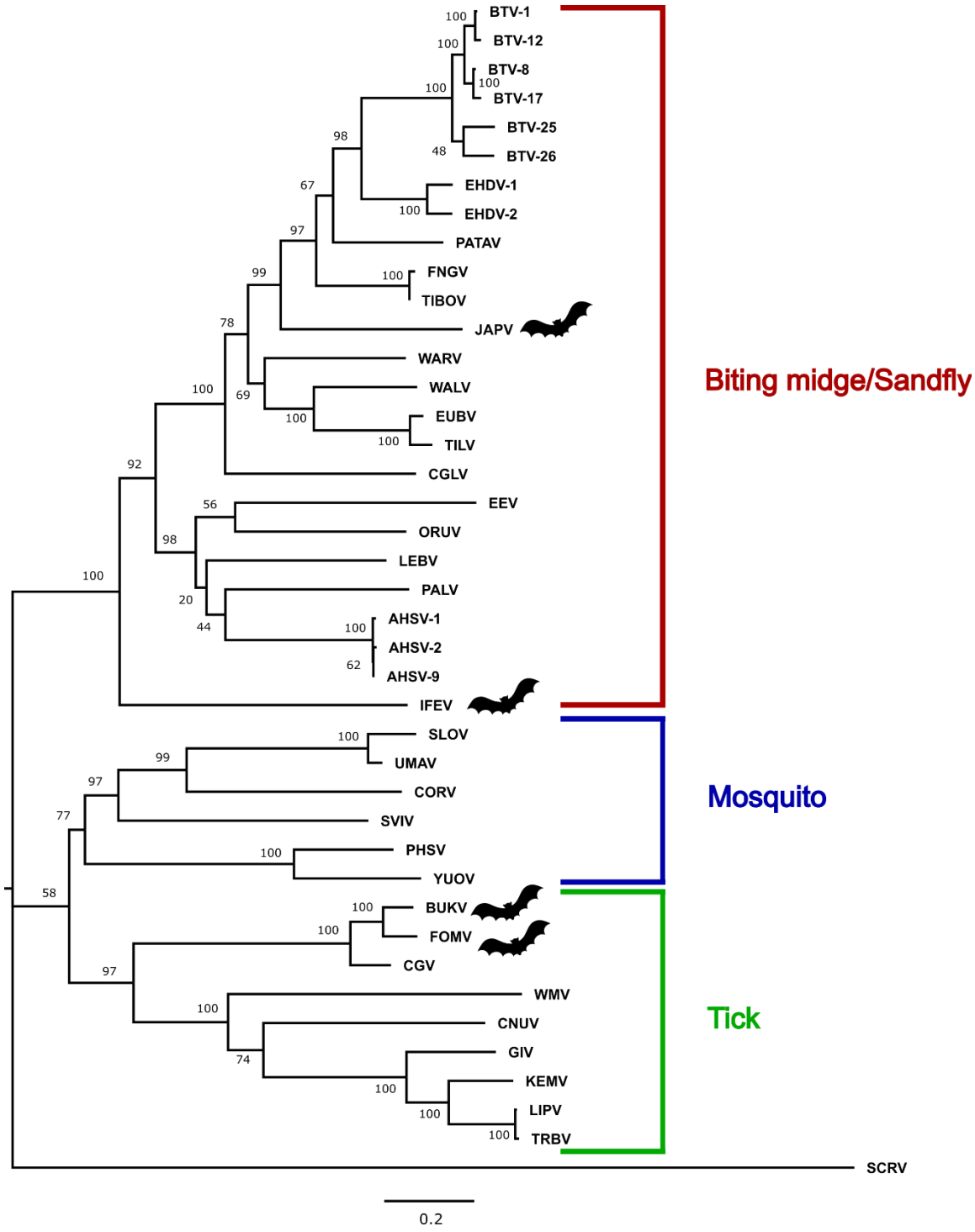


Figure 2.4. Maximum-likelihood phylogenetic tree of the viral polymerase (VP1) of selected orbiviruses (amino acid) constructed in MEGA7 using 1000 bootstrap replicates. The tree is drawn to scale, with branch lengths measured in number of substitutions per site. The tree was rooted with St. Croix River virus (SCRV). Bat symbols identify the viruses sequenced in this study. Full virus names are provided in the Materials and Methods section and accession numbers are provided in Table 2.2.

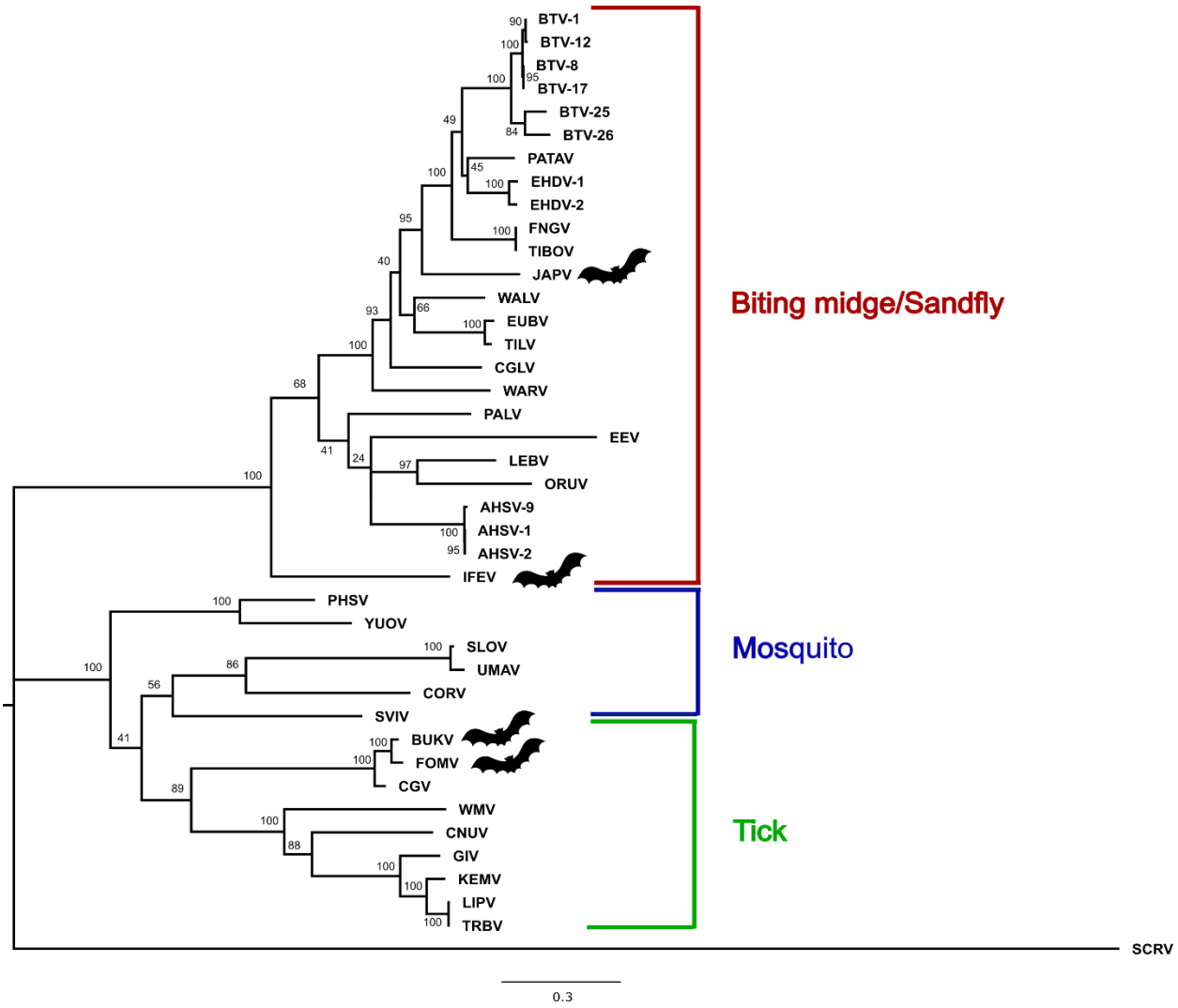


Figure 2.5. Maximum-likelihood phylogenetic tree of the sub-core shell protein (T2) of selected orbiviruses (amino acid) constructed in MEGA7 using 1000 bootstrap replicates. The tree is drawn to scale, with branch lengths measured in number of substitutions per site. The tree was rooted with St. Croix River virus (SCRV). Bat symbols identify the viruses sequenced in this study. Full virus names are provided in the Materials and Methods section and accession numbers are provided in Table 2.2.

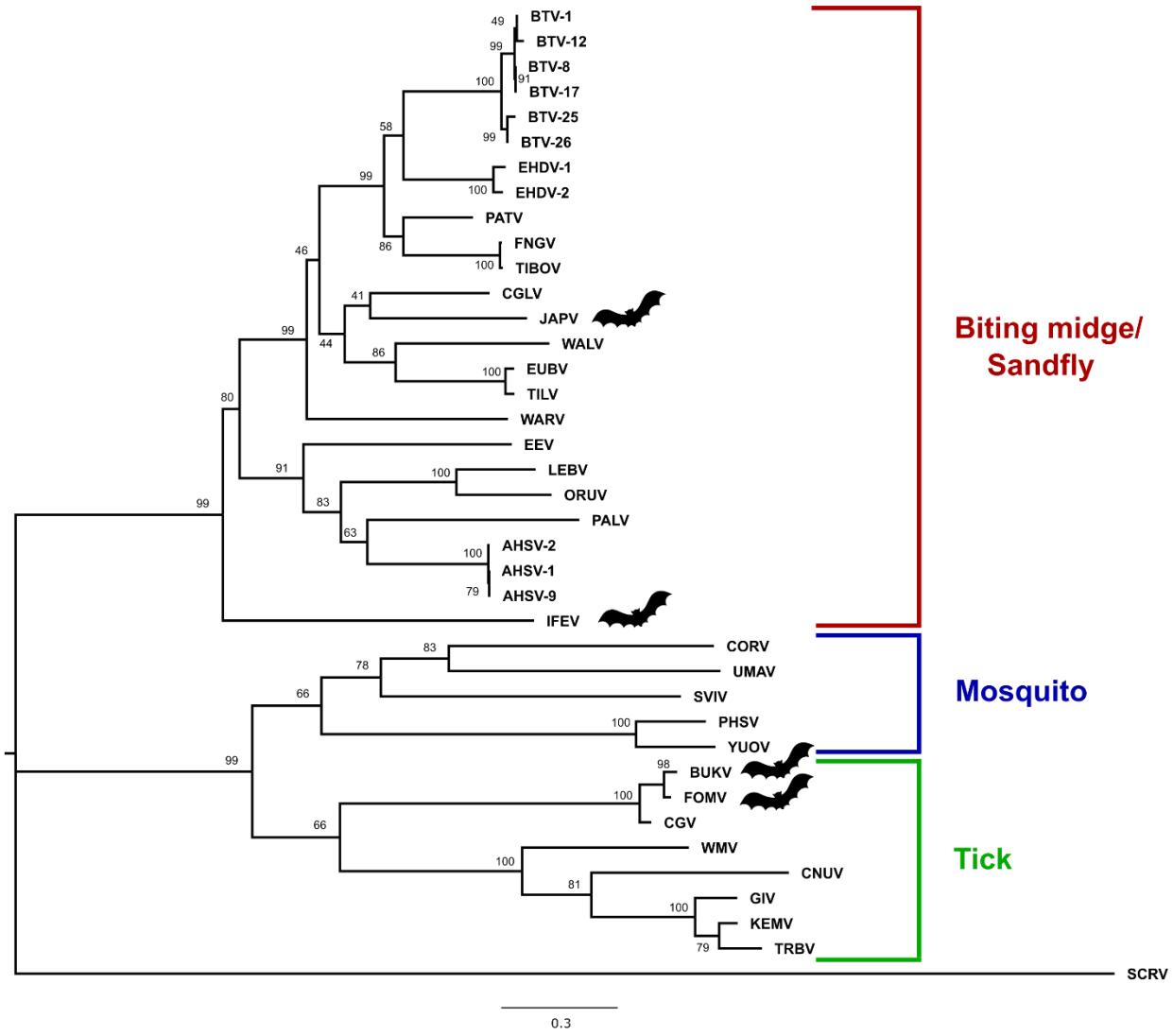


Figure 2.6. Maximum-likelihood phylogenetic tree of the outer core protein (T13) of selected orbiviruses (amino acid) constructed in MEGA7 using 1000 bootstrap replicates. The tree is drawn to scale, with branch lengths measured in number of substitutions per site. The tree was rooted with St. Croix River virus (SCRV). Bat symbols identify the viruses sequenced in this study. Full virus names are provided in the Materials and Methods section and accession numbers are provided in Table 2.2.

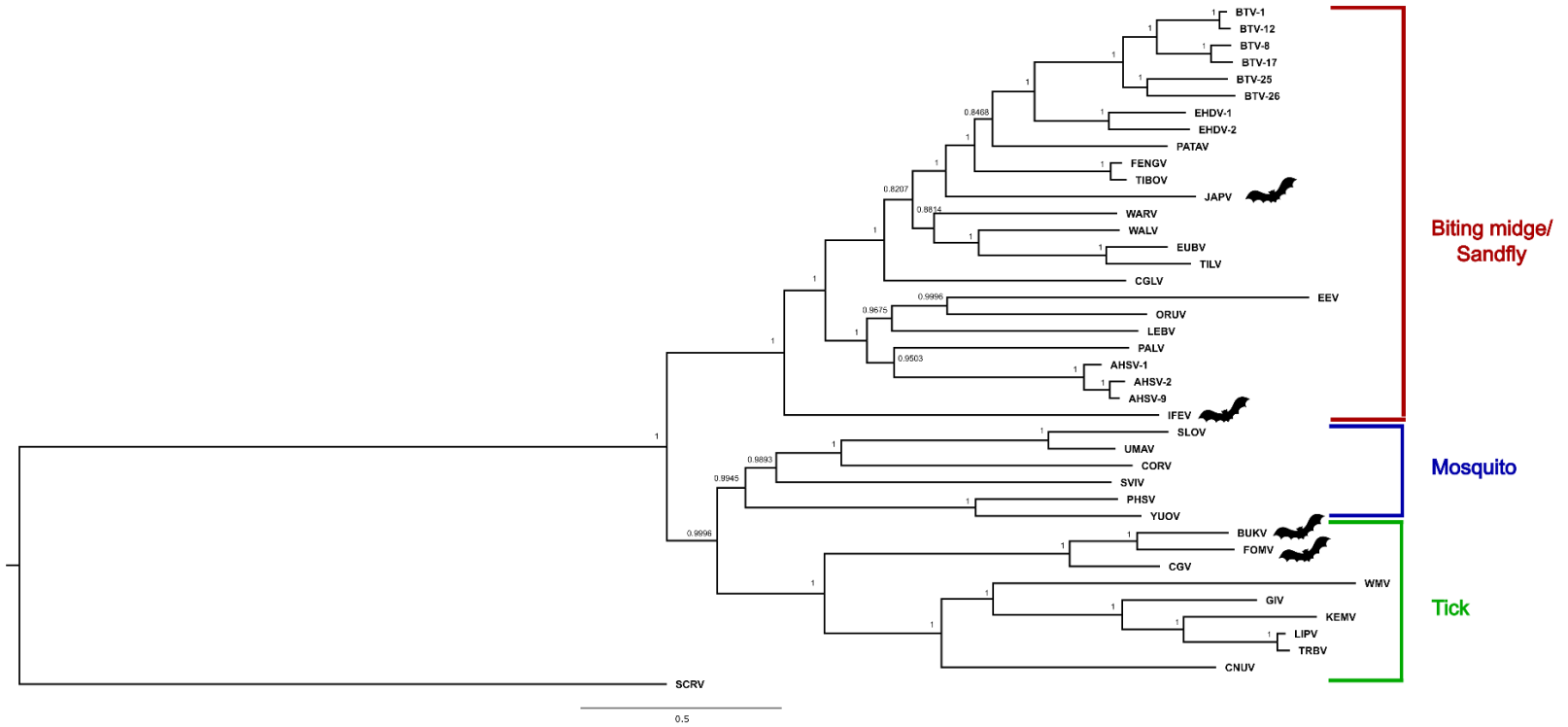


Figure 2.7. Bayesian phylogenetic tree of the viral polymerase gene (VP1) of selected orbiviruses (nucleotide). Branch labels show posterior probability and bar shows substitutions per site.

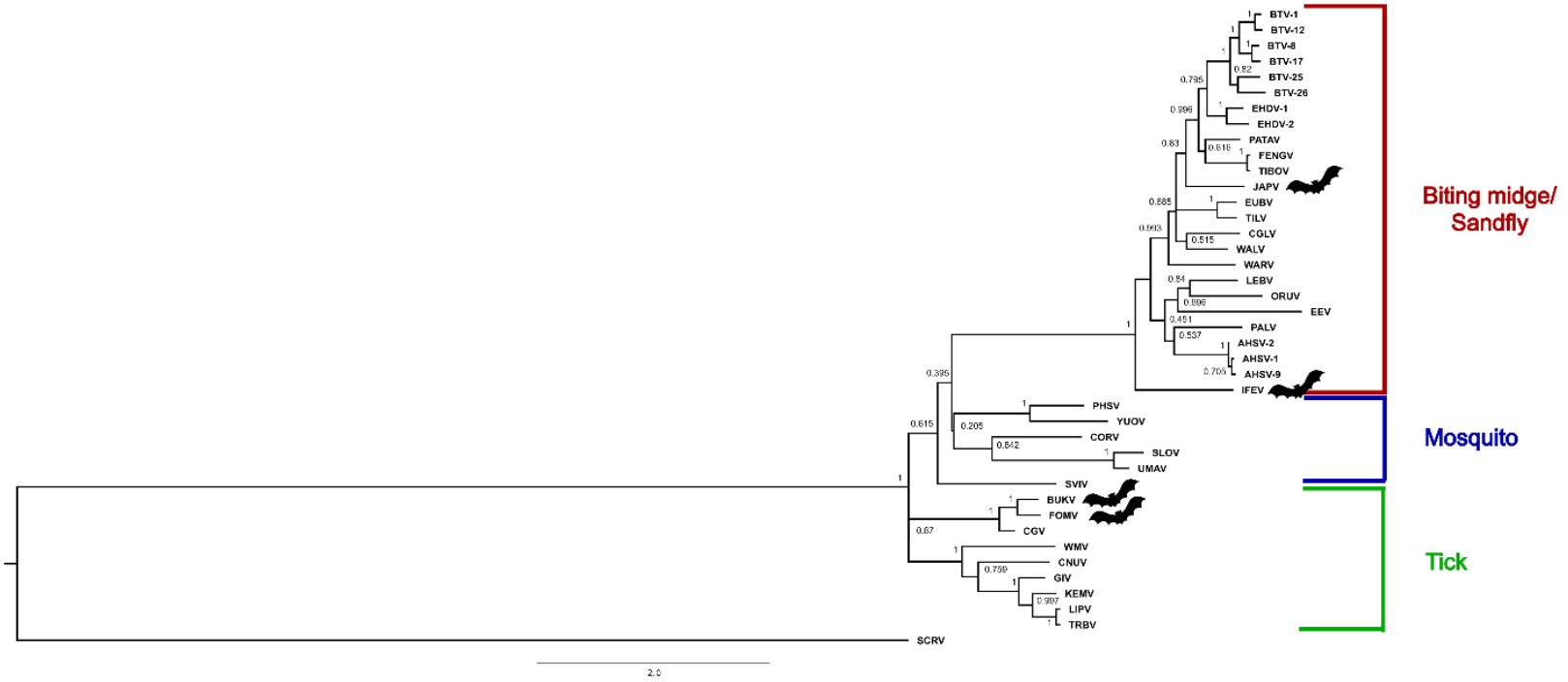


Figure 2.8. Bayesian phylogenetic tree of the gene encoding sub-core shell (T2) of selected orbiviruses (nucleotide). Branch labels show posterior probability and bar shows substitutions per site.

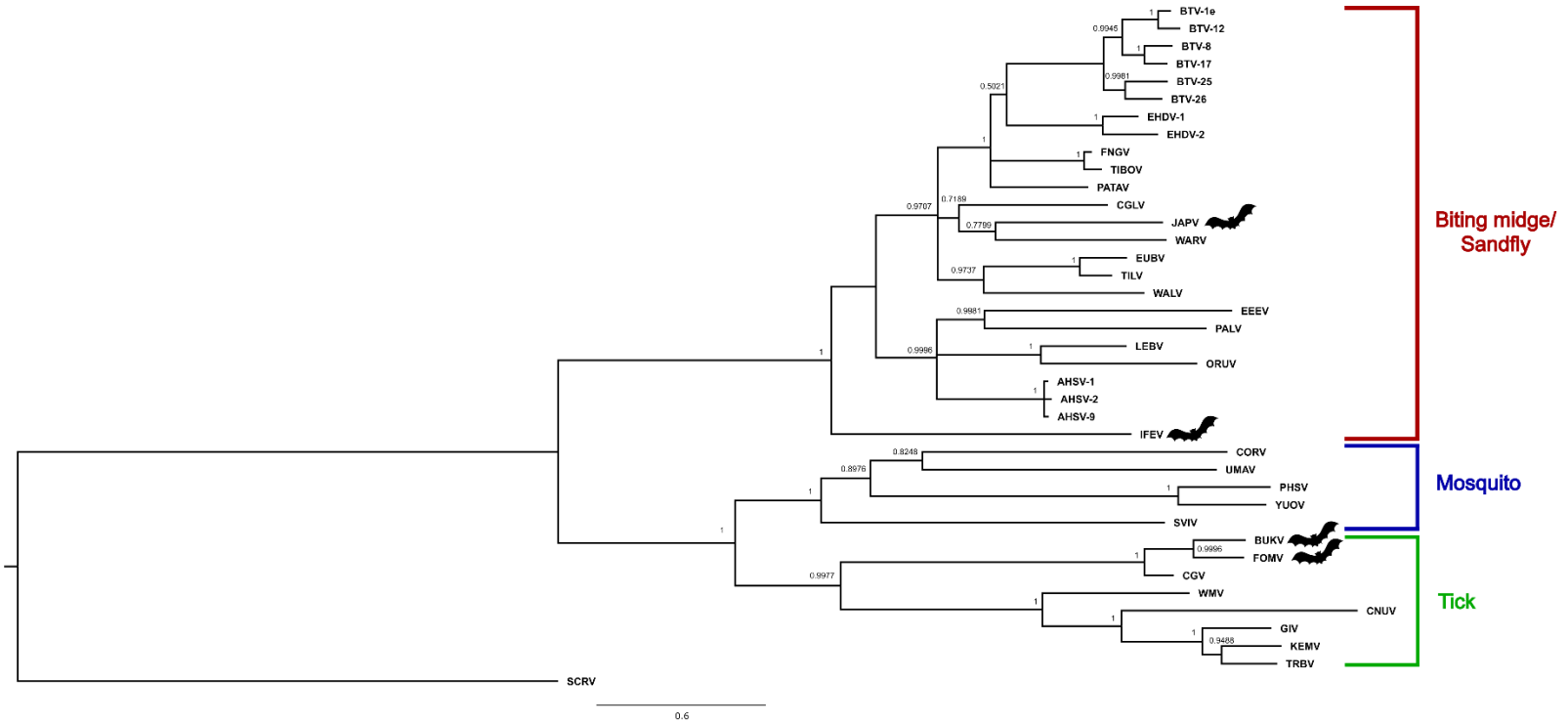
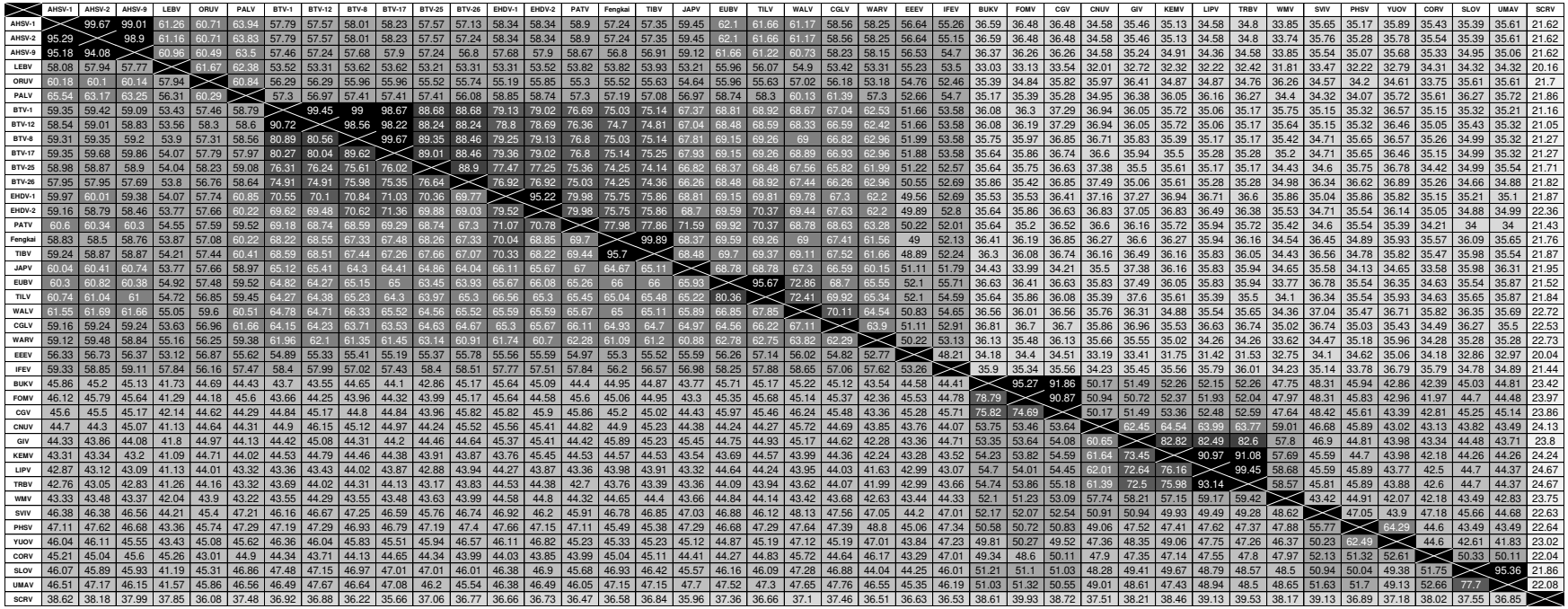
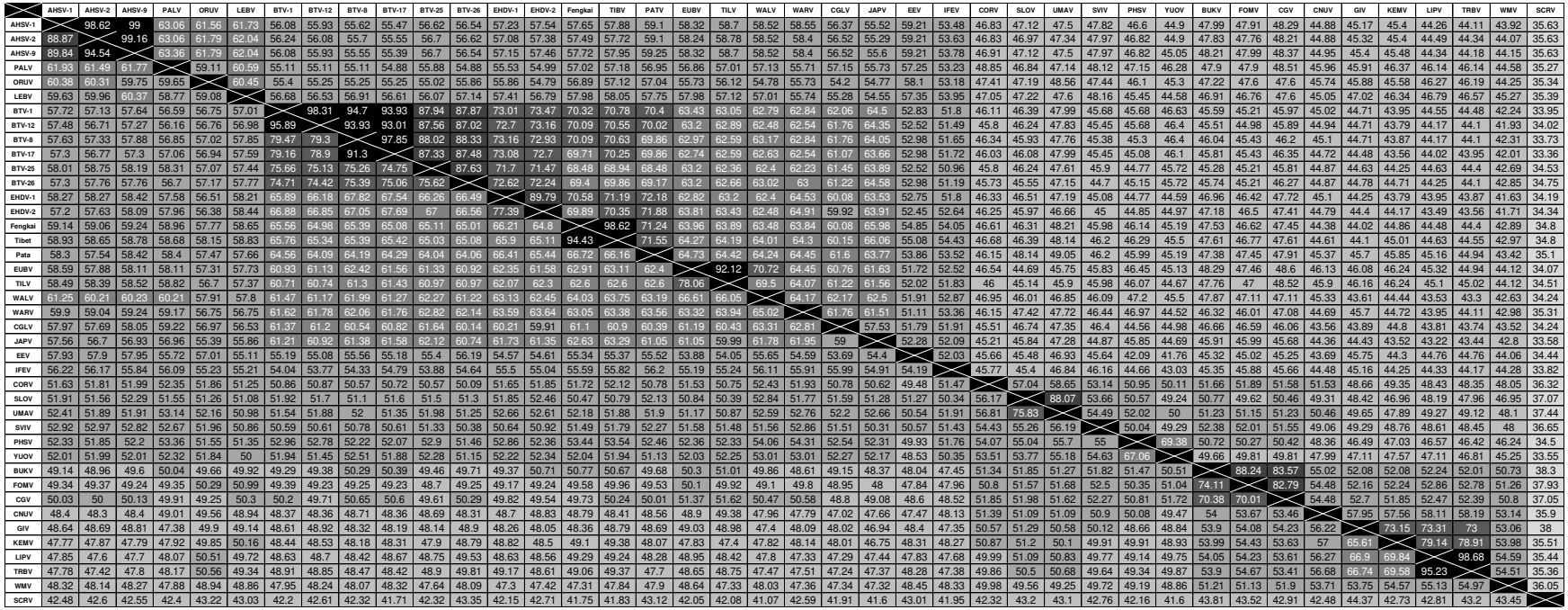


Figure 2.9. Bayesian phylogenetic tree of the gene encoding the outer core protein (T13) of selected orbiviruses (nucleotide). Branch labels show posterior probability and bar shows substitutions per site.



	AHSV-1	AHSV-2	AHSV-9	LEBV	ORUV	PALV	BTV-1	BTV-12	BTV-8	BTV-17	BTV-25	BTV-26	EHDV-1	EHDV-2	PATV	Fengkai	TIBV	JAPV	EUBV	TILV	WALV	CGLV	WARV	EEEV	IFEV	BUKV	FOMV	CGV	CNUV	GIV	KEMV	LIPV	TRBV	WMV	SVIV	PHSV	YUOV	CORV	SLOV	UMAV	SCRV
AHSV-1		99.67	99.01	61.26	60.71	63.94	57.79	57.57	58.01	58.23	57.57	57.13	58.34	58.34	58.9	57.24	57.35	59.45	62.1	61.66	61.17	58.56	58.25	56.64	55.26	36.59	36.48	36.48	34.58	35.46	35.13	34.58	34.8	33.85	35.65	35.17	35.89	35.43	35.39	35.61	21.62
AHSV-2	95.29		98.9	61.16	60.71	63.83	57.79	57.57	58.01	58.23	57.57	57.24	58.34	58.34	58.9	57.24	57.35	59.45	62.1	61.66	61.17	58.56	58.25	56.64	55.15	36.59	36.48	36.48	34.58	35.46	35.13	34.58	34.8	33.74	35.76	35.28	35.78	35.54	35.39	35.61	21.62
AHSV-9	95.18	94.08		60.96	60.49	63.5	57.46	57.24	57.68	57.9	57.24	56.8	57.68	57.9	58.67	56.8	56.91	59.12	61.66	61.22	60.73	58.23	58.15	56.53	54.7	36.37	36.26	36.26	34.58	35.24	34.91	34.36	34.58	33.85	35.54	35.07	35.68	35.33	34.95	35.06	21.62
LEBV	58.08	57.94	57.77		61.67	62.38	53.52	53.31	53.62	53.62	53.21	53.31	53.31	53.52	53.82	53.82	53.93	53.21	55.96	56.07	54.9	53.42	53.31	55.23	53.5	33.03	33.13	33.54	32.01	32.72	32.32	32.22	32.42	31.81	33.47	32.22	32.79	34.31	34.32	34.32	20.16
ORUV	60.18	60.1	60.14	57.94		60.84	56.29	56.29	55.96	55.96	55.52	55.74	55.19	55.85	55.3	55.52	55.63	54.64	55.96	55.63	57.02	56.18	53.18	54.76	52.46	35.39	34.84	35.82	35.97	36.41	34.87	34.87	34.76	36.26	34.57	34.2	34.61	33.75	35.61	35.61	21.7
PALV	65.54	63.17	63.25	56.31	60.29		57.3	56.97	57.41	57.41	57.41	56.08	58.85	58.74	57.3	57.19	57.08	56.97	58.74	58.3	60.13	61.39	57.3	52.66	54.7	35.17	35.39	35.28	34.95	36.38	36.05	36.16	36.27	34.4	34.32	34.07	35.72	35.61	36.27	35.72	21.86
BTV-1	59.35	59.42	59.09	53.43	57.46	58.79		99.45	99	98.67	88.68	88.68	79.13	79.02	76.69	75.03	75.14	67.37	68.81	68.92	68.67	67.04	62.53	51.66	53.58	36.08	36.3	37.29	36.94	36.05	35.72	35.06	35.17	35.75	35.15	35.32	36.57	35.15	35.32	35.21	21.16
BTV-12	58.54	59.01	58.83	53.56	58.3	58.6	90.72		98.56	98.22	88.24	88.24	78.8	78.69	76.36	74.7	74.81	67.04	68.48	68.59	68.33	66.59	62.42	51.66	53.58	36.08	36.19	37.29	36.94	36.05	35.72	35.06	35.17	35.64	35.15	35.32	36.46	35.05	35.43	35.32	21.05
BTV-8	59.31	59.35	59.2	53.9	57.31	58.56	80.89	80.56		99.67	89.35	88.46	79.25	79.13	76.8	75.03	75.14	67.81	69.15	69.26	69	66.82	62.96	51.99	53.58	35.75	35.97	36.85	36.71	35.83	35.39	35.17	35.17	35.42	34.71	35.65	36.57	35.26	34.99	35.32	21.27
BTV-17	59.35	59.68	59.86	54.07	57.79	57.97	80.27	80.04	89.62		89.01	88.46	79.36	79.02	76.8	75.14	75.25	67.93	69.15	69.26	68.89	66.93	62.96	51.88	53.58	35.64	35.86	36.74	36.6	35.94	35.5	35.28	35.28	35.2	34.71	35.65	36.46	35.15	34.99	35.32	21.27
BTV-25	58.98	58.87	58.9	54.04	58.23	59.08	76.31	76.24	75.61	76.02		88.9	77.47	77.25	75.36	74.25	74.14	66.82	68.37	68.48	67.56	65.82	61.99	51.22	52.57	35.64	35.75	36.63	37.38	35.5	35.61	35.17	35.17	34.43	34.6	35.75	36.78	34.42	34.99	35.54	21.71
BTV-26	57.95	57.95	57.69	53.8	56.76	58.64	74.91	74.91	75.98	75.35	76.64		76.92	76.92	75.03	74.25	74.36	66.26	68.48	68.92	67.44	66.26	62.96	50.55	52.69	35.86	35.42	36.85	37.49	35.06	35.61	35.28	35.28	34.98	36.34	36.62	36.89	35.26	34.66	34.88	21.82
EHDV-1	59.97	60.01	59.38	54.07	57.74	60.85	70.55	70.1	70.84	71.03	70.36	69.77		95.22	79.98	75.75	75.86	68.81	69.15	69.81	69.78	67.3	62.2	49.56	52.69	35.53	35.53	36.41	37.16	37.27	36.94	36.71	36.6	35.86	35.04	35.86	35.82	35.15	35.21	35.1	21.87
EHDV-2	59.16	58.79	58.46	53.77	57.66	60.22	69.62	69.48	70.62	71.36	69.88	69.03	79.52		79.98	75.75	75.86	68.7	69.59	70.37	69.44	67.63	62.2	49.89	52.8	35.64	35.86	36.63	36.83	37.05	36.83	36.49	36.38	35.53	34.71	35.54	36.14	35.05	34.88	34.99	22.36
PATV	60.6	60.34	60.3	54.55	57.59	59.52	69.18	68.74	68.59	69.29	68.74	67.3	71.07	70.78		77.98	77.86	71.59	69.92	70.37	68.78	68.63	63.28	50.22	52.01	35.64	35.2	36.52	36.6	36.16	35.72	35.94	35.72	35.42	34.6	35.54	35.39	34.21	34	34	21.43
Fengkai	58.83	58.5	58.76	53.87	57.08	60.22	68.22	68.55	67.33	67.48	68.26	67.33	70.04	68.85	69.7		99.89	68.37	69.59	69.26	69	67.41	61.56	49	52.13	36.41	36.19	36.85	36.27	36.6	36.27	35.94	36.16	34.54	36.45	34.89	35.93	35.57	36.09	35.65	21.76
TIBV	59.24	58.87	58.87	54.21	57.44	60.41	68.59	68.51	67.44	67.26	67.66	67.07	70.33	68.22	69.44	95.7		68.48	69.7	69.37	69.11	67.52	61.66	48.89	52.24	36.3	36.08	36.74	36.16	36.49	36.16	35.83	36.05	34.43	36.56	34.78	35.82	35.47	35.98	35.54	21.87
JAPV	60.04	60.41	60.74	53.77	57.66	58.97	65.12	65.41	64.3	64.41	64.86	64.04	66.11	65.67	67	64.67	65.11		68.78	68.78	67.3	66.59	60.15	51.11	51.79	34.43	33.99	34.21	35.5	37.38	36.16	35.83	35.94	34.65	35.58	34.13	34.65	33.58	35.98	36.31	21.95
EUBV	60.3	60.82	60.38	54.92	57.48	59.52	64.82	64.27	65.15	65	63.45	63.93	65.67	66.08	65.26	66	66	65.93		95.67	72.86	68.7	65.55	52.1	55.71	36.63	36.41	36.63	35.83	37.49	36.05	35.83	35.94	33.77	36.78	35.54	36.35	34.63	35.54	35.87	21.52
TILV	60.74	61.04	61	54.72	56.85	59.45	64.27	64.38	65.23	64.3	63.97	65.3	66.56	65.3	65.45	65.04	65.48	65.22	80.36		72.41	69.92	65.34	52.1	54.59	35.64	35.86	36.08	35.39	37.6	35.61	35.39	35.5	34.1	36.34	35.54	35.93	34.63	35.65	35.87	21.84
WALV	61.55	61.69	61.66	55.05	59.6	60.51	64.78	64.71	66.33	65.52	64.56	65.52	65.59	65.59	65.67	65	65.11	65.89	66.85	67.85		70.11	64.54	50.83	54.65	36.56	36.01	36.56	35.76	36.31	34.88	35.54	35.65	34.36	37.04	35.47	36.71	35.82	36.35	35.69	22.72
CGLV	59.16	59.24	59.24	53.63	56.96	61.66	64.15	64.23	63.71	63.53	64.63	64.67	65.3	65.67	66.11	64.93	64.7	64.97	64.56	66.22	67.11		63.9	51.11	52.91	36.81	36.7	36.7	35.86	36.96	35.53	36.63	36.74	35.02	36.74	35.03	35.43	34.49	36.27	35.5	22.53
WARV	59.12	59.48	58.84	55.16	56.25	59.38	61.96	62.1	61.35	61.45	63.14	60.91	61.74	60.7	62.28	61.09	61.2	60.88	62.78	62.75	63.82	62.29		50.22	53.13	36.13	35.48	36.13	35.66	35.55	35.02	34.26	34.26	33.62	34.47	35.18	35.96	34.28	35.28	35.28	22.73
EEEV	56.33	56.73	56.37	53.12	56.87	55.62	54.89	55.33	55.41	55.19	55.37	55.78	55.56	55.59	54.97	55.3	55.52	55.59	56.26	57.14	56.02	54.82	52.77		48.21	34.18	34.4	34.51	33.19	33.41	31.75	31.42	31.53	32.75	34.1	34.62	35.06	34.18	32.86	32.97	20.04
IFEV	59.33	58.85	59.11	57.84	56.16	57.47	58.4	57.99	57.02	57.43	58.4	58.51	57.77	57.51	57.84	56.2	56.57	56.98	58.25	57.88	58.65	57.06	57.62	53.26		35.9	35.34	35.56	34.23	35.45	35.56	35.79	36.01	34.23	35.14	33.78	36.79	35.79	34.78	34.89	21.44
BUKV	45.86	45.2	45.13	41.73	44.69	44.43	43.7	43.55	44.65	44.1	42.86	45.17	45.64	45.09	44.4	44.95	44.87	43.77	45.71	45.17	45.22	45.12	43.54	44.58	44.41		95.27	91.86	50.17	51.49	52.26	52.15	52.26	47.75	48.31	45.94	42.86	42.39	45.03	44.81	23.42
FOMV	46.12	45.79	45.64	41.29	44.18	45.6	43.66	44.25	43.96	44.32	43.99	45.17	45.64	44.58	45.6	45.06	44.95	43.3	45.35	45.68	45.14	45.37	42.36	45.53	44.78	78.79		90.87	50.94	50.72	52.37	51.93	52.04	47.97	48.31	45.83	42.96	41.97	44.7	44.48	23.97
CGV	45.6	45.5	45.17	42.14	44.62	44.29	44.84	45.17	44.8	44.84	43.96	45.82	45.82	45.9	45.86	45.2	45.02	44.43	45.97	45.46	46.24	45.48	43.36	45.28	45.71	75.82	74.69		50.17	51.49	53.36	52.48	52.59	47.64	48.42	45.61	43.39	42.81	45.25	45.14	23.86
CNUV	44.7	44.3	45.07	41.13	44.64	44.31	44.9	46.15	45.12	44.97	44.24	45.52	45.56	45.41	44.82	44.9	45.23	44.38	44.24	44.27	45.72	44.69	43.85	43.76	44.07	53.75	53.46	53.64		62.45	64.54	63.99	63.77	59.01	46.68	45.89	43.02	43.13	43.82	43.49	24.13
GIV	44.33	43.86	44.08	41.8	44.97	44.13	44.42	45.08	44.31	44.2	44.46	44.64	45.37	45.41	44.42	45.89	45.23	45.45	44.75	44.93	45.17	44.62	42.28	43.36	44.71	53.35	53.64	54.08	60.65		82.82	82.49	82.6	57.8	46.9	44.81	43.98	43.34	44.48	43.71	23.8
KEMV	43.31	43.34	43.2	41.09	44.71	44.02	44.53	44.79	44.46	44.38	43.91	43.87	43.76	45.45	44.53	44.57	44.53	43.54	43.69	44.57	43.99	44.36	42.24	43.28	43.52	54.23	53.82	54.59	61.64	73.45		90.97	91.08	57.69	45.59	44.7	43.98	42.18	44.26	44.26	24.24
LIPV	42.87	43.12	43.09	41.13	44.01	43.32	43.36	43.43	44.02	43.87	42.88	43.94	44.27	43.87	43.36	43.98	43.91	43.32	44.64	44.24	43.95	44.03	41.63	42.99	43.07	54.7	54.01	54.45	62.01	72.64	76.16		99.45	58.68	45.59	45.89	43.77	42.5	44.7	44.37	24.67
TRBV	42.76	43.05	42.83	41.26	44.16	43.32	43.69	44.02	44.31	44.13	43.17	43.83	44.53	44.38	42.7	43.76	43.39	43.36	44.09	43.94	43.62	44.07	41.99	42.99	43.66	54.74	53.86	55.18	61.39	72.5	75.98	93.14		58.57	45.81	45.89	43.88	42.6	44.7	44.37	24.67
WMV	43.33	43.48	43.37	42.04	43.9	43.22	43.55	44.29	43.55	43.48	43.63	43.99	44.58	44.8	44.32	44.65	44.4	43.66	44.84	44.14	43.42	43.68	42.63	43.44	44.33	52.1	51.23	53.09	57.74	58.21	57.15	59.17	59.42		43.42	44.91	42.07	42.18	43.49	42.83	23.75
SVIV	46.38	46.38	46.56	44.21	45.4	47.21	46.16	46.67	47.25	46.59	45.76	46.74	46.92	46.2	45.91	46.78	46.85	47.03	46.88	46.12	48.13	47.56	47.05	44.2	47.01	52.17	52.07	52.54	50.91	50.94	49.93	49.49	49.28	48.62		47.05	43.9	47.18	45.66	44.68	22.63
PHSV	47.11	47.62	46.68	43.36	45.74	47.29	47.19	47.29	46.93	46.79	47.19	47.4	47.66	47.15	47.11	45.49	45.38	47.29	46.68	47.29	47.64	47.39	48.8	45.06	47.34	50.58	50.72	50.83	49.06	47.52	47.41	47.62	47.37	47.88	55.77		64.29	44.6	43.49	43.49	22.64
YUOV	46.04	46.11	45.55	43.43	45.08	45.62	46.36	46.04	45.83	45.51	45.94	46.57	46.11	46.82	45.23	45.33	45.23	45.12	44.87	45.19	47.12	45.19	47.01	43.84	47.23	49.81	50.27	49.52	47.36	48.35	49.06	47.75	47.26	46.37	50.23	62.49		44.6	42.61	41.83	23.02
CORV	45.21	45.04	45.6	45.26	43.01	44.9	44.34	43.71	44.13	44.65	44.34	43.99	44.03	43.85	43.99	45.04	45.11	44.41	44.27	44.83	45.72	44.64	46.17	43.29	47.01	49.34	48.6	50.11	47.9	47.35	47.14	47.55	47.8	47.97	52.13	51.32	52.61		50.33	50.11	22.04
SLOV	46.07	45.89	45.93	41.19	45.31	46.86	47.48	47.15	46.97	47.01	47.01	46.01	46.38	46.9	45.68	46.93	46.42	45.57	46.16	46.09	47.28	46.88	44.04	44.25	46.01	51.21	51.1	51.03	48.28	49.41	49.67	48.79	48.57	48.5	50.94	50.04	49.38	51.75		95.36	21.86
UMAV	46.51	47.17	46.15	41.57	45.86	46.56	46.49	47.67	46.64	47.08	46.2	45.54	46.38	46.49	46.05	47.15	47.15	47.7	47.52	47.3	47.65	47.76	46.55	45.35	46.19	51.03	51.32	50.55	49.01	48.61	47.43	48.94	48.5	48.65	51.63	51.7	49.13	52.66	77.7		22.08
SCRV	38.62	38.18	37.99	37.85	36.08	37.48	36.92	36.88	36.22	35.66	37.06	36.77	36.66	36.73	36.47	36.58	36.84	35.96	37.36	36.66	37.1	37.46	36.51	36.63	36.53	38.61	39.93	38.72	37.51	38.21	38.46	39.13	39.53	38.17	39.13	36.89	37.18	38.02	37.55	36.85	

Figure 2.10. Matrix of T2 pairwise identity percentages of nucleotide (lower left) and amino acid (upper right) between members of the genus *Orbivirus*.



	AHSV-1	AHSV-2	AHSV-9	PALV	ORUV	LEBV	BTV-1	BTV-12	BTV-8	BTV-17	BTV-25	BTV-26	EHDV-1	EHDV-2	Fengkai	TIBV	PATV	EUBV	TILV	WALV	WARV	CGLV	JAPV	EEV	IFEV	CORV	SLOV	UMAV	SVIV	PHSV	YUOV	BUKV	FOMV	CGV	CNUV	GIV	KEMV	LIPV	TRBV	WMV	SCRV
AHSV-1		98.62	99	63.06	61.56	61.73	56.08	55.93	55.62	55.47	56.62	56.54	57.23	57.54	57.65	57.88	59.1	58.32	58.7	58.52	58.55	56.37	55.52	59.21	53.48	46.83	47.12	47.5	47.82	46.6	44.9	47.99	47.91	48.29	44.88	45.17	45.4	44.26	44.11	43.92	35.63
AHSV-2	88.87		99.16	63.06	61.79	62.04	56.24	56.08	55.7	55.55	56.7	56.62	57.08	57.38	57.49	57.72	59.1	58.24	58.78	58.52	58.4	56.52	55.29	59.21	53.63	46.83	46.97	47.34	47.97	46.82	44.9	47.83	47.76	48.21	44.88	45.32	45.4	44.49	44.34	44.07	35.63
AHSV-9	89.84	94.54		63.36	61.79	62.04	56.08	55.93	55.55	55.39	56.7	56.54	57.15	57.46	57.72	57.95	59.25	58.32	58.7	58.52	58.4	56.52	55.6	59.21	53.78	46.91	47.12	47.5	47.97	46.82	45.05	48.21	47.99	48.37	44.95	45.4	45.48	44.34	44.18	44.15	35.63
PALV	61.93	61.49	61.77		59.11	60.59	55.11	55.11	55.11	54.88	55.88	54.88	55.53	54.99	57.02	57.18	56.95	56.86	57.01	57.13	55.71	57.15	55.73	57.25	53.23	48.85	46.84	47.14	48.12	47.15	46.28	47.9	47.9	48.51	45.96	45.91	46.37	46.14	46.14	44.58	35.27
ORUV	60.38	60.31	59.75	59.65		60.45	55.4	55.25	55.25	55.25	55.02	55.86	55.86	54.79	56.89	57.12	57.04	55.73	56.12	54.78	55.73	54.2	54.77	58.1	53.18	47.41	47.19	48.56	47.44	46.1	45.3	47.22	47.6	47.6	45.74	45.88	45.58	46.27	46.19	44.25	35.34
LEBV	59.63	59.96	60.37	58.77	59.08		56.68	56.53	56.91	56.61	56.07	57.14	57.41	56.79	57.98	58.05	57.75	57.98	57.12	57.01	55.74	55.28	54.55	57.35	53.95	47.05	47.22	47.6	48.16	45.45	44.58	46.91	46.76	47.6	45.05	47.02	46.34	46.79	46.57	45.27	35.39
BTV-1	57.72	57.13	57.64	56.59	56.75	57.01		98.31	94.7	93.93	87.94	87.87	73.01	73.47	70.32	70.78	70.4	63.43	63.05	62.79	62.84	62.06	64.5	52.83	51.8	46.11	46.39	47.99	45.68	45.68	46.63	45.59	45.21	45.97	45.02	44.71	43.95	44.55	44.48	42.24	33.95
BTV-12	57.48	56.71	57.27	56.16	56.76	56.98	95.89		93.93	93.01	87.56	87.02	72.7	73.16	70.09	70.55	70.02	63.2	62.89	62.48	62.54	61.76	64.35	52.52	51.49	45.8	46.24	47.83	45.45	45.68	46.4	45.51	44.98	45.89	44.94	44.71	43.79	44.17	44.1	41.93	34.02
BTV-8	57.63	57.33	57.88	56.85	57.02	57.85	79.47	79.3		97.85	88.02	88.33	73.16	72.93	70.09	70.63	69.86	62.97	62.59	63.17	62.84	61.76	64.05	52.98	51.65	46.34	45.93	47.76	45.38	45.3	46.4	46.04	45.43	46.2	45.1	44.71	43.87	44.17	44.1	42.31	33.73
BTV-17	57.3	56.77	57.3	57.06	56.94	57.59	79.16	78.9	91.3		87.33	87.48	73.08	72.7	69.71	70.25	69.86	62.74	62.59	62.63	62.54	61.07	63.66	52.98	51.72	46.03	46.08	47.99	45.45	45.08	46.1	45.81	45.43	46.35	44.72	44.48	43.56	44.02	43.95	42.01	33.36
BTV-25	58.01	58.75	58.19	58.31	57.07	57.44	75.66	75.13	75.26	74.75		87.63	71.7	71.47	68.48	68.94	68.48	63.2	62.36	62.4	62.23	61.45	63.89	52.52	50.96	45.8	46.24	47.61	45.9	44.77	45.72	45.28	45.21	45.81	44.87	44.63	44.25	44.63	44.4	42.69	34.53
BTV-26	57.3	57.76	57.76	56.7	57.17	57.77	74.71	74.42	75.39	75.06	75.62		72.62	72.24	69.4	69.86	69.17	63.2	62.66	63.02	63	61.22	64.58	52.98	51.19	45.73	45.55	47.15	44.7	45.15	45.72	45.74	45.21	46.27	44.87	44.78	44.71	44.25	44.1	42.85	34.75
EHDV-1	58.27	58.27	58.42	57.58	56.51	58.21	65.89	66.18	67.82	67.54	66.26	66.49		89.79	70.58	71.19	72.18	62.82	63.2	62.4	64.53	60.08	63.53	52.75	51.8	46.33	46.51	47.19	45.08	44.77	44.59	46.96	46.42	47.72	45.1	44.25	43.79	43.95	43.87	41.63	34.19
EHDV-2	57.2	57.63	58.09	57.96	56.38	58.44	66.88	66.85	67.05	67.69	67	66.56	77.39		69.89	70.35	71.88	63.81	63.43	62.48	64.91	59.92	63.91	52.45	52.64	46.25	45.97	46.66	45	44.85	44.97	47.18	46.5	47.41	44.79	44.4	44.17	43.49	43.56	41.71	34.34
Fengkai	59.14	59.06	59.24	58.96	57.77	58.65	65.56	64.98	65.39	65.08	65.11	65.01	66.21	64.8		98.62	71.24	63.96	63.89	63.48	63.84	60.08	65.98	54.85	54.05	46.61	46.31	48.21	45.98	46.14	45.19	47.53	46.62	47.45	44.38	44.02	44.86	44.48	44.4	42.89	34.8
Tibet	58.93	58.65	58.78	58.68	58.15	58.83	65.76	65.34	65.39	65.42	65.03	65.08	65.9	65.11	94.43		71.55	64.27	64.19	64.01	64.3	60.15	66.06	55.08	54.43	46.68	46.39	48.14	46.2	46.29	45.5	47.61	46.77	47.61	44.61	44.1	45.01	44.63	44.55	42.97	34.8
Pata	58.3	57.54	58.42	58.4	57.47	57.66	64.56	64.09	64.19	64.29	64.04	64.06	66.41	65.44	66.72	66.16		64.73	64.42	64.24	64.45	61.6	63.77	53.86	53.52	46.15	48.14	49.05	46.2	45.99	45.19	47.38	47.45	47.91	45.37	45.7	45.85	45.16	44.94	43.42	35.1
EUBV	58.59	57.88	58.11	58.11	57.31	57.73	60.93	61.13	62.42	61.56	61.33	60.92	62.35	61.58	62.91	63.11	62.4		92.12	70.72	64.45	60.76	61.63	51.72	52.52	46.54	44.69	45.75	45.83	46.45	45.13	48.29	47.46	48.6	46.13	46.08	46.24	45.32	44.94	44.12	34.07
TILV	58.49	58.39	58.52	58.82	56.7	57.37	60.71	60.74	61.3	61.43	60.97	60.97	62.07	62.3	62.6	62.6	62.6	78.06		69.5	64.07	61.22	61.56	52.02	51.83	46	45.14	45.9	45.98	46.07	44.67	47.76	47	48.52	45.9	46.16	46.24	45.1	45.02	44.12	34.51
WALV	61.25	60.21	60.23	60.21	57.91	57.8	61.47	61.17	61.99	61.27	62.27	61.22	63.13	62.45	64.03	63.75	63.19	66.61	66.05		64.17	62.17	62.5	51.91	52.87	46.95	46.01	46.85	46.09	47.2	45.5	47.87	47.11	47.11	45.33	43.61	44.44	43.53	43.3	42.63	34.24
WARV	59.9	59.04	59.24	59.17	56.75	56.75	61.62	61.78	62.06	61.76	62.82	62.14	63.59	63.64	63.05	63.38	63.56	63.32	63.94	65.02		61.76	61.51	51.11	53.36	46.15	47.42	47.72	46.44	46.97	44.52	46.32	46.01	47.08	44.69	45.7	44.72	43.95	44.11	42.98	35.31
CGLV	57.97	57.69	58.05	59.22	56.97	56.53	61.37	61.2	60.54	60.82	61.64	60.14	60.21	59.91	61.1	60.9	60.39	61.19	60.43	63.31	62.81		57.53	51.79	51.91	45.51	46.74	47.35	46.4	44.56	44.98	46.66	46.59	46.06	43.56	43.89	44.8	43.81	43.74	43.52	34.24
JAPV	57.56	56.7	56.93	56.96	55.39	55.86	61.21	60.92	61.38	61.58	62.12	60.74	61.73	61.35	62.63	63.29	61.05	61.05	59.99	61.78	61.95	59		52.28	52.09	45.21	45.84	47.28	44.87	45.85	44.69	45.91	45.99	45.68	44.36	44.43	43.52	43.22	43.44	42.8	33.58
EEV	57.93	57.9	57.95	55.72	57.01	55.11	55.19	55.08	55.56	55.18	55.4	56.19	54.57	54.61	55.34	55.37	55.52	53.88	54.05	55.65	54.59	53.69	54.4		52.03	45.66	45.48	46.93	45.64	42.09	41.76	45.32	45.02	45.25	43.69	45.75	44.3	44.76	44.76	44.06	34.44
IFEV	56.22	56.17	55.84	56.09	55.23	55.21	54.04	53.77	54.33	54.79	53.88	54.64	55.5	55.04	55.59	55.82	56.2	55.19	55.24	56.11	55.91	55.99	54.91	54.19		45.77	45.4	46.84	46.16	44.66	43.03	45.35	45.88	45.66	44.48	45.16	44.25	44.33	44.17	44.28	33.82
CORV	51.63	51.81	51.99	52.35	51.86	51.25	50.86	50.87	50.57	50.72	50.57	50.09	51.65	51.85	51.72	52.12	50.78	51.53	50.75	52.43	51.93	50.78	50.62	49.48	51.47		57.04	58.65	53.14	50.95	50.11	51.66	51.89	51.58	51.53	48.66	49.35	48.43	48.35	48.05	36.32
SLOV	51.91	51.56	52.29	51.55	51.26	51.08	51.92	51.7	51.1	51.6	51.5	51.3	51.85	52.46	50.47	50.79	52.13	50.84	50.39	52.84	51.77	51.59	51.28	51.27	50.34	56.17		88.07	53.66	50.57	49.24	50.77	49.62	50.46	49.31	48.42	46.96	48.19	47.96	46.95	37.07
UMAV	52.41	51.89	51.91	53.14	52.16	50.98	51.54	51.88	52	51.35	51.98	51.25	52.66	52.61	52.18	51.88	51.9	51.17	50.87	52.59	52.76	52.2	52.66	50.54	51.91	56.81	75.83		54.49	52.02	50	51.23	51.15	51.23	50.46	49.65	47.89	49.27	49.12	48.1	37.44
SVIV	52.92	52.97	52.82	52.67	51.96	50.86	50.59	50.61	50.78	50.61	51.33	50.38	50.64	50.92	51.49	51.79	52.27	51.58	51.48	51.56	52.86	51.51	50.31	50.57	51.43	54.43	55.26	56.19		50.04	49.29	52.38	52.01	51.55	49.06	49.29	48.76	48.61	48.45	48	36.65
PHSV	52.33	51.85	52.2	53.36	51.55	51.35	52.96	52.78	52.22	52.07	52.9	51.46	52.86	52.36	53.44	53.54	52.46	52.36	52.33	54.06	54.31	52.54	52.31	49.93	51.76	54.07	55.04	55.7	55		69.38	50.72	50.27	50.42	48.36	46.49	47.03	46.57	46.42	46.24	34.5
YUOV	52.01	51.99	52.01	52.32	51.84	50	51.94	51.45	52.51	51.88	52.28	51.15	52.22	52.34	52.04	51.94	51.13	52.03	52.25	53.01	53.01	52.27	52.17	48.53	50.35	53.51	53.77	55.18	54.63	67.06		49.66	49.81	49.81	47.99	47.11	47.57	47.11	46.81	45.25	33.55
BUKV	49.14	48.96	49.6	50.04	49.66	49.92	49.29	49.38	50.29	50.39	49.46	49.71	49.37	50.71	50.77	50.67	49.68	50.3	51.01	49.86	48.61	49.15	48.37	48.04	47.45	51.34	51.85	51.27	51.82	51.47	50.51		88.24	83.57	55.02	52.08	52.08	52.24	52.01	50.73	38.3
FOMV	49.34	49.37	49.24	49.35	50.29	50.99	49.39	49.23	49.25	49.23	48.7	49.25	49.17	49.24	49.58	49.96	49.53	50.1	49.92	49.1	49.8	48.95	48	47.84	47.96	50.8	51.57	51.68	52.5	50.35	51.04	74.11		82.79	54.48	52.16	52.24	52.86	52.78	51.26	37.93
CGV	50.03	50	50.13	49.91	49.25	50.3	50.2	49.71	50.65	50.6	49.61	50.29	49.82	49.54	49.73	50.24	50.01	51.37	51.62	50.47	50.58	48.8	49.08	48.6	48.52	51.85	51.98	51.62	52.27	50.81	51.72	70.38	70.01		54.48	52.7	51.85	52.47	52.39	50.8	37.05
CNUV	48.4	48.3	48.4	49.01	49.56	48.94	48.37	48.36	48.71	48.36	48.69	48.31	48.7	48.83	48.79	48.41	48.56	48.9	49.38	47.96	47.79	47.02	47.66	47.47	48.13	51.39	51.09	51.09	50.9	50.08	49.47	54	53.67	53.46		57.95	57.56	58.11	58.19	53.14	35.9
GIV	48.64	48.69	48.81	47.38	49.9	49.14	48.61	48.92	48.32	48.19	48.14	48.9	48.26	48.05	48.36	48.79	48.69	49.03	48.98	47.4	48.09	48.02	46.94	48.4	47.35	50.57	51.29	50.58	50.12	48.66	48.84	53.9	54.08	54.23	56.22		73.15	73.31	73	53.06	38
KEMV	47.77	47.87	47.79	47.92	49.85	50.16	48.44	48.53	48.18	48.31	47.9	48.79	48.82	48.5	49.1	49.38	48.07	47.83	47.4	47.82	48.14	48.01	46.75	48.31	48.27	50.87	51.2	50.1	49.91	49.91	48.93	53.99	54.43	53.63	57	65.61		79.14	78.91	53.98	35.51
LIPV	47.85	47.6	47.7	48.07	50.51	49.72	48.63	48.7	48.42	48.67	48.75	49.53	48.63	48.56	49.29	49.24	48.28	48.95	48.42	47.8	47.33	47.29	47.44	47.83	47.68	49.99	51.09	50.83	49.77	49.14	49.75	54.05	54.23	53.61	56.27	66.9	69.84		98.68	54.59	35.44
TRBV	47.78	47.42	47.8	48.17	50.56	49.34	48.91	48.85	48.47	48.42	48.9	49.81	49.17	48.61	49.06	49.37	47.7	48.65	48.75	47.47	47.51	47.24	47.37	48.28	47.38	49.86	50.5	50.68	49.64	49.34	49.87	53.9	54.67	53.41	56.68	66.74	69.58	95.23		54.51	35.36
WMV	48.32	48.14	48.27	47.88	48.94	48.86	47.95	48.24	48.07	48.32	47.64	48.09	47.3	47.42	47.31	47.84	47.9	48.64	47.33	48.03	47.36	47.34	47.32	48.45	48.33	49.98	49.56	49.25	49.72	49.19	48.86	51.21	51.13	51.9	53.71	53.75	54.57	55.13	54.97		36.05
SCRV	42.48	42.6	42.55	42.4	43.22	43.03	42.2	42.61	42.32	41.71	42.32	43.35	42.15	42.71	41.75	41.83	43.12	42.05	42.08	41.07	42.59	41.91	41.6	43.01	41.95	42.32	43.2	43.1	42.76	42.16	41.6	43.81	43.52	42.91	42.48	44.37	42.73	42.81	43.2	43.45	

Figure 2.11. Matrix of VP1 pairwise identity percentages of nucleotide (lower left) and amino acid (upper right) between members of the genus *Orbivirus*.

	AHSV-1	AHSV-9	AHSV-2	LEBV	ORUV	PALV	BTV-1	BTV-8	BTV-17	BTV-12	BTV-25	BTV-26	EHDV-1	EHDV-2	Fengkai	TIBV	PATV	CGLV	EUBV	TILV	WALV	WARV	JAPV	EEEV	IFEV	BUKV	FOMV	CGV	CNUV	GIV	KEMV	TRBV	WMV	CORV	UMAV	SVIV	PHSV	YUOV	SCRV
AHSV-1		99.43	99.14	51.58	50.00	52.15	42.86	43.14	43.14	42.29	43.71	44.29	44.57	45.43	44.00	44.29	46.86	49.71	45.14	45.43	46.57	44.00	45.14	48.00	40.00	24.93	26.35	25.50	22.88	22.54	22.54	22.82	23.86	23.01	23.14	24.79	19.32	22.60	16.40
AHSV-9	97.05		99.14	51.29	49.71	51.58	42.57	42.86	42.86	42.00	43.43	44.00	44.57	45.43	43.43	43.71	46.86	49.14	45.14	45.43	46.86	44.00	45.43	47.43	40.00	24.93	26.35	25.50	22.88	22.54	22.25	22.82	24.15	23.30	23.43	25.07	19.32	22.60	16.67
AHSV-2	95.91	95.91		51.29	50.00	51.58	42.86	43.14	43.14	42.29	43.71	44.29	45.14	46.29	44.00	44.29	47.14	49.43	45.14	45.43	46.57	44.29	45.43	47.43	39.71	24.93	26.35	25.50	22.88	22.54	22.25	22.82	24.15	23.01	23.43	25.36	19.03	22.32	16.40
LEBV	58.56	58.75	58.56		68.86	48.14	45.43	46.00	45.71	44.29	46.29	46.00	44.29	46.29	43.14	43.43	46.29	43.43	41.43	41.14	43.71	45.71	41.71	44.29	37.43	24.93	25.21	24.08	24.29	22.82	24.23	23.38	23.30	24.43	21.71	23.65	23.58	22.03	19.58
ORUV	56.70	56.03	55.84	63.15		46.57	41.14	40.86	40.57	40.57	41.43	41.71	42.00	42.29	40.00	40.29	42.57	41.14	43.43	42.86	41.43	42.29	41.43	44.00	38.29	25.78	25.78	25.21	23.45	23.38	24.79	23.66	23.58	24.15	20.57	25.64	23.58	23.16	17.46
PALV	57.14	57.14	56.86	53.04	51.28		40.57	40.86	40.86	40.00	41.43	40.86	40.86	42.00	39.43	39.71	41.43	41.14	38.00	36.86	39.14	40.29	40.00	43.14	35.71	24.08	25.50	25.21	22.60	23.10	23.94	23.94	23.30	25.57	25.43	26.21	23.30	22.60	15.87
BTV-1	51.66	51.19	51.38	52.23	50.90	49.19		98.85	98.57	97.71	92.55	94.27	63.32	63.32	60.17	60.17	64.47	51.71	51.71	51.43	48.57	52.86	46.86	41.14	40.86	26.35	26.06	26.35	22.60	20.56	23.10	21.13	23.01	23.58	24.57	20.80	21.88	19.49	16.40
BTV-8	52.23	52.04	52.04	51.57	49.76	50.71	80.19		99.71	97.14	92.84	94.56	63.61	63.61	60.46	60.46	64.76	52.00	52.00	51.71	48.86	52.86	47.14	41.43	40.86	26.91	26.63	26.35	22.60	20.56	23.10	21.13	23.30	23.30	24.57	21.08	21.88	19.49	16.40
BTV-17	52.52	52.23	52.33	52.04	49.10	49.95	80.48	87.14		96.85	92.84	94.56	63.90	63.90	60.46	60.46	64.76	52.00	52.00	51.71	48.86	52.57	47.14	41.14	40.57	26.63	26.35	26.06	22.60	20.85	23.38	21.41	23.30	23.01	24.57	21.08	21.88	19.21	16.40
BTV-12	52.04	51.47	52.14	51.66	50.90	49.38	90.19	80.19	81.71		90.54	92.26	63.04	63.04	59.89	59.89	63.61	50.86	51.43	51.14	48.00	51.71	46.57	40.57	39.71	26.35	26.06	26.35	22.03	20.00	22.54	20.85	22.16	23.58	24.86	20.80	21.31	18.93	16.40
BTV-25	52.04	52.14	52.52	52.04	48.91	49.48	78.48	76.76	77.33	75.62		97.71	64.47	63.90	60.46	60.46	64.18	52.00	53.43	53.14	49.43	53.14	47.43	40.29	40.29	25.21	24.65	24.36	22.60	21.41	22.54	21.13	23.01	22.73	22.86	19.94	20.74	18.93	15.87
BTV-26	52.52	52.90	52.42	52.33	48.91	50.62	78.19	78.76	78.67	76.67	81.24		65.33	64.47	61.32	61.32	64.76	52.57	54.29	54.00	50.00	53.71	48.29	41.14	40.00	25.21	24.65	24.36	22.32	20.85	22.82	21.69	23.30	23.30	23.43	21.08	21.31	19.21	16.14
EHDV-1	53.28	52.99	53.47	51.85	51.00	50.52	62.95	62.48	63.81	61.81	63.91	62.76		94.27	59.89	60.17	65.33	58.00	54.57	54.57	49.43	56.00	54.86	40.86	42.57	23.51	23.23	24.08	23.16	23.38	23.66	24.51	26.14	22.73	22.00	21.37	21.02	20.62	18.25
EHDV-2	53.56	53.47	53.47	52.42	50.24	52.14	62.86	62.57	62.95	62.48	59.91	62.48	78.95		61.03	61.32	66.76	58.57	54.00	54.29	50.57	54.86	56.00	42.29	43.71	23.51	23.23	24.08	22.32	23.38	23.10	24.51	26.71	22.73	22.57	22.22	20.74	21.19	18.25
Fengkai	54.99	54.61	54.51	51.57	50.24	50.24	62.00	62.76	62.67	62.57	62.10	60.95	60.95	61.24		98.85	67.62	52.00	52.57	52.29	53.14	49.71	51.71	42.00	38.57	23.51	23.80	24.36	23.73	23.66	25.07	24.23	25.28	23.30	23.71	21.94	23.30	21.75	17.46
TIBV	55.46	54.80	54.80	51.85	50.33	50.43	62.19	61.52	61.05	61.81	61.62	60.48	60.57	60.67	92.38		67.91	52.00	52.86	52.57	53.14	50.00	51.71	42.29	38.86	23.80	24.08	24.65	24.01	23.94	25.35	24.51	25.28	23.58	24.00	22.22	23.30	21.47	17.46
PATV	54.32	54.42	54.51	52.33	49.29	50.14	63.43	62.57	64.48	62.29	61.43	61.43	63.62	63.71	66.57	65.71		57.14	57.14	56.86	54.57	50.29	54.86	45.14	41.14	25.21	24.65	24.93	21.75	21.69	22.82	23.66	23.86	25.28	25.14	23.36	21.88	20.06	18.52
CGLV	56.41	56.79	56.89	51.38	50.05	50.71	57.36	55.84	56.89	56.79	55.08	55.94	58.03	58.69	57.84	57.55	59.73		56.86	57.14	55.43	54.00	56.57	42.57	39.14	22.10	22.95	23.23	22.03	22.54	23.38	23.10	24.72	22.44	23.43	24.22	19.32	20.62	16.67
EUBV	54.04	53.75	53.47	49.86	50.05	48.81	56.51	55.46	54.23	56.03	56.79	56.51	56.13	55.27	56.51	56.22	57.74	58.22		95.14	57.71	52.00	52.29	38.86	41.43	27.20	27.76	26.91	22.03	24.23	24.51	25.35	26.71	25.85	26.00	25.07	21.31	22.03	19.05
TILV	54.32	54.42	53.66	51.47	50.62	48.43	56.22	55.94	56.79	54.89	58.31	57.36	58.69	55.75	57.46	57.55	58.50	58.31	80.91		58.57	52.57	53.71	40.00	40.86	27.48	28.05	26.35	22.60	23.10	23.38	24.23	26.99	24.43	25.43	25.07	21.02	20.90	18.78
WALV	53.09	53.09	52.99	51.09	49.19	49.76	54.32	52.33	52.33	52.71	53.37	53.18	54.32	54.23	57.27	56.79	58.88	56.32	60.59	59.92		48.29	51.71	40.29	41.14	26.63	27.20	25.78	24.29	23.66	24.23	24.79	26.14	23.30	24.00	24.79	21.59	23.45	17.73
WARV	52.04	51.95	51.85	51.00	49.19	52.42	57.17	56.41	58.03	56.51	57.46	57.55	59.35	58.12	56.03	56.51	56.79	59.35	58.69	58.97	55.18		52.29	42.00	40.00	24.65	24.08	24.08	24.29	22.54	23.10	23.66	25.00	25.28	22.57	22.51	21.02	19.77	17.99
JAPV	53.66	54.13	53.47	51.28	49.00	52.52	52.99	53.85	53.18	51.95	53.56	52.71	56.79	57.46	57.08	56.79	56.70	57.27	56.32	57.46	56.13	58.41		40.57	37.71	21.53	21.81	22.10	20.62	19.72	21.97	21.69	26.42	23.01	21.43	21.94	22.44	19.77	16.14
EEEV	53.94	53.66	54.13	51.09	49.76	51.57	46.91	48.34	47.58	46.63	46.44	47.39	49.19	48.43	48.62	49.00	50.05	48.81	48.34	48.62	48.91	50.05	49.10		39.14	24.08	23.80	23.80	22.88	22.25	23.94	24.23	22.44	23.58	23.71	23.93	23.30	22.03	15.34
IFEV	51.66	51.19	51.19	47.67	47.20	47.01	47.20	48.53	46.91	45.96	47.96	49.00	48.72	48.72	49.95	50.24	49.95	49.48	48.05	47.67	48.43	48.72	47.77	48.62		23.73	23.73	23.16	23.10	22.75	23.03	23.32	24.36	24.65	20.51	21.59	25.78	24.23	15.83
BUKV	37.72	37.72	38.29	38.85	39.32	37.44	39.98	37.91	38.29	38.66	38.85	38.85	36.41	37.07	35.84	35.75	38.01	36.69	39.14	37.91	37.91	37.54	36.78	37.16	36.69		94.93	88.17	32.30	34.17	35.57	34.45	33.24	31.46	30.70	31.55	29.30	28.57	16.27
FOMV	39.14	39.23	39.04	37.16	39.14	38.76	39.14	40.36	39.23	38.48	39.23	39.14	36.69	36.78	36.41	37.16	38.48	37.91	38.85	40.83	38.38	37.35	36.78	37.54	36.60	77.81		89.01	33.15	34.45	36.13	35.01	34.09	31.18	30.42	32.39	29.58	28.85	17.06
CGV	37.16	37.82	38.10	38.29	37.72	39.14	38.38	36.88	38.10	38.10	39.89	38.38	36.97	38.10	36.69	36.03	37.91	35.75	38.38	37.91	37.54	37.16	36.22	37.54	37.25	73.88	74.91		34.27	35.57	35.85	35.29	34.37	33.43	31.55	32.39	27.89	28.57	17.32
CNUV	36.12	36.02	36.12	37.24	38.37	34.43	34.90	34.24	35.46	34.80	34.90	35.84	36.96	36.49	35.65	35.37	34.90	36.68	36.40	35.27	37.05	35.55	35.18	36.49	34.52	42.44	41.62	42.35		52.66	50.42	50.98	47.19	29.97	28.09	29.21	32.58	32.21	15.97
GIV	34.52	34.33	34.52	36.95	36.20	34.52	34.43	35.55	35.45	35.27	36.30	36.30	36.11	37.61	36.11	36.11	33.21	34.61	35.73	35.92	36.02	34.14	31.99	35.17	35.73	45.25	44.23	46.37	52.77		82.07	77.59	51.54	30.45	26.05	28.85	29.41	30.25	15.67
KEMV	36.67	35.36	35.83	35.83	37.98	36.48	35.36	35.08	36.48	35.45	35.83	35.83	36.39	37.33	39.10	39.10	36.02	35.83	37.23	35.45	35.92	36.02	33.96	36.02	35.45	45.44	42.92	44.23	55.04	70.95		86.28	50.42	31.29	26.05	27.45	29.69	29.41	16.45
TRBV	36.39	36.20	36.39	35.08	37.79	35.83	34.52	34.99	34.89	35.17	35.73	36.11	38.07	39.10	37.70	37.79	35.36	36.39	38.82	38.63	38.17	36.67	35.64	36.48	36.02	43.86	44.04	44.60	54.68	70.76	73.93		50.14	30.45	26.05	28.01	28.85	28.57	16.97
WMV	36.89	36.98	36.60	37.83	41.04	36.51	35.09	34.43	35.28	34.62	35.28	35.94	37.74	38.49	37.55	38.11	36.13	36.98	37.36	36.32	39.62	37.08	37.17	37.08	38.49	44.01	42.42	45.41	50.00	55.77	54.94	55.12		32.11	28.53	27.40	31.36	31.46	19.69
CORV	37.74	38.30	38.96	38.30	39.15	38.49	37.08	36.70	36.23	37.08	36.89	36.79	37.36	37.93	39.34	39.15	40.28	39.53	41.89	39.81	38.30	39.34	36.32	38.68	37.55	40.93	40.56	41.21	37.83	39.91	40.09	39.44	41.42		41.24	36.16	33.52	34.17	19.42
UMAV	38.75	38.75	39.13	37.80	35.14	39.20	38.84	39.79	39.51	39.22	38.37	36.56	38.27	37.99	37.70	38.94	40.27	37.51	40.36	40.27	37.80	37.23	38.08	38.08	38.46	42.11	40.43	40.24	37.78	37.60	38.44	37.60	40.17	46.57		36.08	30.60	30.42	15.79
SVIV	40.44	40.06	40.15	39.77	38.26	38.62	38.35	38.54	37.31	39.58	37.22	37.41	38.26	39.11	39.49	39.68	38.26	38.83	39.30	39.39	40.34	36.84	38.16	39.02	38.64	42.42	42.98	42.32	38.98	39.48	39.94	40.13	38.97	44.98	47.88		31.45	29.58	17.63
PHSV	37.65	37.47	36.99	38.79	36.80	38.98	38.32	37.84	38.22	36.61	37.47	37.75	39.36	38.51	39.64	39.92	38.98	37.56	39.92	39.07	40.97	39.17	38.69	38.41	39.92	38.90	39.93	39.46	40.56	37.98	39.65	39.83	41.44	44.13	44.92	47.08		70.99	16.27
YUOV	37.35	37.16	36.97	37.16	36.50	37.63	36.78	36.12	36.88	36.60	36.22	35.84	36.03	35.56	37.25	36.50	36.88	37.91	37.35	38.01	39.79	37.54	36.12	38.10	36.88	39.57	40.97	41.16	42.05	40.30	40.76	41.04	42.48	45.06	42.79	44.01	67.45		17.76
SCRV	30.40	30.14	29.79	32.43	31.20	30.49	30.58	31.63	31.81	31.02	31.63	30.40	30.67	31.37	32.25	32.87	31.63	30.58	32.25	31.55	31.90	30.93	31.55	30.32	31.72	32.12	30.81	31.16	30.37	31.78	32.21	33.33	34.55	32.17	31.85	32.81	30.68	31.51	

Figure 2.12. Matrix of T13 pairwise identity percentages of nucleotide (lower left) and amino acid (upper right) between members of the genus *Orbivirus*.

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Table 2.3. Genetic analysis of BUKV genome segments, predicted ORFs, and predicted proteins
[1]Partial ORF provided as sequencing coverage at 5' end was too low to obtain start codon.

Seg.	Protein encoded	ORF length (nt)	Pred. protein length	%GC Content (ORF)	Top blastx (ORF), accession, pairwise identity	Accession #
1	VP1	3855	1285	51.9	VP1 (Pol) [CGV], YP_009158901, 83.6%	MK359215
2	VP2 (T2)	2730	2730	52.3	VP2 (T2) [CGV], YP_009158902, 91.7%	MK359216
3	VP3 (Cap)	N/A[1]	N/A[1]	N/A[1]	VP3 (Cap) [CGV], YP)009158903, 74.5%	MK359217
4	VP4 (OC1)	1761	587	50.9	VP4 (OC1) [CGV], YP_009158904, 60.1%	MK359218
5	NS1 (TuP)	1575	525	55.3	NS1 (TuP) [CGV], YP_009158905, 80.2%	MK359219
6	VP5 (OC2)	1608	536	53.7	VP5 (OC2) [CGV], YP_009158906, 79.8%	MK359220
7	VP7 (T13)	1068	356	54.1	VP7 (T13) [CGV], YP_009158907, 88.2%	MK359221
8	NS2 (ViP)	1110	370	54.6	NS2 (ViP) [CGV], YP_009158908, 76.7%	MK359222
9	VP6 (Hel)	1041	347	53.2	VP6 (Hel) [CGV], YP_009158909, 54.3%	MK359223
	NS4	717	239	54.4	VP6 (Hel) [CGV], YP_009158909, 54.3%	
10	NS3	621	207	52.3	NS3 [CGV], YP_009158911, 90.3%	MK359224
	NS3a	579	193	52.0	NS3a [CGV], YP_009158912, 90.1%	

Table 2.4. Genetic analysis of FOMV genome segments, predicted ORFs, and predicted proteins.

Seg.	Protein encoded	ORF length (nt)	Pred. protein length	%GC Content (ORF)	Top blastx (ORF), accession, pairwise identity	Accession #
1	VP1	3855	1285	52.7	VP1 (Pol) [CGV], YP_009158901, 82.8%	MK359225
2	VP2 (T2)	2730	910	50.9	VP2 (T2) [CGV], YP_009158902, 90.9%	MK359226
3	VP3 (Cap)	1908	636	53.7	VP3 (Cap) [CGV], YP_009158903, 71.4%	MK359227
4	VP4 (OC1)	1761	587	50.3	VP4 (OC1) [CGV], YP_009158904, 69.3%	MK359228
5	NS1 (TuP)	1575	525	55.7	NS1 (TuP) [CGV], YP_009158905, 78.6%	MK359229
6	VP5 (OC2)	1608	536	54.7	VP5 (OC2) [CGV], YP_009158906, 83.2%	MK359230
7	VP7 (T13)	1068	356	53.2	VP7 (T13) [CGV], YP_009158907, 89.0%	MK359232
8	NS2 (ViP)	1110	370	56.0	NS2 (ViP) [CGV], YP_009158908, 77.2%	MK359231
9	VP6 (Hel)	1041	347	55.1	N/A	MK359233
	NS4	717	239	55.2	NS4 [CGV], YP_009158910, 60.2%	
10	NS3	621	207	53.9	NS3 [CGV], YP_009158911, 88.8%	MK359234
	NS3a	570	190	53.5	NS3a [CGV], YP_009158912, 89.4%	

Table 2.5. Genetic analysis of JAPV genome segments, predicted ORFs, and predicted proteins.

Seg.	Protein encoded	ORF length (nt)	Pred. protein length	%GC Content (ORF)	Top blastx (ORF), accession, pairwise identity	Accession #
1	VP1	3924	1308	38.3	VP1 [TIBOV], APT68074, 66.3%	MK359235
2	VP2 (OC1)	3636	1212	39.2	VP2 (OC1) [BTV-2], CAO79540, 22.1%	MK359236
3	VP3 (T2)	2703	901	40.9	VP3 (T2) [PATV], AFH41521, 72.0%	MK359237
4	VP4 (Cap)	1932	644	40.7	VP4 (Cap) [BTVX], ASV51737, 57.6%	MK359238
5	NS1 (TuP)	1662	554	44.8	NS1 (TuP) [EHDV-7], AIY25176, 30.3%	MK359239
6	VP5 (OC2)	1590	530	43.1	VP5 (OC2) [CGLV], AGZ91948, 54.0%	MK359240
7	VP7 (T13)	1053	351	44.5	VP7 (T13) [CGLV], YP_008719923, 57.1%	MK359241
8	NS2 (ViP)	1011	337	43.8	NS2 (ViP) [CGLV], AGZ91980, 42.6%	MK359242
9	VP6 (Hel)	804	268	44.7	VP6 (Hel) [WALV], YP_008658421, 32.7%	MK359243
	NS4	234	78	48.7	N/A	
10	NS3	786	262	43.6	NS3 [TILV], AFH41508, 39.1%	MK359244
	NS3a	642	214	44.5	NS3 [TILV], AFH41508, 39.1%	

Table 2.6. Genetic analysis of IFEV genome segments, predicted ORFs, and predicted proteins. .

Seg.	Protein encoded	ORF length (nt)	Pred. protein length	%GC (ORF)	Top blastx (ORF), accession, pairwise identity	Accession #
1	VP1	3900	1300	39.7	VP1 [Heramatsu], AGZ62525, 62.0%	MK359245
2	VP3 (T2)	2685	895	42.6	VP3 (T2) [Heramatsu], AGZ62528, 61.9%	MK359246
3	VP2 (OC1)	2523	841	41.7	VP2 (OC1) [Heramatsu], AGZ62527, 29.1%	MK359247
4	VP4 (OC1)	1902	634	42.0	VP4 (Cap) partial [Heramatsu], AGZ62529, 52.7%	MK359248
5	NS1 (TuP)	1581	527	44.7	NS1 (TuP) [Lebombo], YP_009507714, 32.0%	MK359249
6	VP5 (OC2)	1569	523	43.0	VP5 (OC2) [CGLV], AGZ91955, 46.8%	MK359250
7	VP7 (T13)	1047	349	46.2	VP7 (T13) [Wallal], YP_008658420, 41.7%	MK359251
8	NS2 (ViP)	990	330	44.1	NS2 (ViP) [Heramatsu], AGZ62533, 40.5%	MK359252
9	VP6 (Hel)	768	256	47.5	VP6 (Hel) partial [Heramatsu], AGZ62534, 34.7%	MK359253
	NS4	240	80	53.8	None	
10	NS3	612	204	46.6	NS3 [Heramatsu], AGZ62526, 47.1%	MK359254
	NS3a	561	187	46.3	NS3 [Heramatsu], AGZ62526, 47.1%	

2.3.4: Growth Curves for BUKV

Propagation of BUKV was successful in all three mammalian cell lines tested (Vero, BHK-21 [C-13], and R06E cells) with viral titers peaking at 24 hpi in all three cell lines, and the highest titer achieved in BHK-21 [C-13] cells (1.6 × 107 PFU/mL at 24 hpi) (Figure 2.13). Cytopathic effect was detected in all three cell lines between 12 and 24 hpi.

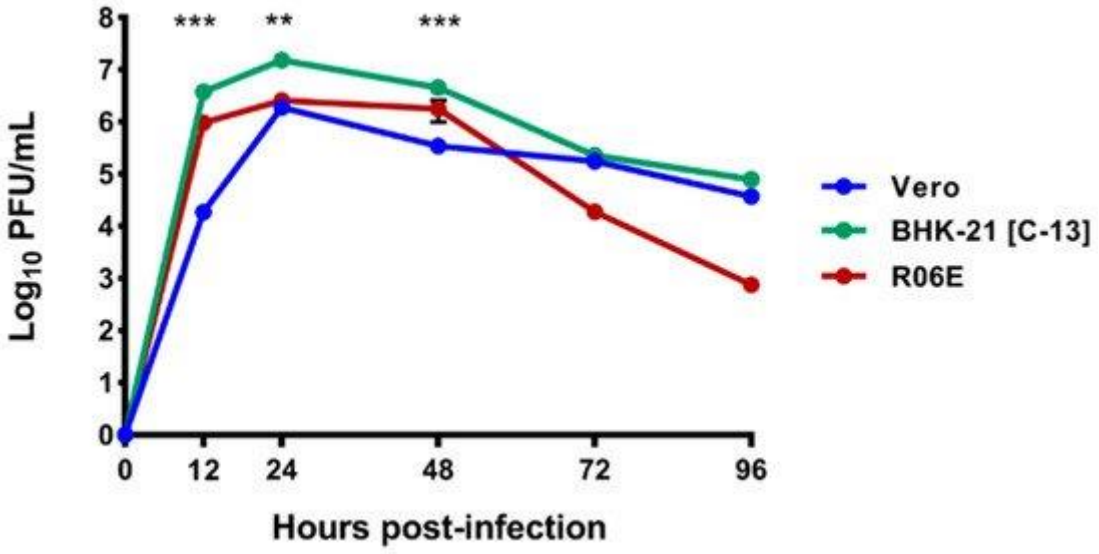

Figure 2.13. Comparison of growth kinetics of BUKV in Vero, BHK-21 [C-13], and R06E cells when infected at MOI 0.01. Means and SEs from three independent replicates are shown. Statistics were performed using a two-way ANOVA with Tukey's correction at each time point. *p*-values: 12 hpi: <0.0001 (Vero vs. BHK-21 [C-13]), 0.0328 (Vero vs. R06E), <0.0001 (BHK-21 [C-13] vs. R06E; 24 hpi: <0.0001 (Vero vs. BHK-21 [C-13]), <0.0001 (BHK-21 [C-13] vs. R06E; 48hpi: <0.0001 (Vero vs. BHK-21 [C-13]), 0.0009 (Vero vs. R06E), <0.0001 (BHK-21 [C-13] vs. R06E). Asterisks in figure indicate the number of two-way comparisons that were significant ($p < 0.05$) at each timepoint.

2.3.5: Testing of Additional Bat and Tick Samples

None of the 171 bat samples screened via qRT-PCR resulted in amplification of BUKV VP1 RNA, though six were suspected or weak positive. None of the six suspect-positive bat samples were confirmed positive for BUKV RNA by nested PCR. Of the 171 samples, GAPDH cDNA was successfully amplified from 86% (147/171) confirming RNA integrity. Of the 513 tick pools tested, 16s rRNA internal extraction control was amplified from 485, but none were positive for BUKV VP1 RNA.

2.4: Discussion

Bats are known to host a number of emerging zoonotic viruses highly pathogenic to humans in the absence of overt pathology within the bat host(Towner et al., 2009; Rahman et al., 2010; Schuh et al., 2017). Limited information exists, however, about the ability of bat species to harbor and transmit medically important arboviruses (Fagre and Kading, 2019). Despite extensive serologic evidence

suggestive of exposure of multiple bat species to arboviruses from different families (*Flaviviridae*, *Togaviridae*, and *Bunyavirales*), few studies have resulted in the isolation of arboviruses from wild-caught bats (Sulkin et al., 1966c; Blackburn et al., 1982; Zhang et al., 1989). Molecular and *in vivo* characterization of novel viruses isolated from wild-caught bats, in addition to enhanced surveillance efforts targeting suspected hosts helps clarify the role of bats as true reservoirs or amplifying hosts for emerging arboviruses.

This study provides complete coding sequences of three previously uncharacterized orbiviruses isolated from bats (JAPV, IFEV, and FOMV) and one novel bat-associated orbivirus, BUKV, isolated from an ERB. Phylogenetic analyses place BUKV and FOMV in the tick-borne orbivirus clade, and IFEV and JAPV cluster with the *Culicoides*/sandfly-borne orbiviruses. This study also provides documentation for *in vitro* propagation of a bat-associated orbivirus in a number of different vertebrate cell lines, one of which was derived from the ERB. The screening of additional archived bat and tick samples resulted in negative findings, but reflects a critical step in the investigation process into the host-vector relationships supported by phylogenetic analyses.

Of the three segments analyzed, the topology of the phylogenetic analysis consistently placed BUKV and FOMV within the tick-borne orbivirus subclade along with Chobar Gorge virus (CGV). Past studies indicated that FOMV is a serotype of the Chobar Gorge virus species based on results of complement fixation, and it is considered as such by the International Committee on the Taxonomy of Viruses (Karabatsos, 1985; Zeller et al., 1989; Attoui et al., 2011). This is consistent with past isolations of FOMV in field-caught Ixodid ticks(Butenko, 1996). CGV has been isolated from *Ornithidoros* spp. ticks in Nepal, and antibodies have been detected in humans and domestic ruminant species in the same region (Centers for Disease Control and Prevention. Arbovirus Catalog: Chobar Gorge). Additionally, the clustering of BUKV, FOMV, and CGV within the same subclade of tick-borne orbiviruses and high degree of nucleotide and amino acid similarity regardless of protein analyzed suggests they are three different serotypes of the same species. Attoui et al. suggested that the amino acid identity for T2 of <91% should be the criteria for designating a species within the genus Orbivirus (Attoui et al., 2001).

According to that criterion BUKV and FOMV viruses are on the border for consideration as new species. BUKV possesses 95.27% amino acid similarity to FOMV and 91.56% similarity to CGV, and Fomede possesses 90.87% amino acid similarity to CGV. Bukakata and Fomede viruses may be ecologically unique from Chobar Gorge in having been isolated from bats, however it is not known whether or not Chobar Gorge virus is also found in bats. Further characterization into the evolutionary relationship of these bat-associated and potentially tick-borne orbiviruses should involve exploration into in vitro growth kinetics in invertebrate cell lines in addition to the potential for serologic cross-reactivity and in vitro reassortment potential.

JAPV and IFEV cluster with the Culicoides/sandfly-borne orbivirus clade and have not yet been approved as species of orbiviruses, but novel genetic sequence obtained during this study indicates that their listing should be revised. Neither JAPV nor IFEV possess the requisite >76% nucleotide identity to any other orbivirus in their conserved T2 gene, indicating they are each their own individual species (Attoui et al., 2011) (Figure 2.10). In the analysis of all three segments, IFEV is very distantly related to all other orbiviruses and may represent its own species due to the low level of nucleotide (maximum 55.7%) and amino acid similarity (maximum 59.3%) to any other orbivirus when analyzing the gene encoding sub-core shell T2 protein (Figure 2.10). Interestingly, the BLASTX results for IFEV virus segments reveal that it is most closely related to Heramatsu orbivirus. Heramatsu virus was obtained from a Japanese eastern long-fingered bat in 1965 and was partially sequenced in 2013 (Miura and Kitaoka, 1977; Zhao et al., 2013). However, due to lack of complete genome information and access to an archive isolate, this virus was not included in our sequencing pipeline or phylogenetic analyses.

Due to their segmented genomes, orbiviruses are known to undergo reassortment during co-infection (Nuttall and Moss, 1989; Shaw et al., 2013). Comparing the placement of JAPV within the VP1 and T2 phylogenies to its placement in the T13 phylogeny suggests that it may have undergone reassortment; however, definitive conclusions surrounding its potential as a reassortant virus are difficult to make due to low bootstrap values and posterior probabilities (Figures 2.4-2.9). Interestingly, our phylogenetic analyses indicate JAPV clusters with the *Culicoides*/sandfly-borne orbiviruses, though it

was isolated from a pool of Culicine mosquitoes in New Guinea (Centers for Disease Control and Prevention. Arbovirus Catalog: Japanaut). Further investigation is required to better characterize potential vector-host associations for JAPV and its potential as a reassortant orbivirus.

BUKV replicated to high titers in all three vertebrate cell lines in which multi-step growth curves were conducted. Two of these cell lines, Vero cells and BHK-21 [C-13] cells, are deficient for the interferon pathway, whereas the R06E pathway has an intact interferon response (Hölzer et al., 2016). The Type I interferon response is the first line of antiviral defense in the mammalian immune system (Perry et al., 2005). Interestingly, viral titers in interferon-competent R06E cells were comparable to interferon-deficient BHK-21 [C13] and Vero cells (Figure 2.13). The immune system of some bat species is highly unique in its constitutive expression of IFN-α (Zhou et al., 2016). A recent study by Pavlovich and colleagues indicate that unlike *Pteropus alecto*, transcriptomic analysis of the ERB does not provide evidence of constitutive interferon expression (Pavlovich et al., 2018). Analysis of interferon expression over the course of infection in bat cells and other interferon-competent vertebrate lines would be an informative way to analyze the presence of this constitutive expression in existing bat cell lines.

None of the 171 bat samples or 513 tick pools tested positive for BUKV viral nucleic acid. However, GAPDH mRNA was tested for each bat sample and samples for which amplification of the GAPDH was not obtained were not included in the denominator of the total tested samples. While all samples were negative for BUKV RNA, some of the bat samples had very high CT values or were nearing the cycle threshold and as such, were considered to be suspect and subjected to a nested PCR protocol. The six suspect samples tested using this nested PCR were also confirmed negative. Samples types screened (spleen and/or liver) are consistent with the organs from which the virus was originally isolated. Testing additional bat species for viral RNA could yield additional information on the circulation of this virus.

While this study provides valuable information regarding potential vector-host associations among the orbiviruses, limitations restrict certain conclusions. Each orbivirus segment contains 1–2 genes, with untranslated regions on either end of the ORF. Due to decreased coverage at the ends of the

reads, variable coverage was achieved throughout the length of each segment and only the complete coding genome of IFEV, JAPV, and FOMV were obtained. The coding complete sequence of segments 1–2 and 4–10 were obtained for BUKV but due to low coverage at the 5′ end of segment 3, the start codon was not obtained (Table 2.3). Individual orbivirus species possess conserved 5′ UTR and 3′ UTR terminal sequences and as such, higher coverage in the untranslated regions would have provided additional information surrounding level of relatedness between these and previously sequenced orbiviruses (Attoui et al., 2011). Field surveillance efforts were opportunistic and retrospective, and only ERB RNA was tested. The testing of additional bat species from nearby geographic areas sharing similar ecological habitats would provide additional information surrounding vertebrate host range. The tick pools tested were also opportunistic and retrospective, and originated in Python Cave, a cave with analogous ecological characteristics to Kasokero Cave, where BUKV was isolated, yet 213 km away (Table 2.1, Figure 2.11).

This study provided coding of the complete genome sequence on three bat-associated orbiviruses that have been discovered to date and coding the complete sequence for nine of ten segments for a newly isolated bat-associated orbivirus. Further, *in vitro* replication kinetics were described in three vertebrate cell lines. From the phylogenetic analyses, inference regarding potential arthropod vector associations can be drawn regarding transmission of bat-associated orbiviruses and supports ticks as a potential vector of BUKV. While the archived bat and tick samples tested for BUKV RNA were negative, this study provides a strong framework for comprehensive viral characterization, including initial discovery, *in vitro* characterization, and the screening of samples collected from the potential vertebrate host and the purported invertebrate vector. The isolation of five orbiviruses from distinct bat species located across space and time and phylogenetically associated with different arthropod vectors indicates a strong association between orbiviruses and bats, and further investigation into the public health impact of these orbiviruses is warranted.

CHAPTER 3: EXPERIMENTAL INFECTION OF JAMAICAN FRUIT BATS WITH BUKAKATA ORBIVIRUS, A NOVEL VIRUS ISOLATED FROM A UGANDAN BAT

3.1: Introduction

In 2013, Bukakata virus (family *Reoviridae*, genus *Orbivirus*) was isolated from the spleen of an Egyptian fruit bat (*Rousettus aegyptiacus*) in Uganda. The bat was apparently healthy at the time of capture and euthanasia, though histopathology was not performed following necropsy (Fagre et al., 2019). Viral discovery efforts often encompass sequencing all or parts of the genome and *in vitro* characterization of growth kinetics, if researchers are able to isolate live virus. However, *in vitro* characterization is limited in its ability to characterize pathogenesis, viremia/pathogen load, and viral shedding from host species of interest. To better inform public health and conservation efforts, the use of animal models provides higher resolution data surrounding the potential of the newly discovered pathogen to threaten the health of humans, domestic animals, or wildlife species.

Bats have been of increasing interest as reservoirs for medically important viruses, and as such, efforts have been made to characterize the pathogenesis and immune response to viral infection in controlled settings (Cogswell-Hawkinson et al., 2012; Amman et al., 2015; Hall et al., 2020). However, few captive bat colonies exist in which to perform such infections owing to high costs associated with housing for free flight requirements in addition to complicated husbandry practices. To date, experimental infections of Old World fruit bats have been performed in fruit bats in genus *Rousettus* with coronaviruses, paramyxoviruses, and filoviruses (Watanabe et al., 2010; Amman et al., 2015, 2020; Jones et al., 2015, 2019; Paweska et al., 2016; Schlottau et al., 2020) and in flying foxes (*Pteropus* spp.) with henipaviruses (Shah and Daniel, 1966; Middleton et al., 2007). Reported experimental infections of New World bats have also been undertaken with varying degrees of success, with experimental infections described so far in Jamaican fruit bats (*Artibeus jamaicensis*), great fruit-eating bats (*Artibeus intermedius*) (Cogswell-Hawkinson et al., 2012; Cabrera-Romo et al., 2014; Munster et al., 2016; Malmlov et al., 2019; Perea-Martinez et al.), big brown bats (*Eptesicus fuscus*) (Davis et al., 2005; Turmelle et al., 2010; Hall et al., 2020), Mexican free-tailed bats (*Tadarida brasiliensis*) (Davis et al.,

2005), and little brown bats (*Myotis lucifugus*) (Reagan et al., 1956; Kolman et al., 1960). A number of challenge studies with lyssaviruses have also been performed in a diverse taxonomic array of bat species (Davis et al., 2005, 2013; Hughes et al., 2006).

Often, assumptions are made about the immunology of bats without considering the vast evolutionary distance between bat genera (Teeling et al., 2002, 2005; Almeida et al., 2011; Zhang et al., 2013). Investigations into immunological mechanisms for innate defenses against viral infection demonstrate marked differences between bats even in the same family. For instance, when considering pteropodid bats, the Australian black flying fox (*Pteropus alecto*) constitutively expresses IFN-α in uninfected tissues (Zhou et al., 2016), while primary cells from the Egyptian fruit bat do not (Pavlovich et al., 2018). Considering the well-characterized co-evolution between bats and some of the viruses they host (e.g. bats in genus *Rhinolophus* with coronaviruses (Cui et al., 2007; Guo et al., 2020)), it is prudent to characterize immune responses in light of the evolutionary and geographic history between vertebrate host and the pathogen in question. However, given geographic spread of arboviruses owing to increasing arthropod ranges and global travel (e.g. Westward expansion of Asian lineage ZIKV)(Besnard et al., 2014; Duong et al., 2017; Hill et al., 2019), evaluation of newly discovered Old World viruses in New World hosts not only allows us to compare host responses to those in the purported Old World reservoir but also to evaluate the potential for species susceptibility and evaluate risk of secondary wildlife reservoirs in the instance of pathogen introduction (Bosco-Lauth et al., 2016, 2018; Hall et al., 2020). Aside from a few reports, very little is known about the pathogenesis and immune responses of New World bats to viruses originating in Old World bats (Munster et al., 2016; Malmlov et al., 2019; Hall et al., 2020).

Four additional orbiviruses have been isolated from bats: two viruses in the bat family *Pteropodidae*, one in family *Nycteridae*, and one in family *Vespertilionidae*. Orbiviruses cluster phylogenetically by the arthropod vector implicated in their transmission cycle, and thus, clades exist for tick-borne orbiviruses, mosquito-borne orbiviruses, and *Culicoides*-borne orbiviruses (Attoui et al., 2011). Both BUKV and the closely related Fomede virus (isolated from the dwarf slit-faced bat (*Nycteris nana)*

in Guinea) appear to be serotypes of Chobar Gorge virus (CGV), which was detected in *Ornithodoros* spp. ticks in Nepal (Fagre et al., 2019; Centers for Disease Control and Prevention. Arbovirus Catalog: Chobar Gorge; Centers for Disease Control and Prevention. Arbovirus Catalog: Fomede). Ife virus was repeatedly isolated from the blood and organs of straw-colored fruit bats (*Eidolon helvum*) in Nigeria, Cameroon, and the Central African Republic in the 1970s, and Japanaut virus was isolated from the Southern blossom bat (*Syconycteris crassa*) in New Guinea around the same time (Centers for Disease Control and Prevention. Arbovirus Catalog: Ife; Centers for Disease Control and Prevention. Arbovirus Catalog: Japanaut). Both Ife and Japanaut viruses cluster phylogenetically with orbiviruses transmitted by biting midges (*Culicoides* spp.) and sandflies) (Fagre et al., 2019). Lastly, Heramatsu virus was isolated in 1965 from the blood of an Eastern long-fingered bat (*Myotis macrodactylus*) and appears to cluster with *Culicoides*-borne orbiviruses (Zhao et al., 2013).

The only evidence for the bat-tick-orbivirus relationship in the New World is the little information available on Matucare virus (MATV), isolated from *Ornithodoros boliviensis* ticks in Bolivia in 1963 (Justines and Kuns, 1970). Serum from *Myotis nigricans* and *Noctilio leporinus* collected close to the site of positive ticks conferred protection in infant mice against infection with Matucare virus. Researchers also discovered larval ticks of *O. boliviensis* parasitizing *Myotis nigricans* and *Molossus major* (Justines and Kuns, 1970). It was suspected that this virus was an orbivirus based on its physical characteristics and as such, its taxonomic placement was investigated and confirmed using a pan-orbivirus assay amplifying a 132-bp fragment of VP1. Phylogenetic analysis of this virus with the other bat-borne orbiviruses indicated that this virus may also be transmitted by ticks (Palacios et al., 2011a).

The tripartite relationship between orbiviruses, bats, and ticks seems to span broad geographic and historical ranges. As such, it provides a valuable opportunity to study the mechanisms driving viral evolution and segmental reassortment, tissue tropism patterns of arboviruses in bats, and the impact of arthropod saliva on infection dynamics. A better understanding of these drivers of viral emergence could help inform risk mitigation and help prevent infection of humans and animals from other tick-borne or bat-borne viruses. The first step is establishing a susceptible Chiropteran host to use in the laboratory. In

this study, we examined the susceptibility of Jamaican fruit bats (*Artibeus jamaicensis)* to Bukakata orbivirus, an orbivirus isolated from an Egyptian fruit bat in 2013.

3.2: Materials and Methods

3.2.1: Virus

Bukakata orbivirus was isolated from the spleen of an Egyptian fruit bat in 2013 (Fagre et al., 2019) and passaged twice on Vero cells (ATCC CCL-81, American Type Culture Collection) prior to collection of supernatant for use in *in vivo* infections.

3.2.2: Pilot study

To initially test for bat susceptibility to infection with BUKV, three sub-adult male Jamaican fruit bats were intraperitoneally inoculated with 10^5 PFU BUKV from Vero cell supernatant under isoflurane anesthesia (IACUC protocol 16-6855A, and Institutional biosafety protocol 16-019B). Following inoculation, plaque assays were performed with the same aliquot of supernatant as was used in infections to confirm titer. Bats were checked twice daily to ensure they were not moribund or distressed, warranting euthanasia. Bats were handled at a maximum one time daily to assess vital signs and reflexes. Following euthanasia, tissues were flash-frozen in liquid nitrogen and stored at -80°C until RNA was Trizol-extracted.

3.2.2.1: Molecular Confirmation of BUKV Infection in Pilot Study Bats

Primers were designed targeting the viral polymerase gene (VP1) (GenBank Accession MK359215.1) (forward: ATT GGC ATC GAT CAC AGC AC, reverse: GCA GAC TGT ATC GCG GAA AG). RNA extracted from the lungs and spleens of infected bats was screened for BUKV VP1 RNA using QIAGEN OneStep reverse-transcription polymerase chain reaction (RT-PCR). Nucleic acid

extracted from cell culture supernatant infected with BUKV was used as a positive amplification control. Results were confirmed by Sanger sequencing.

3.2.3: Timecourse Study

Fourteen Jamaican fruit bats (nine male, five female) were moved from the colony to a large free-flight enclosure two weeks prior to the study start date, in order to allow for acclimation and with the goal of minimizing stress immediately prior to inoculation (IACUC protocol 16-6855A).

Figure 3.1. Free-flight enclosure constructed for timecourse experiment, measuring 10'x10'x7' to allow for bats to move freely about and forage for fruit. The purpose of building this enclosure was to further optimize husbandry conditions and ensure that any background pathology or stress-induced pathology experienced due to housing or transfer from the colony was minimized during the timecourse experiment.

Two weeks following transfer to the free-flight enclosure, one male bat and one female bat were euthanized to serve as negative controls (0 days post-infection (0 dpi)). The remaining ten bats (eight male, four female) were intraperitoneally inoculated with 10^5 PFU viral supernatant under inhalation anesthesia with isoflurane. Owing to the pathology observed in the pilot study bats, the three animals to be held to 15 days post-inoculation (beyond when disease symptoms developed in the pilot study animals)

were moved to Pain Category E under CSU IACUC guidelines, and checked every 8 hours throughout the course of the study to monitor for signs of distress (including moribund state, not roosting properly, trouble moving about the enclosure, etc).

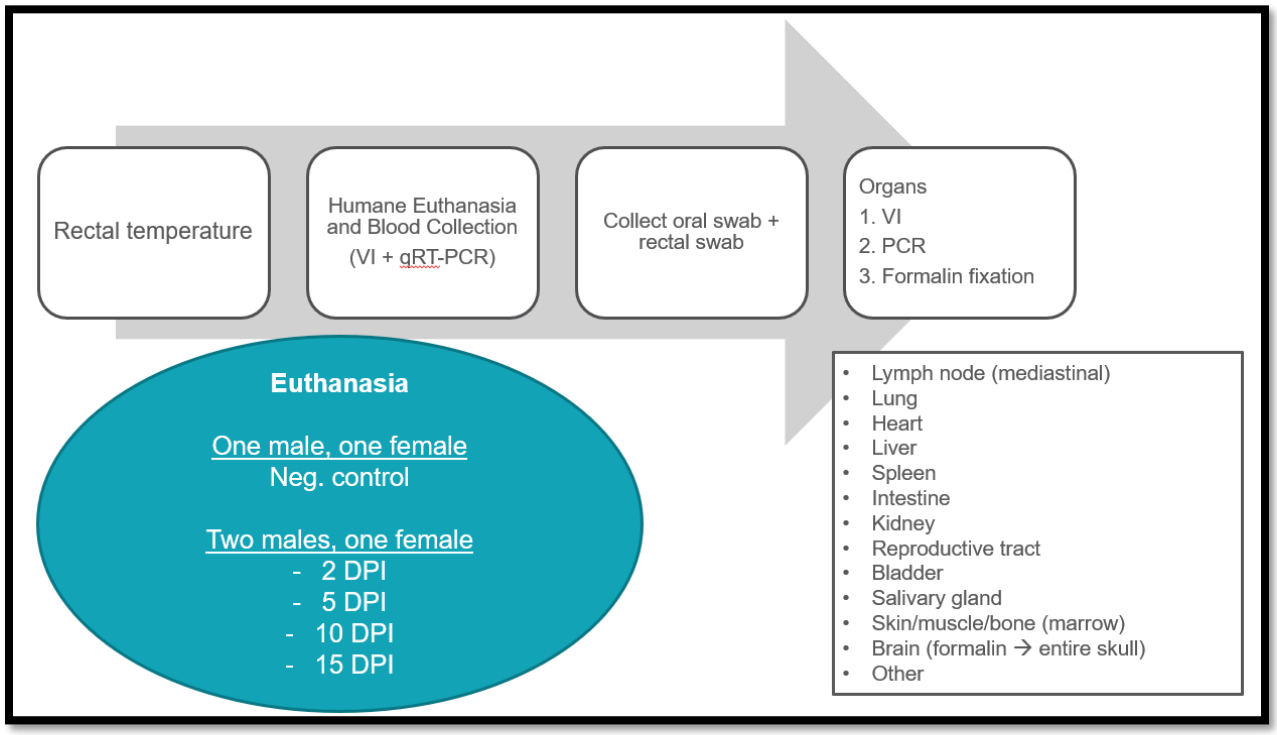

Figure 3.2. Overview of experimental design for BUKV timecourse study, indicating the number of Jamaican fruit bats euthanized at each timepoint and tissues collected for each respective assay.

3.2.4: Euthanasia and sample collection

At 2, 5, 10, and 15 dpi, two male bats and one female bat were anesthetized using isoflurane inhalation anesthesia and euthanized via thoracotomy and cardiac exsanguination. At this time, blood was collected in serum separator tubes and allowed to clot prior to centrifugation and serum collection. Oral and rectal swabs were also collected to assess for viral shedding potential. Tissue samples were divided into three aliquots: one placed into 10% neutral buffered formalin for histopathological analysis, one into viral transport medium (VTM (DMEM with 2% fetal bovine serum and 1% penicillin/streptomycin)) for virus isolation, and one into viral transport medium for RNA extraction. From each bat, the following organs were collected: lung, heart, liver, spleen, mediastinal lymph node, small intestine, kidney,

reproductive tract, bladder, salivary gland, brain, a cross-section of the humerus, and the skull (sagittally sectioned).

3.2.5: Histopathology

Tissues were fixed in 10% neutral-buffered formalin for a minimum of three days prior to being sectioned and trimmed. Slides were stained using hematoxylin and eosin (H&E), and were read by a blinded board-certified veterinary pathologist, who numerically scored inflammation and hemorrhage in addition to providing descriptive pathologic findings. Bone was decalcified for 2 days in formic acid.

3.2.6: Immunohistochemistry

Owing to lack of serologic reagents for BUKV, and the previous phylogenetic placement of BUKV within the Chobar Gorge virus (CGV) serogroup (Fagre et al., 2019), polyclonal antibody in the form of ascites fluid from hyperimmunized mice was obtained from the Centers for Disease Control and Prevention Arbovirus Reference Collection housed at the Division of Vector-borne Diseases Arbovirus Diseases Branch in Fort Collins, CO (Centers for Disease Control and Prevention. Arbovirus Catalog: Chobar Gorge). Polyclonal antibody was diluted 1:500 prior to use. Slides were read by a blinded diplomat of the American College of Veterinary Pathologists, who subjectively scored immunoreactivity in each organ a numerical score, ranging from immunologically unreactive (0) to a high degree of immunoreactivity/staining (3).

3.2.7: Serology

To assess bat serum for the presence of neutralizing antibodies, neutralizing titers were determined using a cutoff of >80% reduction in PFU on Vero cells as previously described (Kading et al., 2013a). Polyclonal ascites fluid from CGV-hyperimmunized mice was used as positive control antisera, as described above.

3.2.8: Virus titration

Plaque assays were performed on Vero cells at 80% confluency. Tissue samples were homogenized and centrifuged, and supernatant from tissue samples and serum was serially diluted (10^{-1} to 10^{-6}) and plated to Vero cells. To allow for viral adsorption, supernatant was added to wells and allowed to incubate at 37°C for one hour, being rocked once every 15 minutes. Subsequently, plates were overlaid with 0.4% agarose on DMEM and incubated for 72 hours prior to fixation with 10% neutral buffered formalin and staining with crystal violet. If results were negative, a second plaque assay was performed with undiluted supernatant to increase sensitivity and detect low amounts of virus (with the exception of liver supernatant, which was diluted at 1:2 to minimize the toxic effects of arginase).

3.2.9: RNA Extraction and qRT-PCR

Samples were extracted using either Trizol or MagBind Viral RNA/DNA Kit on the Kingfisher platform or manually. Each sample was screened for BUKV RNA using the qRT-PCR assay previously described (Fagre et al., 2019) and positive controls extracted from BUKV-infected supernatant collected from Vero cells. A negative template control was included with each qPCR run.

3.2.10: qPCR innate gene microarray

The Primer-BLAST tool was used along with the *Artibeus jamaicensis* genome to design specific primers for 44 innate immune genes as well as 1 housekeeping gene, GAPDH. 2µg RNA (or max possible) from BUKV-infected or control bat spleens was used to generate cDNA using the QuantiNova Reverse Transcription Kit (Qiagen, Cat# 205410). The resulting cDNA was used in the qPCR arrays with the QuantiNova SYBR Green PCR Kit (Qiagen, Cat# 208056) which was cycled using the following parameters: preincubation at 95°C for 120 seconds, 50 cycles of 95°C for 5 seconds, 60°C for 10 seconds.

The fold-change in gene expression was calculated using the ΔΔCT method described previously (Livak and Schmittgen, 2001).

3.3: Results

3.3.1: Pilot Study

At 12dpi, veterinary staff noted that the three inoculated bats displayed hyperemia (reddening, due to increased blood flow to the area) on the abdomen/patagium and rapid shallow breathing suggestive of respiratory distress. In accordance with IACUC guidelines, bats were euthanized and samples collected. Gross pathologic examination revealed severe consolidation in the lungs and congestion on the serosal surface of several abdominal organs, including the intestines and the bladder. Performing two rounds of PCR on lung and spleen samples from two of the pilot study bats revealed the presence of viral nucleic acid in the organs. Histopathologic lesions of perivascular inflammation, hemorrhage, and edema were present in varying degrees of severity in the lungs, liver, and kidney of all three bats. Neutrophilic and eosinophilic gastroenteritis was present in all three bats, and meningeal hemorrhage was present in two of the three bats.

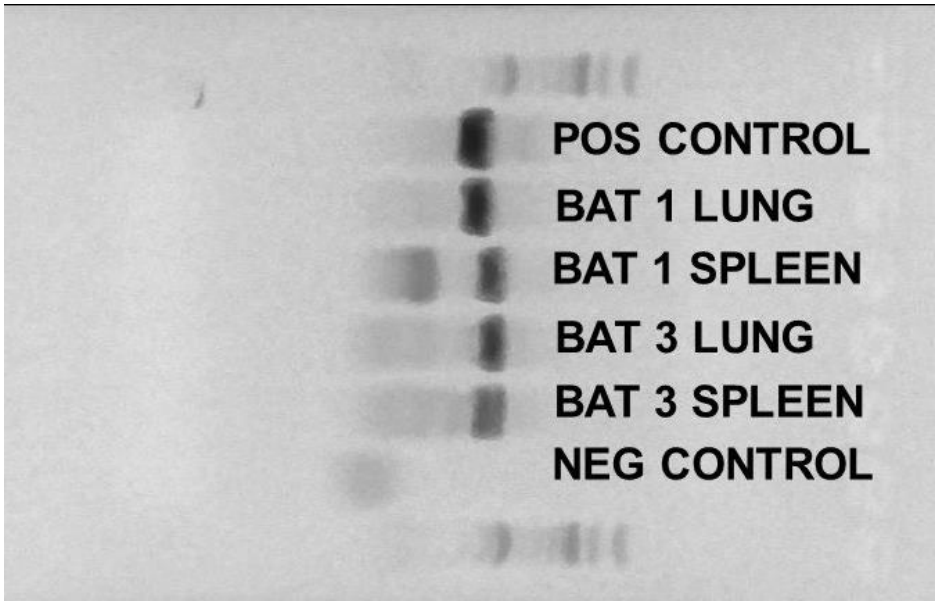

Figure 3.3. Agarose gel displaying amplicon of RT-PCR confirming BUKV RNA amplification from lung and spleen in two of the pilot study bats.

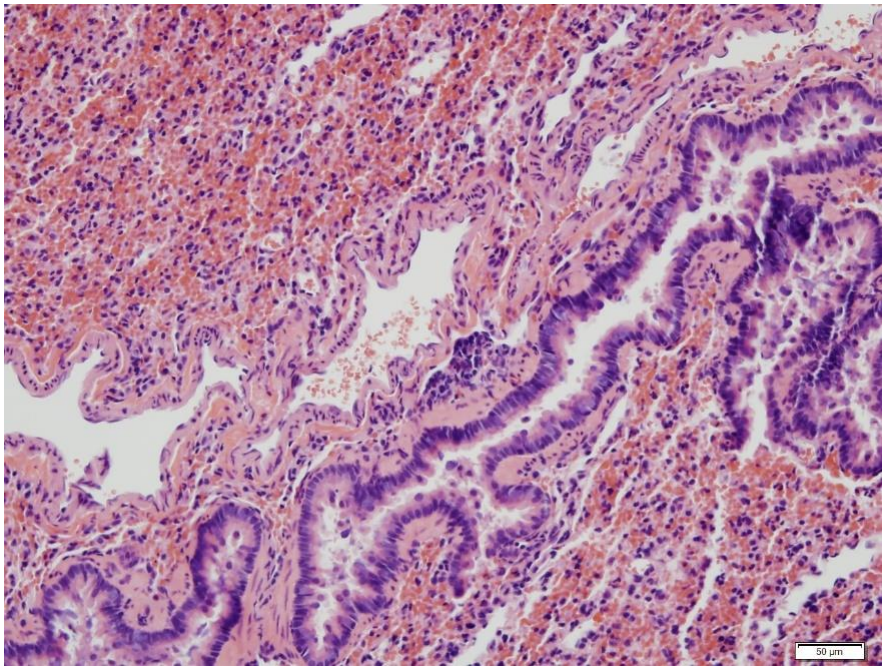

Figure 3.4. Lung of bat 1 with congestion, hemorrhage, atelectasis, and perivascular inflammation.

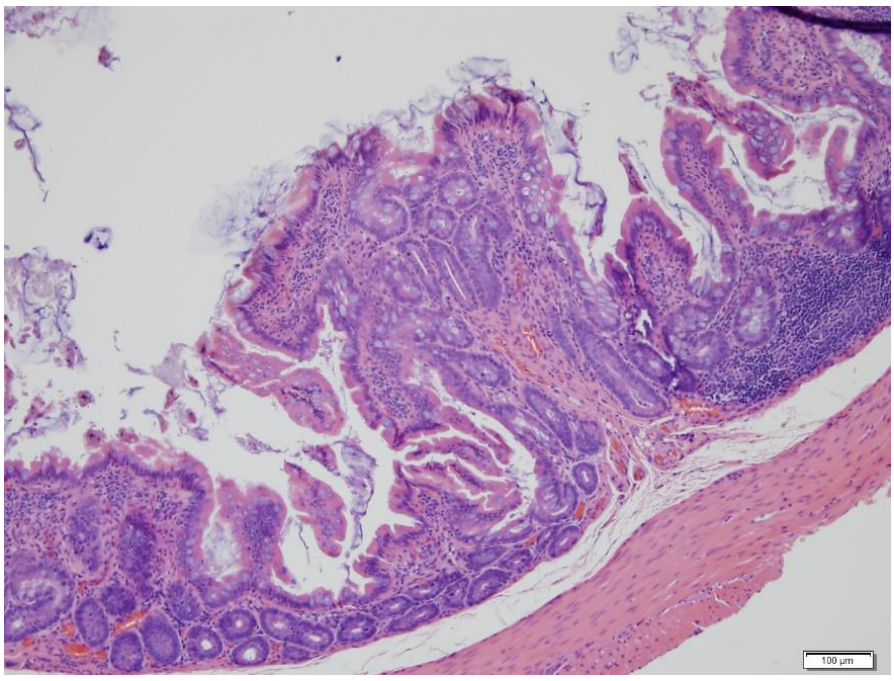

Figure 3.5. Inflammation in the ileum of bat 1 adjacent to a Peyer's patch

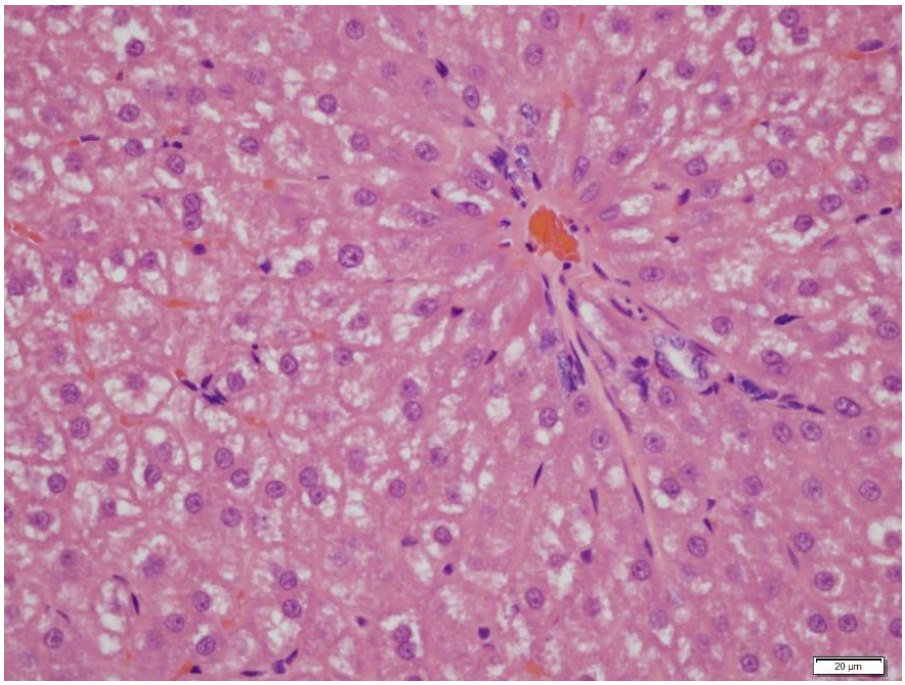

Figure 3.6. Image of the liver of bat 1 with hepatocellularvacuolar change.

3.3.2: Timecourse study

3.3.2.1: Necropsy

Two negative control bats (one male and one female) were euthanized at 0dpi, after being held for two weeks in the free-flight cage. Three intraperitoneally inoculated bats (two males and one female) were euthanized at 2dpi, 5dpi, 10dpi, and 15dpi. At 5dpi, it was noted that one of the female bats appeared pregnant and after consulting with the CSU IACUC, it was decided to keep this bat in the study and euthanize her on the last timepoint (15dpi) to allow investigation into potential vertical transmission, as appreciated in other orbiviruses (Maclachlan and Osburn, 2017).

3.2.2.2: Histopathology

No significant difference was noted in the numerical severity values assigned to the intestinal inflammation, though an increase in pulmonary hemorrhage was appreciated over the course of infection

(Table 3.1). However, mild congestion was also present in the two negative uninfected bats as well. Other histopathologic findings observed in both infected and uninfected bats included mild to moderate lymphoid hyperplasia in the spleen, glycogen changes in the liver, pulmonary congestion and hemorrhage, suppurative and lymphoplasmacytic enteritis.

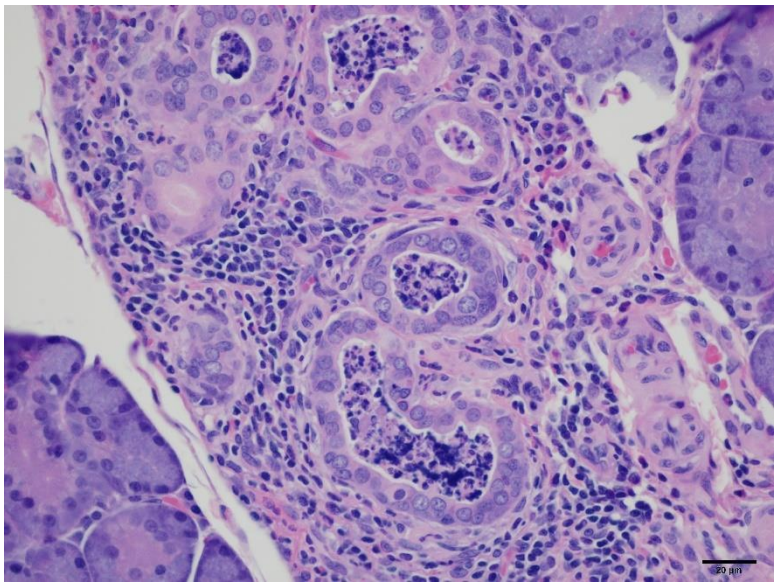

Figure 3.7. Inflammation in the salivary gland (sialoadenitis) appreciated in a male bat euthanized at 10dpi.

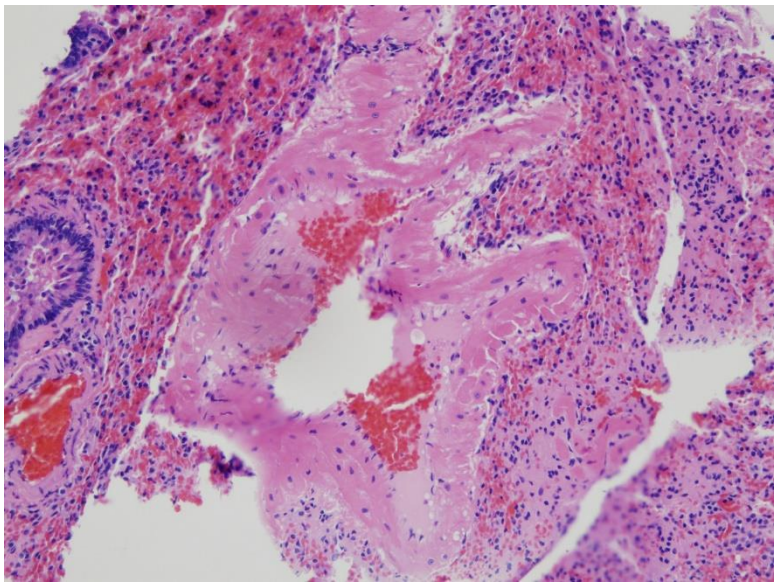

Figure 3.8. Large artery in the lung of bat 14 (pregnant female bat euthanized at 15dpi) showing mild degenerative changes and vacuolation in tunica media.

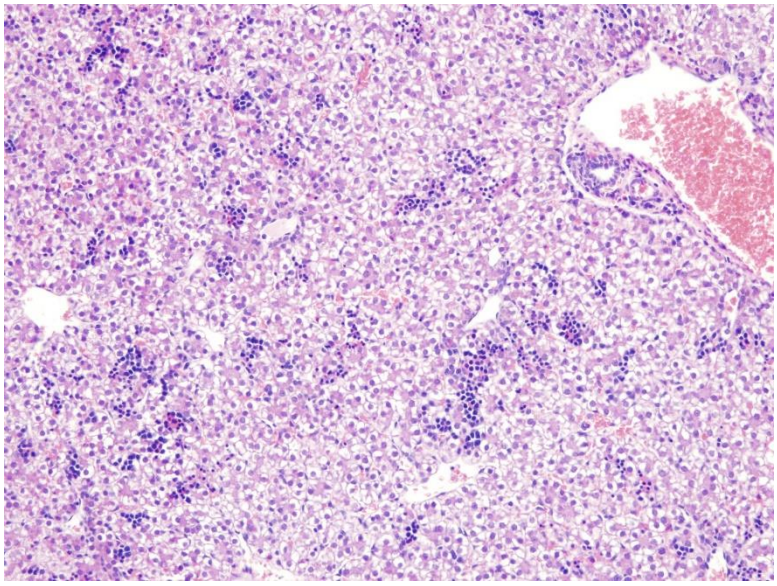

Figure 3.9. Liver of bat 14 (pregnant female bat euthanized at 15dpi) demonstrating diffuse vacuolar change and extramedullary hematopoiesis, which is common in pregnant bats.

3.2.2.3: Immunohistochemistry

Immunoreactivity was observed in several organ systems (see Figure 3.10) but there was no difference in severity between infected (bats #3-15) and uninfected bats (bats #1-2), as scored by our blinded anatomic pathologist, potentially indicative of cross-reactivity or non-specific immunostaining.

Bat ID	Timepoint	Cerebrum	Cerebellum	Brain stem	Spleen	Lung	Heart	Kidney	Liver	Small intestine	Adrenal gland	Skin	Skeletal muscle (Head)	Bone marrow	Tongue (mucosa)	Tonsil/Lymphoid	Oral mucosa	Nasal epithelium	Salivary gland	Uterus	Ovary	Testicle	Epididymis	Prostate
1		0	0	0	0	0	0	1	0	0	2	0	2	1	0	0	0	1	0	NR	NR	1	1	2
2		2	0	0	2	1	0	-	1	3	-	1	1	0	0	1	1	2	1	2	2	NR	NR	NR
3		2	1	2	0	1	0	1	1	2	1	2	2	1	1	-	2	1	1	NR	NR	0	1	2
4		2	0	0	1	1	0	2	1	3	-	1	0	1	-	-	0	1	1	NR	NR	1	1	NR
5		0	0	0	0	1	0	1	1	2	2	-	0	1	-	-	0	0	1	-	-	NR	NR	NR
6		0	1	2	1	1	0	1	1	3	1	-	0	1	-	0	2	1	1	NR	NR	1	1	1
7		1	1	2	1	1	0	1	1	2	-	-	0	1	0	0	0	1	1	NR	NR	1	1	1
8		0	0	1	-	1	0	1	1	2	1	1	0	1	-	0	0	1	0	1	-	NR	NR	NR
9		0	0	1	1	1	1	1	1	1	-	1	0	1	0	0	0	1	1	NR	NR	1	0	0
10		1	1	1	1	1	0	1	1	1	-	1	0	1	0	1	1	1	1	-	-	0	1	2
11		0	1	0	1	1	0	1	1	2	1	-	0	1	0	1	0	1	1	-	-	NR	NR	NR
12		0	0	0	0	1	0	1	1	1	-	0	0	1	0	0	0	0	1	NR	NR	0	0	1
13		0	1	0	-	1	0	1	1	0	0	1	0	1	0	1	0	2	1	NR	NR	1	0	0
14		0	0	0	-	1	0	-	1	2	1	1	0	1	0	1	0	2	0	1	1	NR	NR	NR
Fetus		0	1	1	-	1	1	-	-	1	-	1	0	1	0	1	0	1	1	-	-	-	-	-

Figure 3.10. Immunoreactivity scores, with 3 indicating a high degree of immunoreactivity/staining, and 0 being unreactive/no staining. Bats 1 and 2 represent the uninfected controls. NR=not read.

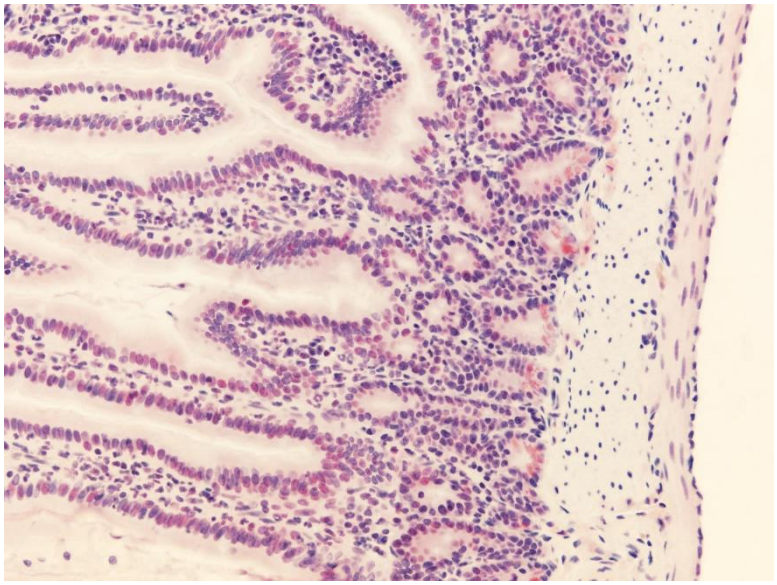

Figure 3.11. Immunoreactivity observed in enterocytes and crypts of small intestine of bat 8 (female bat euthanized at 10 dpi); 20x magnification.

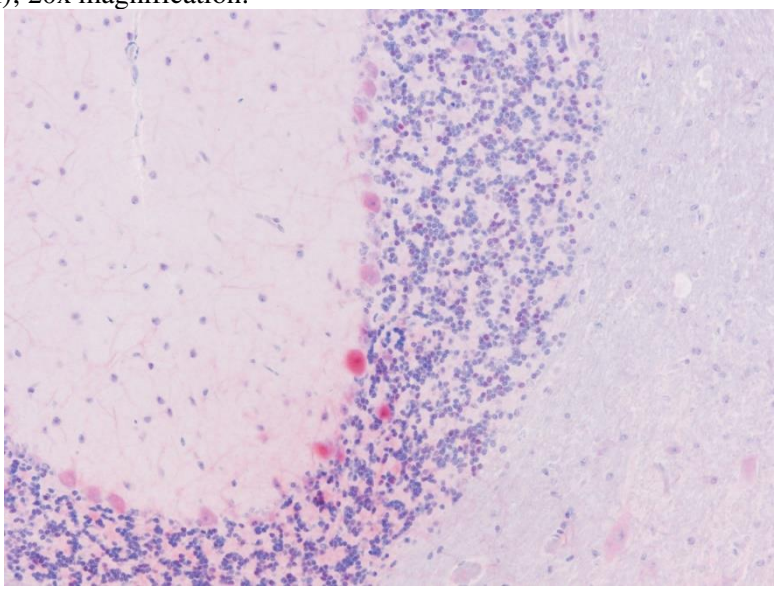

Figure 3.12: Strong immunoreactivity observed in the Purkinje cells and less robust immunoreactivity appreciated in the neurons of the cerebellum of bat 14 (pregnant female bat euthanized at 15 dpi); 20x magnification.

3.2.2.4: Evidence of seroconversion in the absence of BUKV RNA or live virus

RNA was not detected in any tissue samples via qRT-PCR, nor was it detected in the serum, oral swabs, or rectal swabs taken at the time of euthanasia and necropsy. Concurrent with these results, live

virus was not detected in any tissue samples. However, neutralizing antibodies were detected in two bats euthanized at 10dpi (one male bat with a titer of $PRNT_{80}$ = 20 and one female bat with a titer of $PRNT_{80}$ = 10) and from two bats euthanized at 15dpi (one male bat and one pregnant female bat, both with titers of $PRNT_{80}$ = 20) (Table 3.1).

3.2.2.5: qPCR innate gene microarray

Results of the qPCR innate gene microarray indicate up to 2-fold upregulation in some bats for genes related to the antiviral immune response, including TRIM25 and IRF3. However, no significant patterns were noted in the upregulation or downregulation in any group of genes over the course of infection. Further, the RNA from bat 14 (the pregnant female bat euthanized at 15 dpi) was degraded and thus, the majority of genes did not amplify.

Table 3.1. Summary of bats from the timecourse study with results of H&E, IHC, and serum neutralization assay. Immunoreactivity scores, with 3 indicating a high degree of immunoreactivity/staining, and 0 being unreactive/no staining. *pregnant bat

			H&E		IHC		PRNT80
Timepoint	Bat number	Sex	Enteritis	Pulmonary hemorrhage	Lung	Small intestine	Neutralizing titer
	1	M	1	0	0	0	0
0dpi (neg)	2	F	2	0	1	3	0
	3	M	1	0	1	2	0
	4	M	1	0	1	3	0
2dpi	5	F	2	0	1	2	0
	6	M	1	0	1	3	0
	7	M	2	0	1	2	0
5dpi	8	F	2	1	1	2	0
	9	M	2	1	1	1	0
	10	M	2	0	1	1	**20**
10dpi	11	F	2	0	1	2	**10**
	12	M	1	2	1	1	0
	13	M	1	0	1	0	**20**
	14	F*	2	2	1	2	**20**
15dpi	fetus	unknown	1	1	1	1	N/A

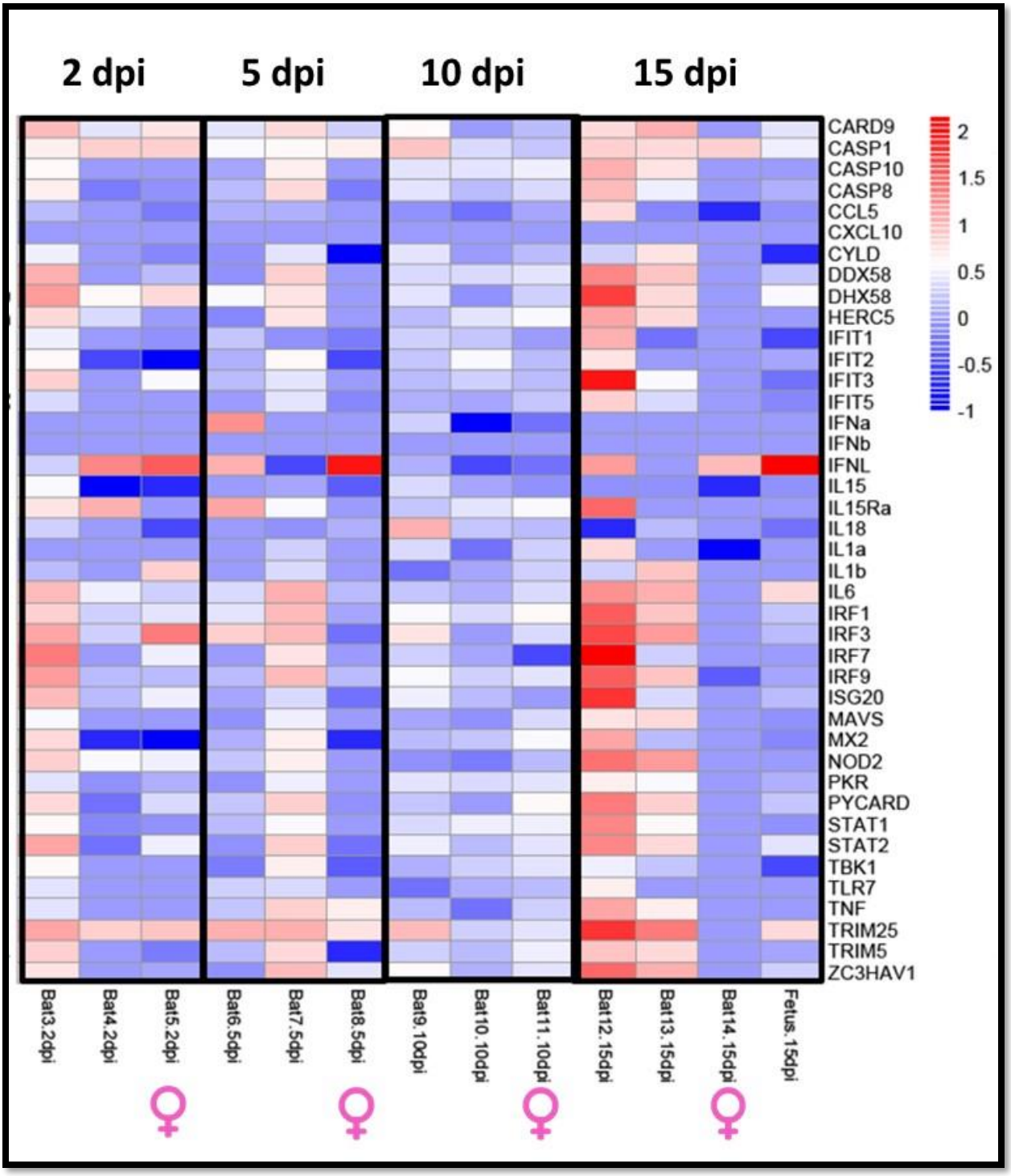

Figure 3.14. Results of the innate immune gene microarrays performed using SYBR green assay (log_{10} scale). The color spectrum runs from red = two-fold upregulation to blue = one-fold down regulation, and white being neutral compared with the housekeeping gene GAPDH.

3.4: Discussion

The discovery of novel viruses is often founded upon molecular surveillance efforts supplemented by whole or partial genome sequencing. The data gleaned from molecular characterization are valuable and allow for *in silico* receptor modeling and phylogenetic placement of viruses to infer potential host/vector associations. However, without a viral isolate, it is impossible to understand viral growth kinetics *in vitro* or *in vivo* (barring generation of an infectious clone (Bhat and Rao, 2020)). We previously determined BUKV replicates to high titers on cells derived from the Egyptian rousette bat (*Rousettus aegyptiacus*), the host from which the virus was initially isolated (Fagre et al., 2019). Challenging live bats with the virus facilitates analyses into pathogenesis, immune kinetics, and forward transmission potential by direct contact or arthropod-borne transmission mechanisms that *in vitro* analyses do not permit, and is a valuable component to better understanding the potential threat of novel pathogens to the health of humans, domestic animals, and wildlife.

In this study, we sought to characterize the experimental susceptibility of the Jamaican fruit bat (*Artibeus jamaicensis*) to a newly characterized orbivirus isolated from an Egyptian rousette bat (Fagre et al., 2019). Taken together, the results from our molecular, serologic, and imaging analyses indicate that the Jamaican fruit bat does not succumb to infection with BUKV, but rather mounts a virus-neutralizing immune response sufficient to clear virus (Borrow, 1997). These results reflect those of another susceptibility study performed in Jamaican fruit bats in which they were challenged with either high or low doses (10^6 and 10^4 $TCID_{50}$ equivalents, respectively) of Tacaribe virus (TACV) (Order: *Bunyavirales*, Family *Arenaviridae*) intranasally or subcutaneously. In this previous study, the group of bats inoculated with the low dose of virus seroconverted and developed neutralizing antibodies in the absence of viral RNA detection or morbidity/mortality, suggesting clearance of the virus (Cogswell-Hawkinson et al., 2012). The congruency of these results with two viral agents in different families suggests an immunological immune mechanism in bats acting to promote infection tolerance and warrants further investigation.

Other experimental animal infections investigating orbivirus susceptibility and pathogenesis have demonstrated seroconversion in the absence of viremia in sheep infected with bluetongue virus-TOGV (van Rijn et al., 2016). This observation could speak to the host specificity of many orbiviruses, as differential morbidity/mortality is observed between ruminants of different families (e.g. goats and sheep challenged under same circumstances demonstrate markedly variable clinical outcomes, with goats often acting as a reservoir (Erasmus, 1975)). Interestingly, BUKV was isolated from an Old World bat, which is taxonomically distant from New World Phyllostomid bats, such as the Jamaican fruit bat (Teeling et al., 2005; Agnarsson et al., 2011). Orbiviruses from the same serogroup (Chobar Gorge virus serogroup) have been isolated from bats in Nigeria (FOMV) and Uganda (BUKV), while the third virus in the serogroup (CGV) has been isolated from *Ornithodoros* spp. ticks in Nepal. Isolation of both FOMV and BUKV from bats in taxonomically distinct families (*Nycteridae* and *Pteropodidae*, respectively) could be indicative of broader patterns of host-virus-arthropod co-evolution, and future studies into this serogroup including the susceptibility of soft-bodied ticks to viral infection is warranted.

In addition to host specificity, the impacts of age, sex, and immune status on infection outcomes are poorly understood in bats. Looking to variability in RNAemia and shedding in established viral reservoir systems, 17 grey-headed fruit bats (*Pteropus poliocephalus*) experimentally inoculated with Nipah virus (Order: *Mononegavirales*, Family: *Paramyxoviridae*) mounted a neutralizing antibody response throughout a subclinical course of infection marked only by intermittent isolation of virus from the urine of one bat and two bats with positive organ samples (kidney and uterus) (Middleton et al., 2007). In humans, factors like age and immune status have been well-characterized in their ability to restrict viral replication. A recent study analyzing variability in SARS-CoV-2 infection outcomes within a single family demonstrated that while both parents had confirmed symptomatic infection, all three children developed neutralizing antibodies (titers of 10-20) despite never testing positive via nasopharyngeal swab (Tosif et al., 2020). Authors attribute these results to an immune response sufficiently robust to restrict viral replication. Across vertebrate species and viral families, examples exist of the host mounting an immune response sufficiently robust to restrict viral replication and promote

clearance of infection. Unique aspects of both the innate and adaptive arm of the bat's immune system are likely at play in effective viral clearance of BUKV and many other viruses.

Our study had a number of limitations, many of which stem from nuances and uncertainties that arise when working with less frequently modeled animal species. Baseline parameters collected from healthy animals (often referred to as reference intervals) are published for many of the most commonly used species, including hematology (complete blood count and blood chemistry), blood gas analysis, and other clinically relevant parameters (Friedrichs et al., 2012). Published hematology profiles now exist for captive Jamaican fruit bats (Strumpf et al., 2020), laying an important foundation upon which to build future knowledge and compare hematological profiles in these animals for future experimental infections. Since the establishment of the captive Jamaican fruit bat colony at Colorado State University in 2016, optimization of husbandry conditions during infections and other *in vivo* studies has been ongoing with the goals of animal welfare and minimization of background changes. It is possible that transfer from the larger colony to smaller enclosures for experimental inoculations and animal handling could explain some of the findings observed in both uninfected and infected bats. For example, endogenous glucocorticoids could lead to the hepatic vacuolar change (glycogen accumulation). It is also possible that, owing to the native habitat (Central America and the Caribbean islands) of these Neotropical bats (Ortega and Arita, 1999), microclimatic differences (e.g. temperature and humidity) between the colony room and the space in which bats are maintained for experimental infections have contributed to differences between animals. These background changes are present in any animal species, despite excellent health status. Because of this, differentiating the significance of lymphoid hyperplasia in control animals versus challenge animals facing antigenic stimuli is difficult given the similarities across groups. Similarly, it is unclear whether the presence of pulmonary changes in control bats is related to euthanasia, but the severity of pulmonary findings in infected bats suggests an association with infection. Establishing a baseline of normal histopathologic variation in any bat species intended for extensive research purposes would be invaluable for future work.

Another limitation of our study is our inability to contextualize our results in light of pathogenesis, immune kinetics, and viremia profiles in experimentally challenged Egyptian rousette bats, the purported natural host of BUKV (Fagre et al., 2019). To better ascertain the difference in immune response between species that naturally host viruses and those taxonomically divergent from the purported host, comparative pathology studies between the Old World Egyptian fruit bat and the Jamaican fruit bat, a distantly related New World fruit bat, would be invaluable. The Egyptian rousette bat from which BUKV was isolated was apparently healthy, but as the virus was discovered in a large biosurveillance study, tissue samples were not saved in formalin for each bat to investigate pathology caused by any viruses detected during these studies. As very few captive colonies of Egyptian rousette bats exist (Amman et al., 2015; Jones et al., 2015), performing side-by-side infections of BUKV in cell lines derived from Egyptian rousette bats as well as other Old World fruit bats and New World fruit bats to allow for comparison in a cell culture system would allow for a comparative analysis of growth kinetics and innate immune gene expression profiling (Gerrard et al., 2017; Arnold et al., 2018). Analyzing infection dynamics across a broad taxonomic range of bat families would also permit investigation into orbivirus host-pathogen co-evolution in orbiviruses through examining differential impacts of host phylogeny on viral replication kinetics.

Another important consideration for the development of arboviral infection models that is often neglected is investigation into what extent arthropod saliva potentiates infection. Many studies demonstrate the important role that arthropod salivary components play in exacerbating infection, altering pathology, and even changing tissue tropism (Schneider and Higgs, 2008; Hermance and Thangamani, 2015). Phylogenetic analysis places BUKV in the tick-borne orbivirus clade (Fagre et al., 2019), and its close relative Chobar Gorge virus was isolated from *Ornithodoros* spp. ticks in Nepal (Centers for Disease Control and Prevention. Arbovirus Catalog: Chobar Gorge). The potentiation of viral infection in the presence of tick saliva on BUKV infection, both *in vitro* and *in vivo*, would lend support to the notion that this is a tick-transmitted pathogen, and possibly influence the infection outcome.

The susceptibility of Jamaican fruit bats to experimental infection across a diverse range of viral families has been demonstrated in past studies (Tacaribe virus, family *Arenaviridae*; Zika virus, *Flaviviridae*; family MERS-CoV, subfamily *Coronavirinae,* family *Coronaviridae*) (Cogswell-Hawkinson et al., 2012; Munster et al., 2016; Malmlov et al., 2019). Attempts at inoculation of the Jamaican fruit bat with a novel orbivirus isolated from an Egyptian fruit bat resulted in seroconversion but failure to detect live virus or viral RNA in the tissues, serum, or oral and rectal swabs. These results are consistent with viral clearance, potentially as a result of enhanced autophagy or changes in baseline levels of interferon signaling (Zhou et al., 2016; Laing et al., 2019). An *in vitro* study examining host-virus interactions during infection of flying fox cells with Australian bat lyssavirus demonstrated that elevations in baseline autophagy resulted in cellular clearance of the virus (Laing et al., 2019). Future studies should examine the impact of tick saliva on the outcome of infection, as well as investigation into the *in vitro* and *in vivo* susceptibility of the host from which the virus was initially isolated, the Egyptian fruit bat.

CHAPTER 4: DETECTION OF SUBGENOMIC FLAVIVIRUS RNA IN ARCHIVED SAMPLES FROM UGANDAN BATS

4.1: Introduction

Zika virus (ZIKV), a historically sylvatic virus known to circulate between mosquitoes and non-human primates (Dick et al., 1952; Haddow et al., 1964), has emerged in the last decade to become a pathogen of global health importance. ZIKV is a positive-sense RNA virus of the viral family *Flaviviridae*, and is transmitted to vertebrates by mosquitoes in the genus *Aedes* (Haddow et al., 1964), though it can also be transmitted sexually and vertically between infected vertebrates (D'Ortenzio et al., 2016; Wu et al., 2016a). As efforts have been made to better characterize ZIKV pathogenesis for preventive and therapeutic purposes, knowledge gaps remain surrounding the epidemiology and ecology of the virus (Kading and Schountz, 2016; Ragan et al., 2017). While non-human primates and mosquitoes are already implicated in the transmission cycle of ZIKV, the role of other vertebrates in sylvatic transmission, particularly in areas of introduction where non-human primates are absent, must be considered. Bats (order *Chiroptera*) have been increasingly implicated as reservoirs for many medically important groups of viruses including lyssaviruses, henipaviruses, filoviruses and coronaviruses (Chua et al., 2000; Leroy et al., 2005; Li et al., 2005; Calisher et al., 2006; Towner et al., 2009; Rupprecht et al., 2011; Goldstein et al., 2018). However, field studies investigating their role as reservoirs of arboviruses including ZIKV are lacking (Fagre and Kading, 2019). A better understanding of the sylvatic ecology of ZIKV could provide insights into viral evolution and host adaptation, in addition to informing risk analysis and strategies to prevent viral transmission.

The MR766 strain of ZIKV was originally isolated from a sentinel rhesus macaque in the canopy of Zika forest in 1947 (Dick et al., 1952), where bats also reside. While the importance of canopy-dwelling mosquitoes in Zika forest (Uganda) was initially recognized in the context of yellow fever virus (YFV) transmission, Haddow et al. (1964) later reported numerous isolations of ZIKV from the arboreal sylvan mosquito, *Aedes (Stegomyia) africanus* (Haddow et al., 1964). We hypothesized that if sylvatic ZIKV is being transmitted by arboreal mosquito vectors in the forest canopy among non-human primates,

then bats congregating at this forest stratification to roost and/or feed on fruits would also be exposed to feeding mosquitoes. Other studies have demonstrated cohabitation of bats and mosquitoes in caves, and mosquito bloodmeals taken from frugivorous bats have been confirmed in Uganda (Crabtree et al., 2013).

Early studies examined the potential for ZIKV MR766 to infect experimentally-inoculated bats (African straw-colored fruit bat (*Eidolon helvum*), Angolan rousette bat (*Lissonycteris angolensis*), and Egyptian rousette bat (*Rousettus aegyptiacus*)) and assessed seroprevalence in free-ranging bats (Little free-tailed bat (*Chaerephon pumila*), African straw-colored fruit bat, Angolan free-tailed bat (*Mops condylurus*), and Egyptian rousette bat from Uganda (Shepherd and Williams, 1964; Simpson et al., 1968). A more recent field study indicated the widespread presence of flavivirus antibodies in both frugivorous and insectivorous Ugandan bats (Kading et al., 2018). Experimental infection of Jamaican fruit bats (*Artibeus jamaicensis*) with the strain causing the 2015-2016 epidemic (ZIKV PRVABC59) resulted in seroconversion and the detection of viral nucleic acid in multiple tissues (Malmlov et al., 2019), suggesting the potential for New World fruit bats to contribute to the sylvatic transmission of ZIKV PRVABC59 in the Americas. Additional studies describing flaviviruses in bats have been reviewed in detail elsewhere (Kading and Schountz, 2016; Fagre and Kading, 2019).

Kading et al. (2018) demonstrated the presence of non-specific flavivirus antibodies in both frugivorous and insectivorous Ugandan bats, yet no nucleic acid was detected in the spleens of seropositive bats when tested with pan-flavivirus primers targeting NS5 (Kuno et al., 1998; Kading et al., 2018). It is likely that the bats had been infected in the past, and viral nucleic acid was no longer present due to nucleic acid degradation by host cell enzymes (Tourriere et al., 2002). mRNA degradation in cells is a well-characterized phenomenon that regulates steady-state RNA levels. RNA viruses have evolved mechanisms to combat degradation by these enzymes and protect viral nucleic acid for replication and other purpose (Dickson and Wilusz, 2011). Among these mechanisms, subgenomic flaviviral RNA (sfRNA) in the 3' untranslated region (UTR) is known to persist at high levels in host tissue, due to its ability to stall exoribonuclease-1 (XRN-1) on the complex hairpin structures characteristic of viral 3' sequences. This stalling results in incomplete degradation of viral transcripts and subsequent

accumulation of these short, subgenomic sequences (sfRNA) in cells and tissue 23-25 (Roby et al., 2014; Akiyama et al., 2016; Michalski et al., 2019). For this reason, ZIKV sfRNA was selected as a detection target to confirm the past exposure of wild-caught bats to ZIKV, operating under the hypothesis that while the majority of viral RNA will have been degraded, these residual fragments of RNA would provide a longer window of opportunity to detect past viral infection. This study provides the first published report of ZIKV RNA in free-ranging bats, representing a strain that most closely aligns with strains in the Asian lineage. It also describes the application of sfRNA as a target for detection of residual viral RNA in free-ranging wildlife.

4.2: Materials and Methods

4.2.1: Confirmation of RNA Integrity via Amplification of Housekeeping Gene

For each sample, 50 ng cDNA was tested on the QX200™ Droplet Digital (ddPCR™) System (Bio-Rad Laboratories, Hercules, CA, United States) using previously published primers demonstrated to amplify GAPDH from two Old World bat species (black flying fox and Egyptian rousette bat) and one New World bat species (common vampire bat) (forward: GTC GCC ATC AAT GAC CCC TTC and reverse: TTC AAG TGA GCC CCA GCC) (Stasiak et al., 2018). For samples with undetectable RNA concentration on the Qubit RNA HS assay, 6 µL cDNA was used as input.

4.2.2: Droplet Digital PCR

Each reaction consisted of 50 ng cDNA, 125 nM F' primer, 125 nM R' primer, and 10 µL QX200™ ddPCR™ EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA, United States). Following reaction preparation, 20 µL of reaction and 60 µL of QX200 Droplet Generation Oil for EvaGreen (Bio-Rad Laboratories, Hercules, CA, United States) were loaded into a DG8 Cartridge for droplet generation in the QX200 Droplet Generator (Bio-Rad Laboratories, Hercules, CA, United States). Following droplet

generation, plates were sealed in the PX1 PCR Plate Sealer (Bio-Rad Laboratories, Hercules, CA, United States). PCR was performed on a T100 Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, United States), and cycling parameters were as follows: 95˚C for 5 min, 40 cycles of 95˚C for 30 sec followed by 60˚C for 1 min, 4˚C for 5 min, 90˚C for 5 min, and held at 4˚C until reading. Plates were read on the QX200 Droplet Reader (Bio-Rad Laboratories, Hercules, CA, United States). Analysis by two individuals were performed using QuantaSoft Software (Bio-Rad Laboratories, Hercules, CA, United States) to determine results of ddPCR using GAPDH primers prior to subjecting these samples to ddPCR testing with ZIKV primers.

4.2.3: Optimization of ddPCR Assay.

Gradient PCR was performed to select the optimal annealing temperature using the 3' UTR ZIKV primers, with a selected annealing temperature of 57.5°C (Figure 4.1). At this annealing temperature, the ddPCR reaction using the 3' UTR primers successfully amplified ZIKV strains MR766, DakAR41525, and PRVABC59 (Figure 4.2). As an additional and biologically relevant sample type, the ddPCR assay was tested using 50 ng cDNA from the organs of mice experimentally infected with ZIKV PRVABC59. Successful amplification was obtained from mouse kidney and spleen using a primer set targeting ZIKV sfRNA (3' UTR) (Figure 4.2).

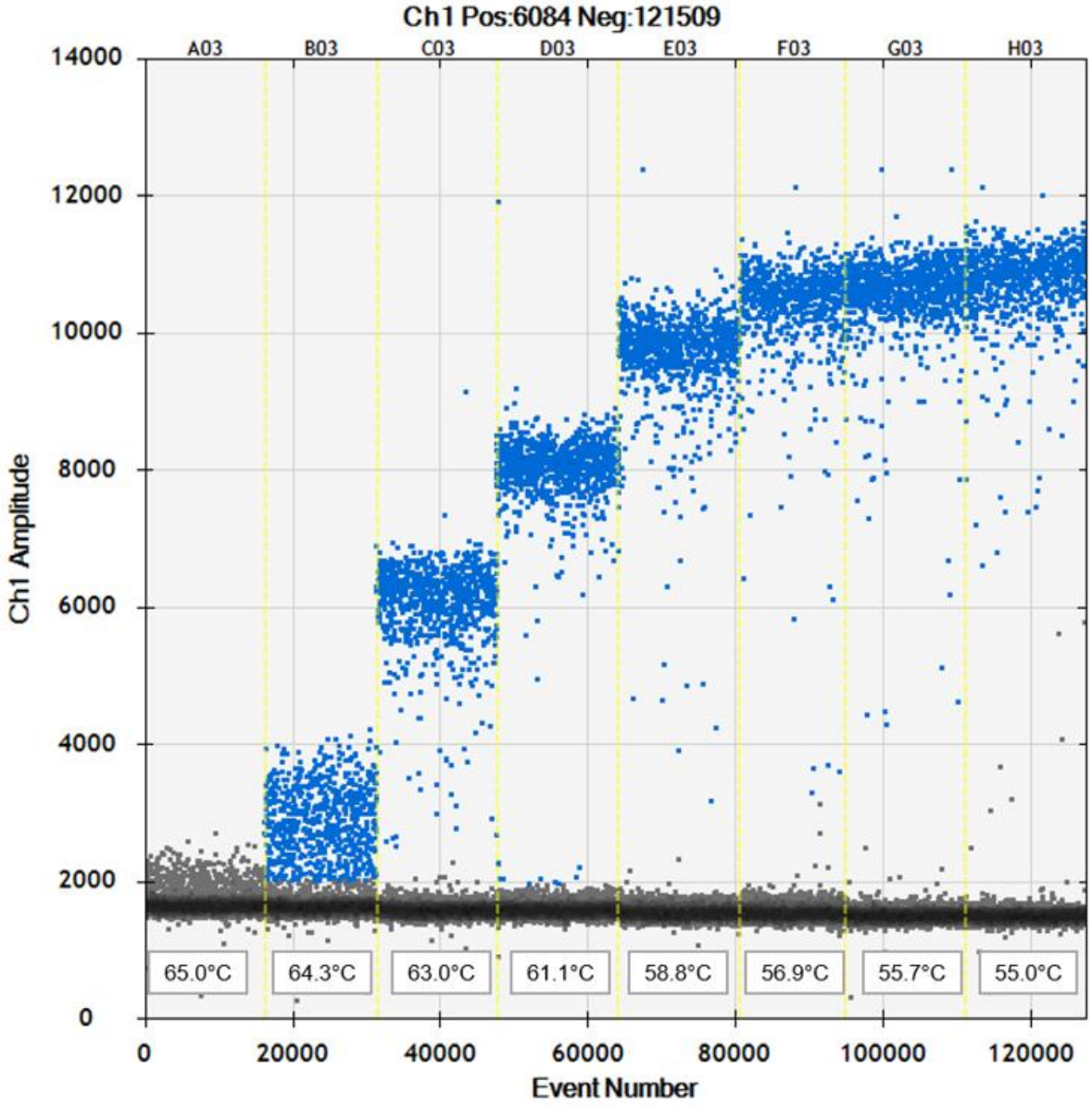

Figure 4.1. Gradient PCR performed on ddPCR platform to select optimal annealing temperature.

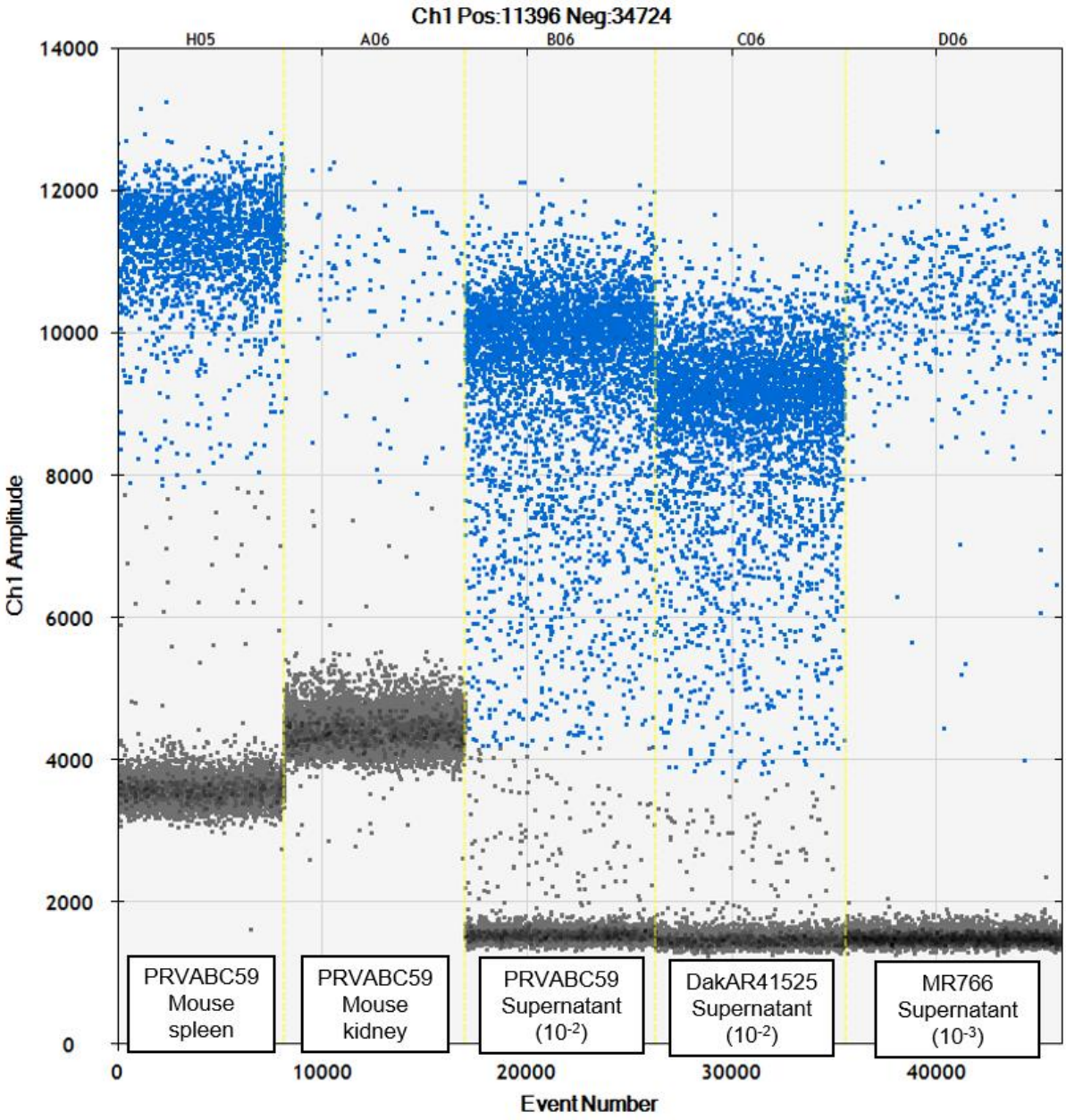

Figure 4.2. Validation of ddPCR assay against three strains of ZIKV as well as RNA extracted from the spleen and kidney of an A129 mouse infected with ZIKV strain PRVABC59

4.2.4: Confirmation of RNA Integrity

To confirm specific amplification of GAPDH sequence for each of the 8 Old World species and 4 New World species, the same primers were used in a conventional PCR assay using GoTaq HotStart Polymerase (Promega corporation, Madison, WI, United States). Cycling parameters were as follows: 95˚C for 2 min; 35 cycles of 95˚C for 1 min, 57.5˚C for 1 min, and 72 ˚C for 30 seconds; followed by 72˚C for 5 min and samples were held at 4˚C until being analyzed for the presence of a 248-bp amplicon via gel electrophoresis. Amplicons were verified by Sanger sequencing (GENEWIZ, Inc., South Plainfield NJ, United States). Results obtained from Sanger sequencing were subjected to quality analysis prior to aligning forward and reverse reads, and the consensus read was subjected to a BLAST search.

4.2.5: Preparation of Positive Controls for Molecular Testing

ZIKV strains MR766, PRVABC59, and DakAR41525 were propagated on Vero cells (ATCC CCL-81) and cell supernatant was harvested 72 hpi. Due to undetectable RNA concentration, the maximum input volume of 11 µL was used for cDNA generation using the SuperScript™ IV First-Strand Synthesis System (Thermo Fisher Scientific, Waltham, MA, United States). A ten-fold dilution series of RNA was generated for each strain to validate detection of phylogenetically divergent strains of ZIKV using our primer set. For ddPCR and cPCR of experimentally-infected and field-caught bats, 3 µL of 10-3 of MR766 was used experimentally as the positive control. Propagation of ZIKV was conducted under CSU biosafety protocol 17-059B.

4.2.6: Infection Protocol, RNA Extraction, and cDNA Synthesis for Experimentally Infected Mice.

Three sub-adult male A129 mice were obtained from the breeding colony at Colorado State University, with all procedures approved by the Colorado State University Institutional Animal Care and Use Committee (protocol 15-6677AA). The study was carried out in accordance with institutional regulations and ARRIVE guidelines Animals were subcutaneously inoculated with 1 x 10^3 PFU

supernatant from PRVABC59-infected Vero cells. At 7 dpi, organs were harvested. Mag-Bind Viral DNA/RNA 96 kit (Omega Bio-Tek Inc., Norcross, GA, United States) on the KingFisher Flex Magnetic Particle Processor (Thermo Fisher Scientific, Waltham, MA, United States). RNA was eluted in 30 µL nuclease-free water. Promega GoTaq Probe 1-Step RT-qPCR System Time (Promega corporation, Madison, WI, United States) was used on RNA extracted from blood and tissue to quantify ZIKV RNA according to manufacturers' instructions. Standards were generated using a forward primer containing a T7 promoter, and a non-modified reverse primer was used to amplify ZIKV strain PRVABC59 viral RNA. cDNA was generated as described above for bat samples, and 50 ng cDNA was used as input for a biological positive control using RNA from kidney and brain.

4.2.7: Infection Protocol, RNA Extraction, and cDNA Synthesis for Experimentally-Infected Jamaican Fruit Bats.

Three female Jamaican fruit bats (*Artibeus jamaicensis*) were obtained from the breeding colony at Colorado State University, with all procedures approved by the Colorado State University Institutional Animal Care and Use Committee (protocol 15-6677AA). The study was carried out in accordance with institutional regulations and ARRIVE guidelines. Animals were subcutaneously inoculated with 7.5×10^5 PFU supernatant from Vero cells infected with PRVABC59, MR766, and DakAR41525. The bat infected with ZIKV strain MR766 was euthanized at 28 dpi, and the two bats infected with strains PRVABC59 and DakAR41525 were euthanized at 45 dpi. Organs and blood were harvested and placed into DMEM supplemented with 1% penicillin/streptomycin (Thermo Fisher Scientific, Waltham, MA, United States) and 10% FBS (Atlas Biologicals, Fort Collins, CO, United States) and stored at -80˚C until RNA extraction and sample processing. cDNA was generated as described above for bat samples, and 50 ng cDNA was used as input for a biological positive control using RNA from brain, heart, lung, liver, spleen, kidney, bladder, ovary, uterus, and blood.

4.2.8: Screening of Bat Samples for ZIKV using ddPCR.

For samples determined to be positive or suspect-positive for GAPDH, 50 ng cDNA (or 6 µL if RNA level was undetectable via Qubit RNA HS assay) was tested in duplicate on the QX200 Droplet Digital (ddPCR™) System (Bio-Rad Laboratories, Hercules, CA, United States) using primers targeting the 3' UTR of multiple strains of ZIKV (forward: TTC CCC ACC CTT YAA TCT GG and reverse: TGG TCT TTC CCA GCG TCA AT) resulting in an amplicon of 123 bp. Each reaction consisted of 50 ng cDNA, 125 nM F' primer, 125 nM R' primer, and 10 µL QX200™ ddPCR™ EvaGreen Supermix (Bio-Rad Laboratories, Hercules, CA, United States). Droplet generation was performed as described above, and cycling parameters on the T100 Thermal Cycler were as follows: 95˚C for 5 min, 40 cycles of 95˚C for 30 sec followed by 57.5˚C for 1 min, 4˚C for 5 min, 90˚C for 5 min, and held at 4˚C until reading the plate. Appropriate annealing temperature was determined by gradient PCR (Figure 4.1). Plates were read on the QX200 Droplet Reader and data were analyzed using QuantaSoft Software.

4.2.9: Capture and Sampling of Ugandan bats.

This study utilized archived tissue samples from bats previously captured in Uganda from 2009–2013 (Amman et al., 2012; Kading et al., 2018) (Table 4.1). Bats were identified using a field guide specific to East African bats (Patterson and Webala, 2012).

Table 4.1. All bat species and trap sites collected from 2009-2013, Uganda. Numbers in parentheses indicate the number of individuals from which spleen and liver were collected and analyzed separately (n=6), for a total of 439 bats sampled and 445 samples processed. (Species codes as follows: ROAE = *Rousettus aegyptiacus*, CHPU = *Chaerephon pumila*, EIHE = *Eidolon helvum*, EPLA = *Epomophorus labiatus*, HIRU = *Hipposideros ruber*, LIAN = *Lissonycteris angolensis*, MOCO = *Mops condylura*, SCHI = *Scotoecus hindei*).

			BAT SPECIES								
			ROAE	**CHPU**	**EIHE**	**EPLA**	**HIRU**	**LIAN**	**MOCO**	**SCHI**	**TOTAL**
Location	**Latitude**	**Longitude**									
Python cave	-0.26667	30.05000	71	-	-	-	-	-	-	-	**71**
Kasokero cave	-0.34214	31.96627	55	-	-	-	3	-	-	-	**58**
Tutum cave	1.28333	34.46667	45	-	-	-	-	-	-	-	**45**
Banga, Nakiwogo	0.08333	32.45000	-	28	-	-	-	-	26	-	**54**
Kawuku	0.13487	32.53392	-	63	-	51	-	-	-	-	**114**
Kisubi	0.11826	32.53017	-	6	-	-	-	-	-	-	**6**
Namasuba	0.29778	32.81861	-	17	-	-	-	-	-	-	**17**
Zika forest	0.11667	32.53333	-	2	-	-	-	-	1	1	**4**
Bugonga	0.05000	32.46667	-	-	7 (5)	-	-	-	-	-	**7**
Jinja	0.41667	33.20000	-	-	8	-	-	-	-	-	**8**
Buwaya Lugonjo	0.08333	32.43333	-	-	-	23	-	-	-	-	**23**
Kasange	0.15000	32.40000	-	-	-	4	-	-	-	-	**4**
Kikaaya	0.37017	32.58932	-	-	-	16 (1)	-	-	-	-	**16**
Kapkwai cave	1.33333	34.41667	-	-	-	-	3	9	-	-	**12**
Species sum			**171**	**116**	**15 (5)**	**94 (1)**	**6**	**9**	**27**	**1**	**439 (6)**

4.2.10: RNA Extraction and cDNA Synthesis for Field-Caught Bat Samples.

RNA was extracted from frozen tissue homogenates using the MagMax 96 total RNA isolation kit (Applied Biosystems, Foster City, CA, United States). Each RNA sample was quantified using the Qubit RNA HS Assay (ThermoFisher Scientific, Waltham, MA, USA), and 1 µg total RNA was used as input for cDNA generation using the SuperScript™ IV First-Strand Synthesis System (ThermoFisher Scientific, Waltham, MA, USA). For samples with an undetectable RNA concentration, 11 µL was used as input (maximum input volume).

4.2.11: Confirmatory PCR testing and sequencing.

Samples deemed 'suspect' via screening on the ddPCR™ system with ZIKV 3' UTR primers were subjected to additional PCR and Sanger sequencing using the same primer set targeting the 3' UTR of ZIKV. ZIKV strain MR766 was used as a positive control in these assays. Samples were considered 'suspect' if 1) the automatically-defined threshold yielded >1 positive droplet in the same 1D amplitude as the positive control cDNA (ZIKV MR766) or 2) the negative droplet populations existed in the same 1D amplitude region of positive control droplets and thus, precluded the ability to differentiate positive and negative populations. The cDNA from these samples was amplified using the GoTaq HotStart system (Promega corporation, Madison, WI, United States), with each reaction consisting of 50 ng cDNA, 25 µL GoTaq HotStart Master Mix, 400 nM F' primer, 400 nM R' primer, and 1 M Betaine. Cycling parameters were as follows: 95˚C for 2 min; 35 cycles of 95˚C for 1 min, 57.5˚C for 1 min, and 72 ˚C for 30 seconds; followed by 72˚C for 5 min and samples were held at 4˚C until being analyzed for the presence of a 123-bp amplicon via gel electrophoresis. Positive samples were verified by Sanger sequencing (GENEWIZ, Inc., South Plainfield NJ, United States). Results obtained from Sanger sequencing were subjected to quality analysis prior to aligning forward and reverse reads in Geneious v11.1.5 (www.geneious.com), and the consensus read was aligned to the 3' UTR of MR766.

4.2.12: Comparison of Detection Sensitivity Between sfRNA and NS5 in Field-Caught Samples.

The four samples from which ZIKV sfRNA was amplified were subjected to PCR amplification with primers designed for this study targeting NS5 from MR766, PRVABC59, and DakAR41525 in order to compare detection sensitivity (F: TGC CGC CAC CAA GAT GAA CT, R: CAT TCT CCC TTT CCA TGG ATT GAC C). Cycling parameters were as follows: 95˚C for 2 min; 35 cycles of 95˚C for 1 min, 57.5˚C for 1 min, and 72 ˚C for 30 seconds; followed by 72˚C for 5 min and samples were held at 4˚C. cDNA from ZIKV MR766 was used as a positive control. Results were sent for Sanger sequencing if a band was present.

4.3: Results

4.3.1: Confirmatory PCR and Sanger sequencing of samples from bats experimentally infected with ZIKV.

Experimentally challenged bats were screened for ZIKV sfRNA using the droplet digital PCR (ddPCR) platform, and then confirmed with conventional PCR (cPCR). Successful amplification and Sanger sequencing of ZIKV 3'UTR sfRNA, but not NS5, from multiple organs (brain, heart, lung, spleen, kidney, bladder, and uterus) and blood from Jamaican fruit bats subcutaneously inoculated with ZIKV (Table 4.2) further supports our hypothesis that sfRNA is a more sensitive detection target than NS5 due to XRN1 stalling.

Table 4.2. Organs from which ZIKV sfRNA was amplified and sequenced obtained from subcutaneously-inoculated female Jamaican fruit bats (n=3). One animal was inoculated with each of three strains of ZIKV and euthanized at varying timepoints (e.g. female bat inoculated with MR766 euthanized at 28 dpi).

	MR766 (28dpi)	PRVABC59 (45dpi)	DakAR41525 (45dpi)
Brain		X	X
Heart	X		X
Lung	X		X
Liver			
Spleen	X		
Kidney	X		X
Bladder	X		X
Ovary			X
Uterus	X	X	
Blood	X	X	

4.3.2: Detection and Sanger Sequencing of ZIKV RNA in Field-Caught Bat Samples.

RNA quality was confirmed by amplification of GAPDH in 198/445 archived samples, and 49/198 of these resulted in suspect results using ddPCR and were subjected to confirmatory cPCR. Of these, four of the 49 field samples from three species of bats (African straw-colored fruit bat (n=2), Egyptian rousette bat (n=1), and Ethiopian epauletted bat (*Epomophorus labiatus*) (n=1)) at three separate sites throughout Uganda were confirmed positive for ZIKV RNA by cPCR and Sanger sequencing (123-bp amplicon) (Figures 4.4-4.8). Sequences were deposited in Genbank. Sequence alignment suggest that the sfRNA detected is most closely related to Asian lineage ZIKV (Table 4.3).

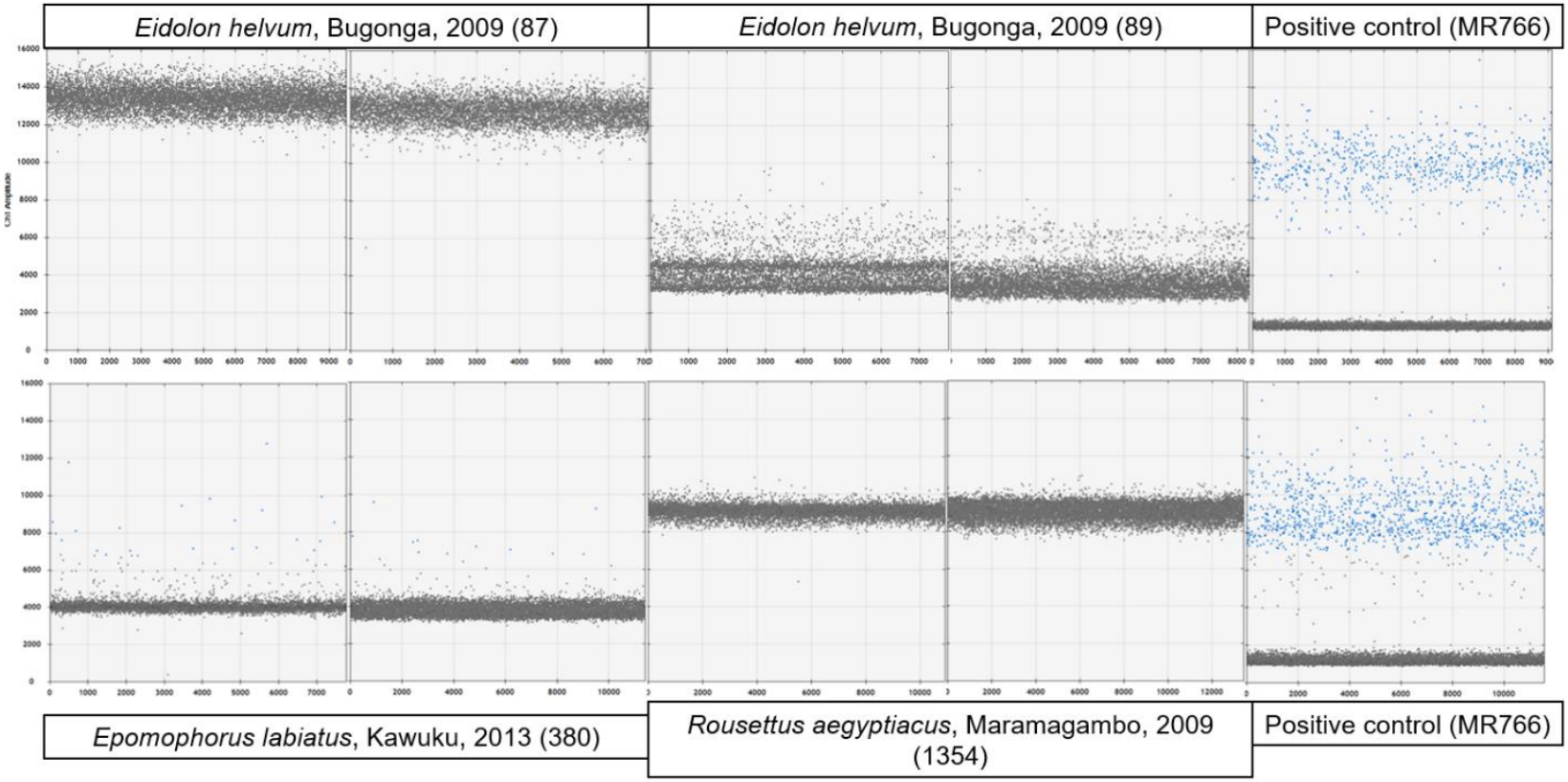

Figure 4.3. ddPCR results from all positive bats.

Table 4.3. Nucleotides present at various positions in 3' UTR of field-caught bat samples indicating that sequences align most closely to the Asian strain of ZIKV. Positions are numbered based on PRVABC59 (MH158237). For each sample, bat species, identification number, and Genbank accession number are provided.

Numbered according to PRVABC59 (MH158237)	**10,636**	**10,637**	**10,645**	**10,652**	**10,654**	**10,658**	**10,664**
PRVABC59 (MH158237)	**T**	**C**	**G**	**A**	**A**	**G**	**A**
DakAR41525 (KU955591)	**T**	**T**	**A**	**G**	**C**	**A**	**A**
MR766 (AY632535)	**C**	**T**	**A**	**A**	**C**	**G**	**A**
E. helvum (bat 87, MT482106)	T	C	G	A	A	G	G
E. helvum (bat 89, MT482107)	T	C	G	A	A	N	A
E. labiatus (bat 380, MT482108)	T	C	G	A	A	G	A
R. aegyptiacus (bat 1354, MT482109)	T	C	G	A	A	G	A

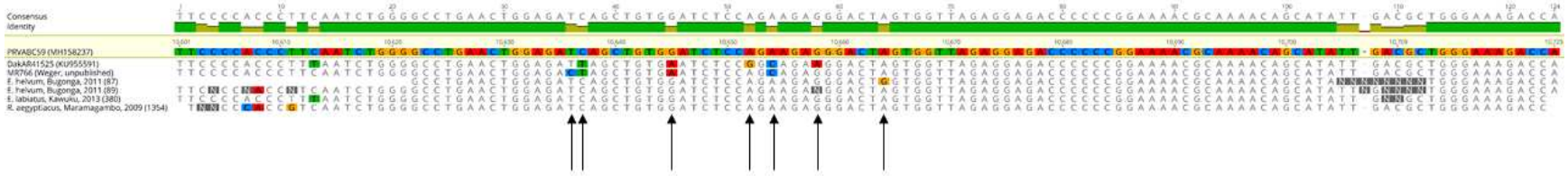

Figure 4.4. Alignment of all positive bat samples with three strains of ZIKV.

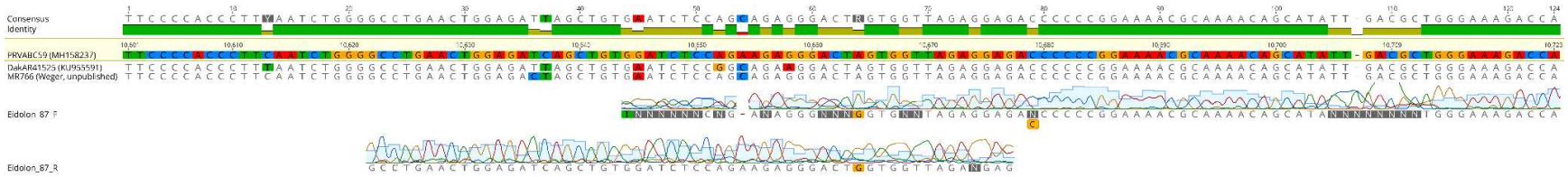

Figure 4.5. Alignment of .ab1 file from positive straw-colored fruit bat (*Eidolon helvum)* (bat #87) with three strains of ZIKV.

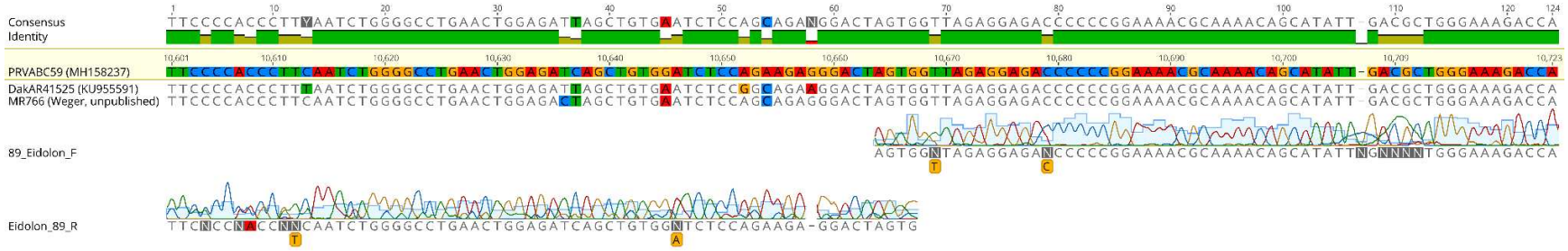

Figure 4.6. Alignment of .ab1 file from positive straw-colored fruit bat (*Eidolon* helvum) (bat #89) with three strains of ZIKV.

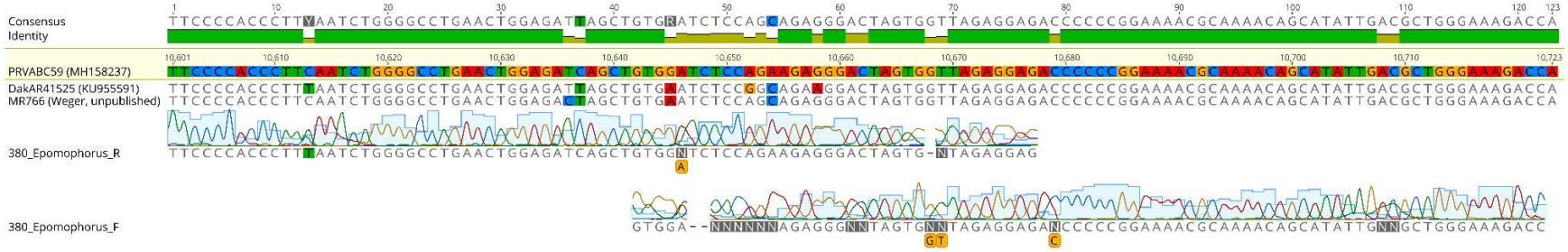

Figure 4.7. Alignment of .ab1 file from positive epauletted fruit bat (*Epomophorus labiatus*) with three strains of ZIKV.

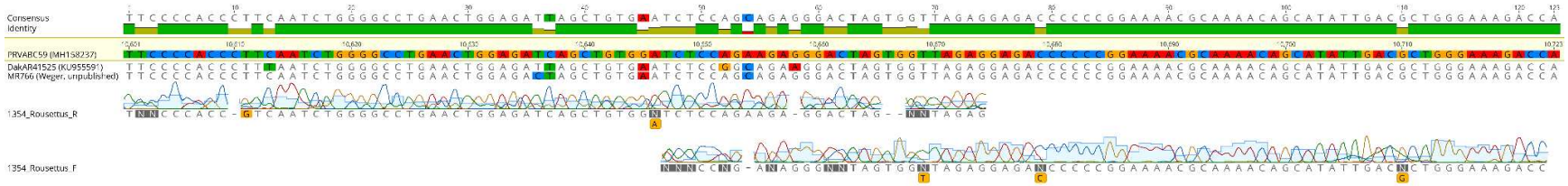

Figure 4.8. Alignment of .ab1 file from positive Egyptian fruit bat (*Rousettus aegyptiacus)* with with three strains of ZIKV

4.3: Discussion

We confirmed the presence of 3' UTR ZIKV RNA from free-ranging bats across Uganda. Targeting the viral 3'UTR represents a novel and sensitive approach for detection of residual flavivirus RNA in previously-infected animals. NS5 is degraded by the XRN-1 exonuclease; therefore, we hypothesized that samples positive for ZIKV 3'UTR sfRNA would be negative for NS5 (Akiyama et al., 2016), which was true in both experimentally-inoculated bats as well as naturally-infected bats from Uganda. The four bats from which ZIKV RNA was amplified comprised three species (African straw-colored fruit bat (n=1), Egyptian rousette bats (n=2), and Ethiopian epauletted fruit bat (n=1)), all in family *Pteropodidae*, captured from three separate locations across Uganda, between 2009 and 2013 (Fig 1). The viral 3'UTR sequence from all four of these bats is most closely aligned with Asian lineage ZIKV (Table 4.2, Figure 4.3). Recent reports describe detection of Asian lineage ZIKV in human cases from Cabo Verde and Angola, and genomic analysis indicates that the first introduction of Asian lineage ZIKV to Angola was between July 2015 and 2016 (Hill et al., 2019). These findings corroborate serologic evidence of flavivirus circulation within Ugandan fruit bat populations and suggest either an earlier introduction of Asian lineage ZIKV to Africa than previously thought or genetic divergence of ZIKV strains prior to the eastward spread of ZIKV to Malaysia (Duong et al., 2017; Kading et al., 2018), though further investigation is warranted.

The natural infection of ZIKV among diverse frugivorous bat species is intriguing and suggests a more widespread exposure of wildlife to sylvatic arboviruses than currently understood. Cave-dwelling Egyptian rousette bats naturally inhabit forested areas, whereas Ethiopian epauletted fruit bats and African straw-colored flying foxes are more adapted to disturbed environments, dwelling in man-made structures and tall trees, respectively. The latter two species may visit forested habitats, raising questions regarding the exposure of each of these bats to mosquito vectors of Zika virus. Previously, mosquito blood meals matching Egyptian rousette and African straw-colored fruit bats have been confirmed from Maramagambo and Semliki forests, Uganda (Crabtree et al., 2013). Serological detection of anti-ZIKV neutralizing and IgM antibodies in febrile human patients were later confirmed between 2014 and 2017,

documenting active ZIKV circulation in Uganda and the first laboratory-confirmed human case in decades (Kayiwa et al., 2018). Investigation into the interactions between bats and arthropods transmitting viruses of medical importance in different ecological systems is warranted.

The use of MR766 strain as a positive control throughout the ddPCR and cPCR testing of samples from our experimentally infected and field-caught bats rules out contamination as a possible explanation of these results. A natural SNP was also detected in the 3'UTR ZIKV sequence amplified from one of the two African straw-colored fruit bats (Table 4.22, Figure 4.5). Further, the amplification of sfRNA in the absence of NS5 amplification in both field-caught and experimentally infected bats illustrates the potential for this portion of the genome to act as a valuable and sensitive biosurveillance target, due to its stalling of XRN-1.

We describe the use of GAPDH as a proxy for RNA integrity, as we successfully amplified this gene from all eight of our field-caught bat species. These primers were adapted from equine GAPDH primers and have previously been demonstrated to amplify the GAPDH gene in two Old World bat species (Egyptian rousette bats and the black flying fox (*Pteropus alecto*)) and one New World bat species (the common vampire bat (*Desmodus rotundus*)) (Zhang et al., 2009; Stasiak et al., 2018). This is a valuable tool for future biosurveillance efforts as it allows determination of viable RNA and subsequent prioritization of screening from a number of both New World and Old World bat species.

This is the first published application of ddPCR to viral surveillance of wildlife samples, though other studies have used the tool for pathogen detection and for environmental monitoring (Simmons et al., 2015; Mahon and Jerde, 2016; Wu et al., 2017). Using the ddPCR platform, we amplified bat GAPDH and ZIKV RNA from field-caught bat samples, ZIKV NS5 and sfRNA in experimentally-infected mouse samples, and ZIKV sfRNA in the blood and organs of experimentally-infected bats from which we were unable to amplify NS5. However, variable levels of background fluorescence in all assays precluded us from using quantitative algorithms for defining the number of copies/µL, such as 'ddPCRquant' and 'definetherain' (Jones et al., 2014; Trypsteen et al., 2015). Additional limitations of the study include the age and quality of the archived field-samples tested. The tissue samples were homogenized in cell culture

media and frozen at -80˚C for 6-10 years prior to this study. Only 44.4% (198/445) of the samples contained detectable levels of GAPDH by ddPCR, indicating that a majority of the samples had poor RNA integrity as a result of RNA degradation. Ultimately, while our assays were readily adaptable to a ddPCR platform, ddPCR did not present a significant advantage over traditional amplification methods, but rather the persistence of 3'UTR sfRNA provided a diagnostic advantage for detecting small amounts of residual viral RNA.

This study illustrates the use of sfRNA as a novel and highly sensitive biosurveillance target and lays the groundwork for future in vivo and field studies. Our results demonstrate the application of this molecular target for flavivirus biosurveillance in field samples from which coding RNA is no longer detectable and may be especially useful for samples with questionable or non-specific flavivirus serology results. Future studies should perform a comparative analysis of coding RNA and sfRNA levels over the course of infection to quantify the rate of degradation and determine the extent to which sfRNA persists and accumulates in tissues.

CHAPTER 5: DIAGNOSTIC STRATEGIES FOR NON-LETHAL AND MINIMALLY INVASIVE SAMPLING OF FREE-RANGING BATS

5.1: Review of current diagnostic strategies employed for infectious disease surveillance

At present, many surveillance studies analyzing the presence or prevalence of infectious organisms routinely euthanize free-ranging bats to collect spleen or other internal organs (Young and Olival, 2016). Additionally, operating under the rule that only 1% of body weight may safely be collected in blood volume, many bat species must be euthanized via cardiac exsanguination to obtain enough blood for traditional serology assays or molecular testing (Kading et al., 2018). Some groups do not conduct thorough speciation of bats in the field prior to euthanasia, but rather obtain molecular identification confirmation after returning to the laboratory (Wang et al., 2017; Luo et al., 2018) or obtain oral and anal swabs post-euthanasia without further processing of tissue samples (Carrington et al., 2008). This undoubtedly results in unnecessary euthanasia of bats, some of which have uncharacterized population statuses. Of the 1400+ species of bats, over 200 species in 60 countries are considered threatened (Critically Endangered, Endangered, or Vulnerable) by the International Union for the Conservation of Nature (The IUCN Red List of Threatened Species), so sampling bats in such a way that is non-destructive but still meets sensitivity and detection needs for surveillance, would be ideal for reconciling public health surveillance as well as wildlife conservation priorities.

Few analyses investigate the sensitivity or relative detection rates for different viral families between tissue types (e.g. non-terminal fecal collection vs. euthanasia and intestine collection), though technologies to detect markers of infection in non-terminal sample types are growing (e.g. proteomics, metagenomics from feces, enhanced molecular detection from fecal and oral swabs, etc) (Young and Olival, 2016; Giles et al., 2018). With this in mind, the tissue tropism of certain viruses dictates sampling strategy for targeted biosurveillance efforts and in some cases (Amman et al., 2012; Fagre et al., 2019), terminal sampling is necessary to characterize risk of spillover from bats to human and animal populations at a high resolution.

A comparison of studies focused on comparing lethal vs. non-lethal sampling for purposes of coronavirus detection showed that non-lethal sampling is effective in detecting samples shed in feces and saliva. However, in the absence of side-by-side comparison of lethal vs. non-lethal sample from the same bat for each pathogen of interest, it is impossible to generalize the loss of detection sensitivity when collecting non-lethal samples and as such, euthanasia considerations for each field study must be analyzed on a case-by-case basis. In some papers which did perform side-by-side comparisons of feces vs. intestinal sampling, screening of intestinal samples did not result in markedly higher detection rates (and in some instances, even lower detection rates than fecal samples), emphasizing the importance of optimizing surveillance strategies so as to minimize unnecessary sacrifice and ideally, disruption to bat populations (Shirato et al., 2012).

While not all sampling and diagnostic strategies in this chapter are focused on viral surveillance, exploration into different sample types is important and may be applied across different groups of pathogens. In this chapter, I explore the use of FTA cards to store blood samples from field-caught bats in an effort to detect haemosporidian parasites as a collaborative effort focused on the conservation of the endangered Indiana bat (*Myotis sodalis*). I also describe application of two widely used qRT-PCR assays for detection of SARS-CoV-2 for fecal screening of bats in rehabilitation centers prior to their release.

5.2: Investigation of FTA cards for detection of haemosporidian parasites as a health marker in North American bats

5.2.1: Introduction

Exploration into proxies for characterization of bat population health status is necessary to enhance our ability to acutely detect changes to these populations. While many expeditions focused on viral discovery conduct terminal sampling to obtain tissue samples, conservationists and wildlife health professionals prioritize non-lethal sampling so as not to skew or disrupt population dynamics. Some commonly collected non-lethal sample types include saliva/oral swabs, feces/rectal swabs, roost guano, hair, wing punches, and blood. Blood collected in the field is often collected and stored on FTA cards as a means of nucleic acid preservation, and many research groups amplify viral, bacterial, and parasitic pathogens from these cards. Identifying the presence of blood-borne pathogens in bat populations would allow for direct comparison of microorganism prevalence between populations in disturbed or undisturbed areas, and could serve as a valuable conservation tool for wildlife health agencies to monitor the response of bat populations to land use change and other anthropogenic stressors.

Haemosporidian parasites of bats exist within at least four genera – *Plasmodium*, *Hepatocystis*, *Nycteria*, and *Polychromophilus*. Of these four, only the last (*Polychromophilus* spp.) have been identified in both Old and New World bats, with the other three genera detected only in bats of the Old World. Within *Polychromophilus* spp., *P. melanipherus* infects bats of the family Miniopteridae and *P. murinus* infects bats of the family Vespertilionidae. Reviewed in Perkins & Schaer (2016), *Bioccala* is a putative subgenus within *Polychromophilus murinus*. The recognized species is *Bioccala deanei* (Perkins and Schaer, 2016).

Bioccala deanei has been microscopically identified in the US as well as Central & South America among bats in the family Vespertilionidae (Table 5.1). The only molecular detection of *Polychromophilus* parasites in the New World was from a Panamanian *Myotis* spp. bats, so any positive samples that we obtain would be the first molecular detection of the parasite in North America. As

previous studies have demonstrated that haemosporidian parasites may adversely impact host health, it was decide to investigate the presence of *Polychromophilus Bioccala deanei* in our study populations.

Table 5.1. Review of *Polychromophilus murinus* (or more specifically *Polychromophilus Bioccala deanei* as indicated by an asterisk) in the New World and the temperate zones of the Old World.

Bat species	Location	Prevalence	Molecular/physical	Ref.
Myotis daubentonii	Switzerland	51% (65/127)	Molecular	(Megali et al., 2011)
Myotis myotis	Switzerland	4% (2/47)	Molecular	
Eptesicus serotinus	Switzerland	11% (2/18)	Molecular	
Nyactalus noctula	Switzerland	7% (1/15)	Molecular	
Myotis nigricans	Panama	Single detection	Molecular	(Borner et al., 2016)
Myotis nigricans	Brazil	Single detection	Microscopic ID*	(Garnham et al., 1971)
Glossophaga soricina	Brazil	Single detection	Microscopic ID*	(Garnham, 1973)
Eptesicus fuscus	Colombia	Single detection	Microscopic ID*	(Marinkelle, 1995)
Myotis austroriparius	Florida (US)	Single detection	Microscopic ID	(Foster, 1979)
Antrozous pallidus	California (US)	Single detection	Microscopic ID	(Wood, 1952)
Pipistrellus hesperus	Texas (US)	Single detection	Microscopic ID	

5.2.2: Materials and Methods

5.2.2.1: Elution of Blood from Filter Papers & DNA Extraction

Blood spots from 30 Indiana bats (*M. sodalis*) and 5 big brown bats (*E. fuscus*) (35 total) were tested. A standardized area of the filter paper was removed & eluted in RNA Rapid Extraction solution overnight at 4˚C. The next day, total nucleic acid extraction was performed using the MagBind Viral RNA/DNA kit (Omega Bio-tek) on the KingFisher Flex extraction robot (ThermoFisher Scientific). As a negative control, an empty piece of filter paper stored with the bat samples was collected and processed in parallel with all bat samples. As a positive control, a piece of filter paper with human blood was collected and processed in parallel with all bat samples.

5.2.2.2: Nested PCR Protocol for Detection of *Polychromophilus murinus* DNA

A nested PCR protocol was designed by Duval and colleagues to amplify a 709-bp region of the cytochrome *b* gene (Duval et al., 2007). These primers are specific to order Haemosporidia, and do not result in non-specific amplification of host or other Apicomplexan parasite DNA. This protocol was used in the detection of *P. murinus* in multiple species of Swiss bats (family *Vespertilionidae*) (Megali et al., 2011). As a positive control, a synthetic oligonucleotide 489 bp in length (sequence available upon request) was designed for three reasons:

1. Calculate limit of detection (as few as 20 copy numbers were easily detectable).
2. Differentiate contamination from true positive prior to receiving Sanger sequencing results (due to difference in amplicon size).
3. We did not have access to a naturally occurring positive control (e.g. *P. murinus*)

Table 5.2. Primers used for nested PCR, as described in Duval et al 2007.

	Forward (5' – 3')	Reverse (5' – 3')
Outer	GAGAATTATGGAGTGGATGGTG (**PLAS1**)	GTGGTAATTGACATCCWATCC (**PLAS2**)
Nested	GGTGTTTYAGATAYATGCAYGC (**PLAS3**)	CATCCWATCCATARTAWAGCATAG (**PLAS4**)

5.2.2.3: PCR Protocol for Detection of *CytB* DNA as a Proxy for Nucleic Acid Integrity

To evaluate whether the DNA had been degraded, we opted to use a set of conserved primers are designed to amplify a 358 bp fragment of the vertebrate mitochondrial cytochrome b gene in vertebrate species using primers described in Meece et al. 2005 (Listed 5' – 3': forward: CCC CTC AGA ATG ATA TTT GTC CTC A and reverse: CCA TCC AAC ATC TCA GCA TGA TGA AA) (Meece et al., 2005).

As a positive control, DNA extracted from human blood eluted off of a Whatman filter card at the same time as the bat samples was used. This acted as both a positive extraction control (to ensure the extraction was successful) and a positive amplification control (to ensure the PCR assay amplified target DNA).

5.2.3: Results

5.2.3.1: Nested PCR for *Polychromophilus murinus* DNA

Of the 35 samples tested, no samples resulted in amplification of *Polychromophilus murinus* nucleic acid. As a positive control for PCR, the synthetic barcoded oligonucleotide (482 bp in length) was amplified (Figure 5.1).

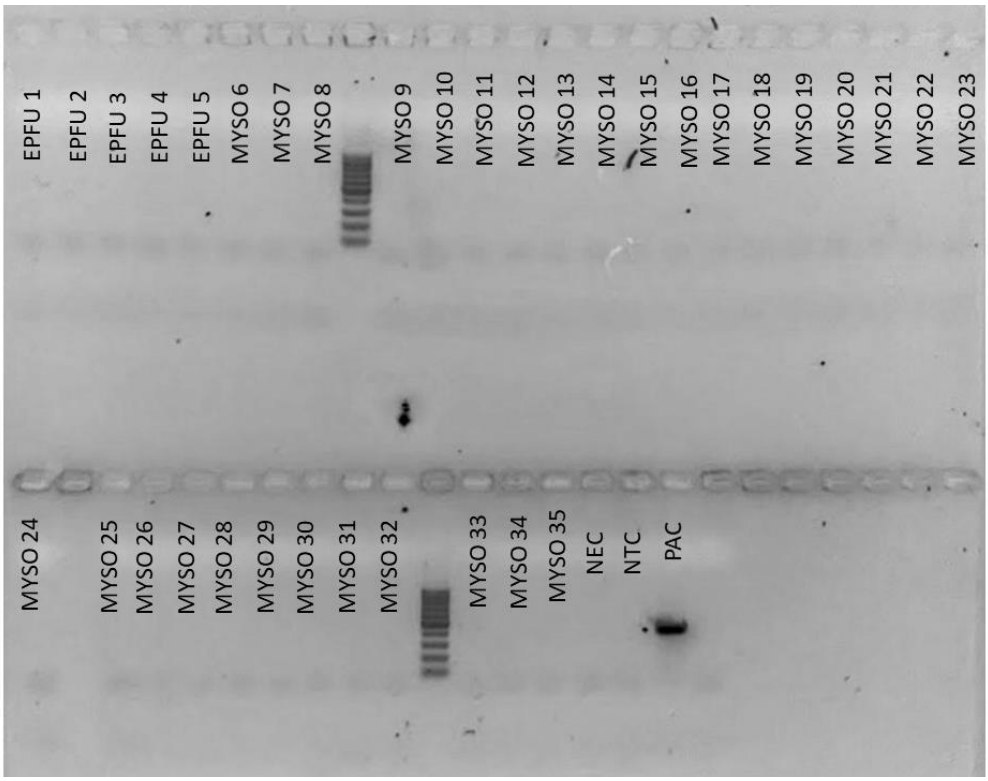

Figure 5.1. Gel showing results of DNA run with primers against the haemosporidian parasite *Polychromophilus murinus*. NEC = no extraction control. NTC = no template control. PAC = positive amplification control. DNA ladder demarcates 100 nucleotide size increments. A 710 bp amplicon is expected for positive samples. The amplicon size of the synthetic PAC is 489 bp.

5.2.3.2: Testing of *CytB* indicative of Suboptimal Nucleic Acid Integrity for Many Samples

To assess DNA integrity/quality, all samples were also tested using primers targeting vertebrate *cytochrome b* (Fig 5.2)(Meece et al., 2005). Of the 35 samples, *cytb* was only amplifiable from 10 samples. Of the samples from which *cytb* was amplified, the amplicon was very faint compared to the positive control (Fig 5.2). As a positive control, DNA extracted from the human blood spot was used and

cytb was successfully amplified. These results suggest that DNA degradation likely occurred in the field samples, possibly due to exposure of samples to nuclease activity in between sample collection and sample processing. It is also possible that the Whatman filter paper used during collection did not allow for adequate nucleic acid preservation.

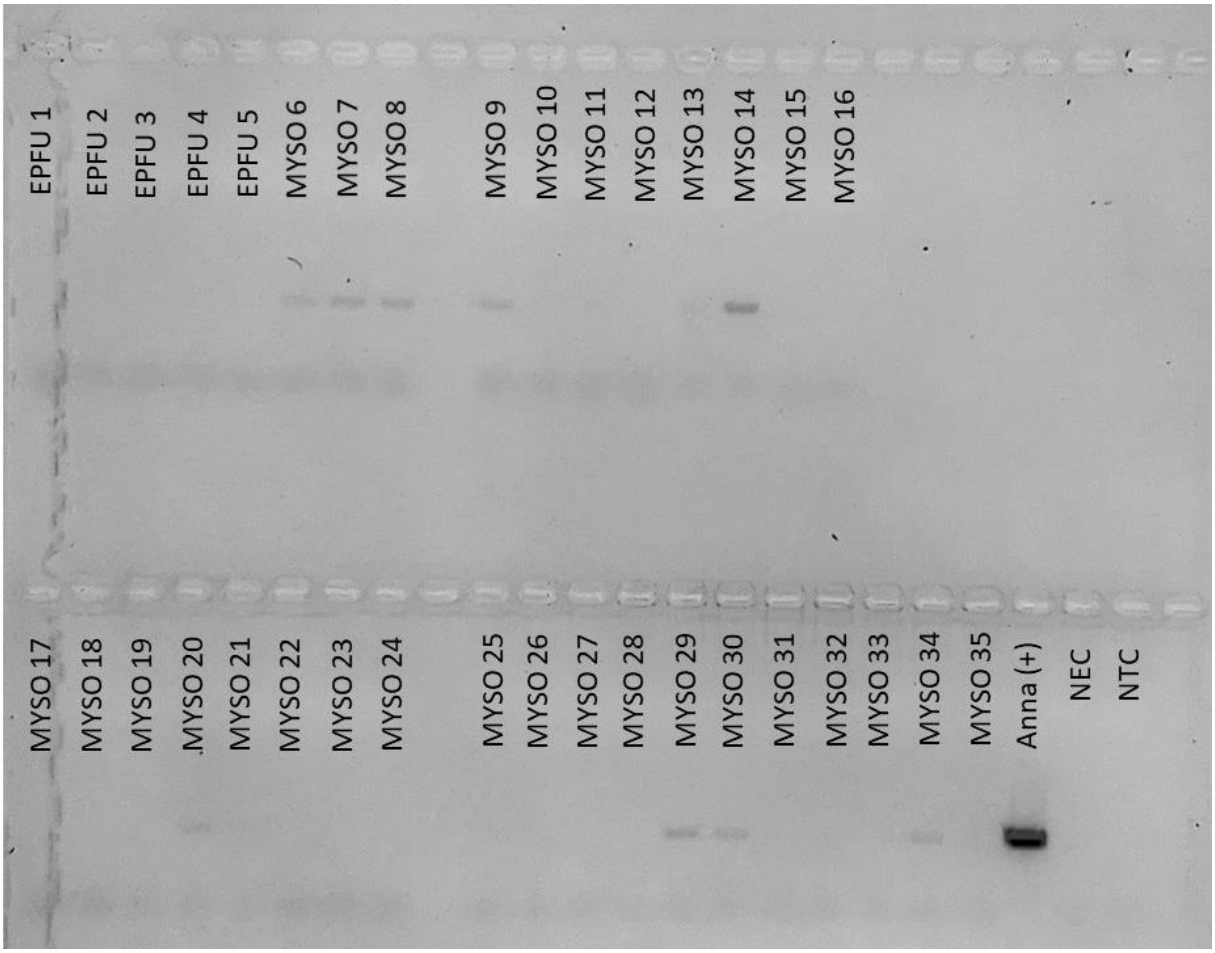

Figure 5.2: Gel showing results of DNA run with primers targeting vertebrate cytochrome b gene. Anna (+) = positive control blood extraction from filter paper. NEC = no extraction control. NTC = no template control. An amplicon of the expected size is present from Anna (+) as well as 10 field samples.

5.2.4: Discussion

Characterizing of haemosporidian parasites in many animal species has revealed that the presence of this pathogen has an impact on population health (Perkins and Schaer, 2016). To date, scant information is available on the presence of haemosporidian parasites in New World bat populations. With the increase of urbanization and land use change impacting New World bat populations, in addition to

other pathogen threats (e.g. white nose syndrome), identifying minimally invasive metrics by which to analyze population health changes is critical. We investigated the presence of North American bat populations, including the endangered Indiana bat (*Myotis sodalis*) for the presence of haemosporidian parasites in genus *Polychromophilus*.

None of the 35 samples we tested for haemosporidian parasites were positive, and *cytb* was amplified in only 10 of them (28.56%). Because of this result, the true denominator of our tested samples is only 10. Therefore, our sample size is too small to rule out the presence of *Polychromophilus murinus* circulating in these bat populations. However, the molecular test for the blood-borne parasite of bats is now validated for future use in assessing parasite prevalence in North American bats. Areas worth exploring for assay optimization are side-by-side comparisons of extraction kits and the impact that different types of Whatman paper have on sample integrity and nucleic acid preservation. In the future, we will investigate the use of other extraction kits to maximize sensitivity.

Suggestions to our collaborators to help improve sample preservation included moving samples to a -20˚C freezer as quickly as possible, and to avoid touching the filter papers with ungloved hands due to the presence of nucleases on human skin that will degrade sample DNA. As a future direction, we will also explore specific types of Whatman paper to use for blood collections, as different types of filter paper have different treatment for nuclease protection. Human blood was eluted off of a Whatman FTA card and strong amplification was achieved. We may want to pursue using only these cards in the future for preservation of field samples, as this is the filter card type used in other successful detection of haemosporidian parasites (Perkins and Schall, 2002; Lutz et al., 2016)

Testing for haemosporidian parasites was used as a proof of concept for future testing of blood spots for important pathogens that could impact bat health. Another pathogen group whose geographic range has increased to higher latitudes owing to climate change are trypanosomes (Hodo et al., 2016). Moving forward, exploration into the prevalence of *Bartonella* spp. in these populations would also be an interesting next step. There is no indication that these bacteria cause morbidity or mortality in Chiropteran hosts, but the high prevalence and genetic diversity of *Bartonella* spp. bacteria have been used in many

studies to ascertain bat evolution and geographic spread and could lend further insights into bat movement as species distributions change owing to land use change and other anthropogenic impacts (Morse et al., 2012; McKee et al., 2016).

5.3: SARS-CoV-2 Surveillance at the Bat-Human Interface to Monitor for the Occurrence of Spillback using North American Bats Managed in Rehabilitation Facilities

5.3.1: Introduction

Release of the SARS-CoV-2 genome in January 2020 at the beginning of the pandemic revealed phylogenetic association with SARS-CoV and other bat-borne betacoronaviruses. Since the likely evolutionary origin of SARS-CoV-2 in bats was suggested (Lau et al., 2020), questions have arisen about the susceptibility of other bat species outside of Asia, ultimately resulting in establishment of a wildlife reservoir(s) of the disease (Olival et al., 2020). The high prevalence and asymptomatic transmission of SARS-CoV-2 in the human population has resulted in multiple spillback events of both domestic animals and captive wildlife in zoos (family *Felidae* and gorillas) and farms (minks) (Bartlett et al., 2020; Koopmans, 2020; Gibbons, Ann, 2021). Owing to the direct and rapid nature of SARS-CoV-2 spread, any activities resulting in close contact between infected humans and animals could result in cross-species transmission (Garigliany et al., 2020). Widespread moratoria were placed on bat field research in 2020 owing to uncertainties surrounding the susceptibility of New World bats to SARS-CoV-2 (Donahue, 2020). As spillback of other pathogens has been documented in wildlife rehabilitation centers in the past (Krawczyk et al., 2015; McDougall et al., 2019), groups focused on bat rehabilitation and healthcare also realized the need to act proactively in ensuring zooanthroponotic transmission did not occur with animals in their care. The International Union for the Conservation of Nature (IUCN) Bat Specialist Group also released guidelines for bat rehabilitation facilities, in which the make specific recommendations to minimize exposure of bats to SARS-CoV-2 and protect bats from becoming infected by handlers (Jolliffee et al., 2020). We present a framework for diagnostic testing for rehabilitation groups providing healthcare to bats prior to animal release – a framework useful not only to monitor zooanthroponotic transmission of SARS-CoV-2, but other pathogens carried by humans as well.

5.3.2: Materials and Methods

5.3.2.1: Sample Collection

As a collaborative effort with Colorado Parks and Wildlife, fecal samples were obtained from 21 bats from two different local rehabilitation centers/bat rescues (in family *Vespertilionidae*: 17 big brown bats (*Eptesicus fuscus*), 2 little brown bats (*Myotis lucifugus*), 1 silver-haired bat (*Lasionycteris noctivagans*) and in family *Molossidae*: 1 Mexican free-tail bat (*Tadarida brasiliensis*)). Prior to the release of these bat pups in October to find their winter hibernacula, 1-2 fecal pellets from each pup were collected twice (once timepoint 1 and two weeks later at timepoint 2) to confirm repeated negative rest result for SARS-CoV-2 RNA. Feces were stored in RNAlater stabilization solution (ThermoFisher Scientific, Waltham, MA) at 0°C for no longer than one week prior to sample processing.

5.3.2.2: Nucleic Acid Extraction

Fecal pellets were removed from RNAlater solution and moved to a tube containing 500 µL sterile water. They were then homogenized, centrifuged, and supernatant was collected for RNA extraction using the MagMax™ Pathogen RNA/DNA Isolation Kit (Applied Biosystems™, ThermoFisher Scientific, Waltham, MA) according to manufacturer protocol.

5.3.2.3: SARS-CoV-2 qRT-PCR

All qRT-PCR diagnostics were performed on a QuantStudio 3™ Real-Time PCR System (Applied Biosystems™, Thermofisher Scientific, Waltham, MA). The Charité/Berlin (WHO) protocol primer and probe panel and the CDC assays for SARS-CoV-2 detection ("2019-nCoV Research Use Only kit" - specifically primer sets N1 and N2) were used as previously described (Integrated DNA Technologies, Coral City, IA)(CDC, 2020; Corman et al., 2020). TaqMan Fast Virus 1-Step Master Mix

(Applied BioSystems™, ThermoFisher Scientific, Waltham, MA) was used according to manufacturer protocol, with 5 µL supernatant used for each sample. A ten-fold dilution series was generated for each positive control, using the 2019-nCoV_E Positive Control for the Berlin-Charlité assay and 2019-nCoV Plasmid Control for the CDC assay. A dilution series was run in duplicate for initial optimization and for generation of standard curves on each run afterwards. Water was used as a negative control. Samples were preliminarily screened using the WHO E gene assay, and suspect or positive samples were confirmed using the CDC N1 assay.

5.3.3: Results

5.3.3.1: Optimization of qRT-PCR

For each assay, the standard curve generated by ten-fold dilution series of the manufacturer-provided control resulted in appropriate number of cycles between dilutions (ideally 3.3 cycles for each log dilution) with negligent background noise and validation of the negative control.

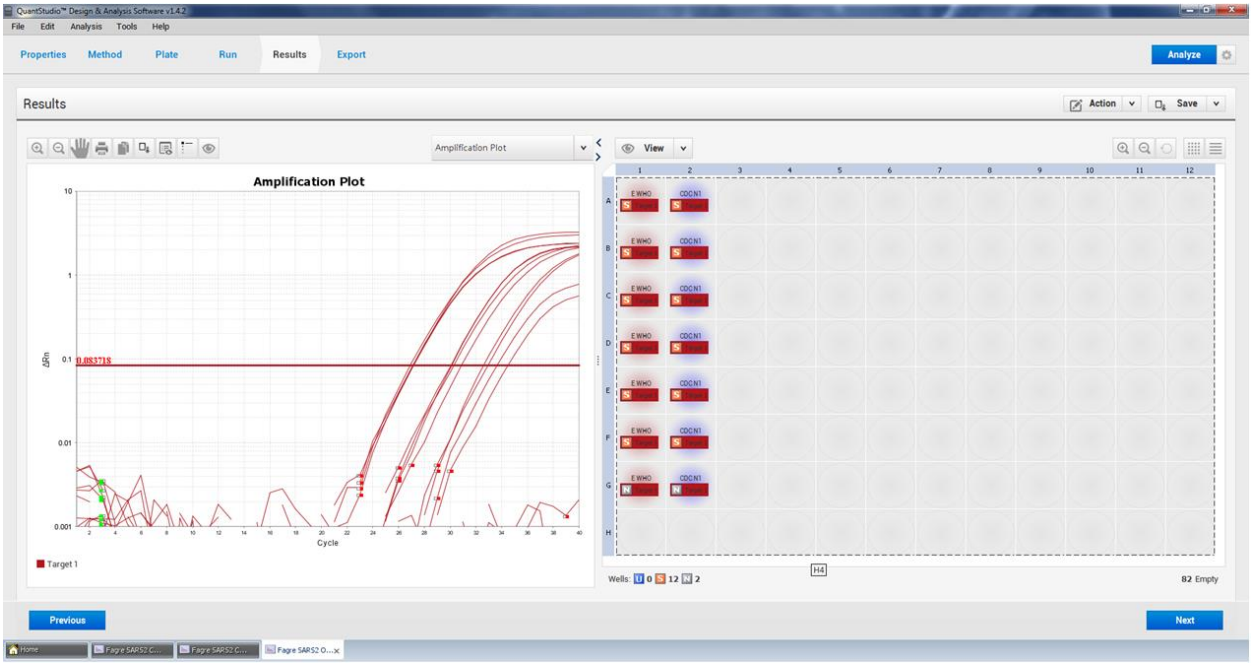

Figure 5.3. qRT-PCR amplification plot for standard curves generated for each assay in use for biosurveillance; a ten-fold dilution series was generated for each positive control. Water was used as the negative control.

Table 5.3. Cycle threshold values for the dilution series generated for both the WHO E gene assay (Berlin/Charlité)(Corman et al., 2020) and the CDC N1 assay(CDC, 2020).

Genome equiv.	WHO E assay		CDC N1 assay	
10^4	26.91	27.22	27.11	27.17
10^3	30.08	30.14	30.90	30.29
10^2	34.44	33.63	32.93	32.62
NTC	0	0	0	0

5.3.3.2: Testing of Fecal Samples from Bats in Rehabilitation Centers

Samples were tested using the Charlité/Berlin E gene assay for first line screening and all bat samples were negative at both timepoint 1 and timepoint 2. Positive and negative controls were validated for each run.

5.3.4: Discussion

In the absence of well-characterized risk for zooanthroponotic transmission of pathogen X, it is prudent to act out of an abundance of caution and treat every species as if they were susceptible to said pathogen (Nuñez et al., 2020). The susceptibility of North American bats to SARS-CoV-2 has not yet been fully characterized owing to the broad taxonomic diversity of bats and varying pathogen susceptibility between bat species. To date, only big brown bats have been confirmed refractory to infection experimentally (Hall et al., 2020), though it is critical to consider that bats in the family *Vespertilionidae* are not a monolith, and other bat families are present in North America (*Molossidae, Phyllostomidae,* and *Mormoopidae*). Species differences have been demonstrated between bats from the same family. For instance, although both in family *Pteropodidae*, bats in genus *Pteropus* support robust viral replication for Nipah virus(Middleton et al., 2007; Rahman et al., 2010), while Egyptian fruit bats do not (Seifert et al., 2020). Stringent biosecurity (e.g. N95, gloves, frequent disinfection) and biosurveillance protocols are critical to keeping both bats and humans safe in instances where interspecies

contact is frequent and intense (e.g. wildlife rehabilitation, zoos, interactions between bats and researchers during seasonal field work) (Nuñez et al., 2020; Olival et al., 2020; Banerjee et al., 2021).

While SARS-CoV-2 is the first coronavirus to cause a pandemic, the taxonomy of *Coronavirinae* (family *Coronaviridae*) is rich, diverse, and deeply intertwined with order *Chiroptera* (Cui et al., 2007). Alphacoronaviruses and most betacoronaviruses are thought to have their evolutionary origins in bats, and recent genomic analyses and high-throughput wildlife sampling indicates that many holes remain within the phylogeny of this viral family (Anthony et al., 2017). Bats harbor viruses closely related not only to sarbecoviruses and marbecoviruses, but close relatives of the common cold (human coronaviruses 229E, NL63, OC43, and HKU1). Owing to the ready nature of cross-species transmission, genetic recombination, and host adaptation mechanisms employed by viruses in *Coronavirinae*, longitudinal monitoring of viral diversity in both vertebrate hosts (humans and bats) at these interfaces is warranted. Surveillance using pan-coronavirus primers would not only allow for characterization of viral diversity and evolution, but it would allow us to more closely monitor the potential for both zoonotic and zooanthroponotic transmission of a group of viruses with a wide range of susceptible hosts and tissue tropism.

Finally, coronaviruses are not the only group of pathogens transmitted between humans and bats. Often, discussions surrounding bats and the pathogens they carry center around reducing spillover events into human populations. However, zooanthroponotic transmission owing to tourism or management of wild animals in captivity has resulted in significant morbidity and mortality events (Palacios et al., 2011b). Transmission of bacterial and protozoal pathogens from humans to bats in wildlife rehabilitation centers and zoos has been documented (Espinosa-Gongora et al., 2012; Schiller et al., 2016; Feßler et al., 2018), and the introduction of novel pathogens to a naïve bat population upon reintroduction of rehabilitated individuals could result in unforeseen consequences.

CHAPTER 6: CONCLUDING REMARKS

6.1: Existing Gaps in the Assessment of Vector-borne Disease Transmission Between Humans and Bats

The WHO definition of arbovirus outlines two roles the vertebrate host may play in transmission – the amplifying host or the reservoir. A review discussing the potential for vertebrates to serve as reservoirs of arboviruses by Kuno and colleagues discusses how the distinction between these two terms has become interchangeable in the literature, and as reviewed in Chapter 1 of this body of work, many knowledge gaps remain in the role that bats play in the transmission of arboviruses (Kuno et al., 2017).

While my dissertation has focused on characterizing the potential role that bats play in the transmission of arboviruses and other vectorborne diseases, it is important to remember that the presence of a pathogen in a given vertebrate or arthropod population does not necessarily indicate ongoing transmission or pathogen dependence on this particular host to complete its lifecycle. While chapters 2 and 4 of my dissertation focused largely on describing the presence or prevalence of arboviruses in bat populations (a novel orbivirus and detection of ZIKV sfRNA, respectively), chapter 3 focused on an important aspect of characterizing novel pathogens – assessing the presence of viremia, pathology, and seroconversion in an *in vivo* system.

In fulfilling criteria for classifications of infectious disease etiology and epidemiology, Koch's postulates were historically used to confirm the causal link between a pathogen and its clinical outcome. Similarly, the nine causal guidelines put forth by Bradford Hill in 1965 provide a framework for causal inference in epidemiology, but are infrequently used in assessing causal associations in arbovirology (barring an analysis of microcephaly and ZIKV in light of these guidelines (Awadh et al., 2017). Of relevance to molecular surveillance, Fredericks and Relman propose postulates related to prevalence and magnitude of sequence-based detection. And more recently, Wolfe and colleagues have proposed a framework for identifying the five stages a pathogen undergoes as it adapts from animal pathogen to human pathogen, which is frequently cited in literature surrounding spillback and disease ecology (Wolfe

et al., 2007). While these frameworks are helpful for contextualizing cross-species transmission events and risk at the human-animal interface, countless nuances associated with vectorborne disease and establishing viral reservoirs for arboviruses are not recognized when we adhere strictly to the aforementioned frameworks.

To properly ascertain transmission cycles of vectorborne diseases in nature and assess presence of a wildlife reservoir, a new set of postulates or criteria must be put into place. Kuno et al. has outlined a set of empirically determined conditions which a vertebrate must fulfill in order to be considered the reservoir of an arbovirus. Below, I have outlined these conditions with considerations for bat research and how best to approach addressing these criteria for any given tripartite (bat-arthropod-virus) relationship (Table 6.1).

Table 6.1. Criteria for vertebrate reservoirs of arboviruses as outlined by Kuno et al (2017), with additional considerations for their application to field and experimental studies in bats.

Criterion proposed (Kuno et al., 2017)	Field studies **Free-ranging bats**	Experimental studies ***in vivo***	Primary limitation(s)
Chronic or persistent infection in the absence of pathology or compromised function	Requires subjective clinical assessment by veterinarian in the field	Close monitoring of clinical status is ideal for ascertaining any impacts to animal health or function (including hematology and post-mortem histopathology)	Requires establishment of reference intervals or health parameters for each species in question
Shedding or viremia sufficient to source infection to additional vertebrates or arthropods	Requires detection of live virus, which imposes difficulties in regard to export and sample preservation	Collection of serial samples from the same individual is ideal, though terminal blood draws at serial timepoints is more realistic	Requires full understanding of viral titer sufficient to infect arthropod host taking bloodmeal from bat
Continual or intermittent isolation of the arbovirus from the suspected vertebrate during overwintering period of arthropod (or complete cessation of feeding activity for 2+ months)	Repeated isolation of pathogen X from free-ranging bats requires continued sampling and as such, a means of marking individual animals (RFID or otherwise) for rapid identification upon re-capture	Experimental conditions allow for long-term studies to assess viral RNA levels in blood or excreta	Husbandry conditions of bats must be optimized to permit long-term studies without compromising animal health and nutrition
No newly introduced infected vertebrates or vectors during the aforementioned feeding cessation period	Difficult to determine addition of new individuals owing to movement of bats between roosts and large roost sizes	Experimental conditions allow for full control of this criterion	Primarily a limitation in field studies owing to long distances traveled by bats in addition to gregarious roosting behavior
Detection of *infectious* virus from blood following completion of the 2+ month cessation period	Relies on repeated sampling of the same population and high re-capture rates to confirm true longitudinal sampling	Collecting blood from bats held in longitudinal studies may warrant euthanasia, so this may be only an endpoint sample	If unable to isolate virus on Vero cells, acquisition of appropriate bat-derived cell lines is warranted

6.2: Limitations in the Ways that Questions Surrounding Viral Ecology in Bats is Assessed across Scales

With regard to experimental infections, one must take into account the method of inoculation (e.g., intracerebral, subcutaneous, intraperitoneal, intramuscular), the inoculating titer, as well as whether or not vector bites were associated with the infection. In analyzing serologic results, it is important to differentiate whether the assay is detecting neutralizing (e.g. PRNT80) or non-neutralizing antibodies (e.g. ELISA), and consider the dynamics of antibody decay which may impact detection sensitivity (Baker et al., 2014). Arthropod saliva has been shown to potentiate arbovirus infection, affecting not only the magnitude of peak viremia but also the viral loads in certain organs (Schneider and Higgs, 2008). The inoculating viral strain used is also important, as it should align geographically with where the species under study resides to truly characterize their potential as a reservoir species. Komar et al. (2003) presented detailed methods for estimating the reservoir competence of vertebrate species for mosquito-borne viruses (Komar et al., 2003), including calculating a reservoir competence index based on the susceptibility to infection, mean daily infectiousness, and the duration of infectiousness. This reservoir competence index is interpreted as the relative number of infectious vectors that derive from a particular vertebrate species, and would be easily transferrable to evaluating the reservoir competence of bats. Further, bats are very long-lived (up to 25 years) compared to other small mammals, potentially increasing the chances of exposure and subsequent transmission to other vertebrates or invertebrate vectors (Calisher et al., 2006). Cell culture also offers a viable starting point for initial assessments of viral replication in a particular host species, particularly when in vivo studies are not feasible. To date, many cell lines derived from the organs of bats have been established (Banerjee et al., 2018).

Importantly, the intent of reviewing the presence of arboviruses in bats is not to vilify the order, but to analyze the existing literature and its support for bats as arboviral hosts, in addition to identifying areas for future study. Bats provide vital ecosystem services, such as arthropod suppression, pollination, and seed dispersal (Kunz et al., 2011). Past depopulation efforts in response to viral outbreaks resulted in increased viral spillover, and are not a viable means of disease control and have even resulted in higher

virus infection rates in the bat species when colonies repopulated from neighboring roosts(Amman et al., 2014). Further work should encompass directed field surveillance complemented by both in vitro and in vivo approaches, with field surveillance efforts focused on optimization of non-lethal and non-invasive sampling (Puechmaille and Petit, 2007; Boston et al., 2012; Young and Olival, 2016; Giles et al., 2018). When possible, longitudinal sampling of identifiable animals through use of marking or tagging allows for monitoring of seroconversion and viremia persistence, providing a chance to better characterize transmission periodicity and seasonal changes in viremia profiles.

To truly elucidate the role of bats as reservoirs for arboviruses, field surveillance studies documenting natural infection and transmission dynamics among vector and vertebrate species must be supplemented with experimental infections to characterize viremia profiles and infectiousness to vectors, virus-induced pathology, and immune kinetics following infection. With bats, these tasks are not trivial, and carry significant challenges in both the field and the lab. These challenges are evidenced by the few arboviruses for which there are substantial field and laboratory data involving the infection of bats. While many studies have presented serologic data indicative of past exposure of free-ranging bats to arboviruses of medical and veterinary importance, these studies should be followed up with laboratory assessments of reservoir host competence to shed light on the true epidemiological significance of the field data. Further, the detection of viral nucleic acid in free-ranging bats does not necessarily implicate the species as an arboviral reservoir. Rather, recovery and isolation of live virus at biologically relevant titers, and demonstrating the persistence of the pathogen in nature among connected populations of a potential reservoir species is more definitive. Unfortunately, few established bat colonies exist for use in vivo viral pathogenesis studies, limiting the achievability of these studies.

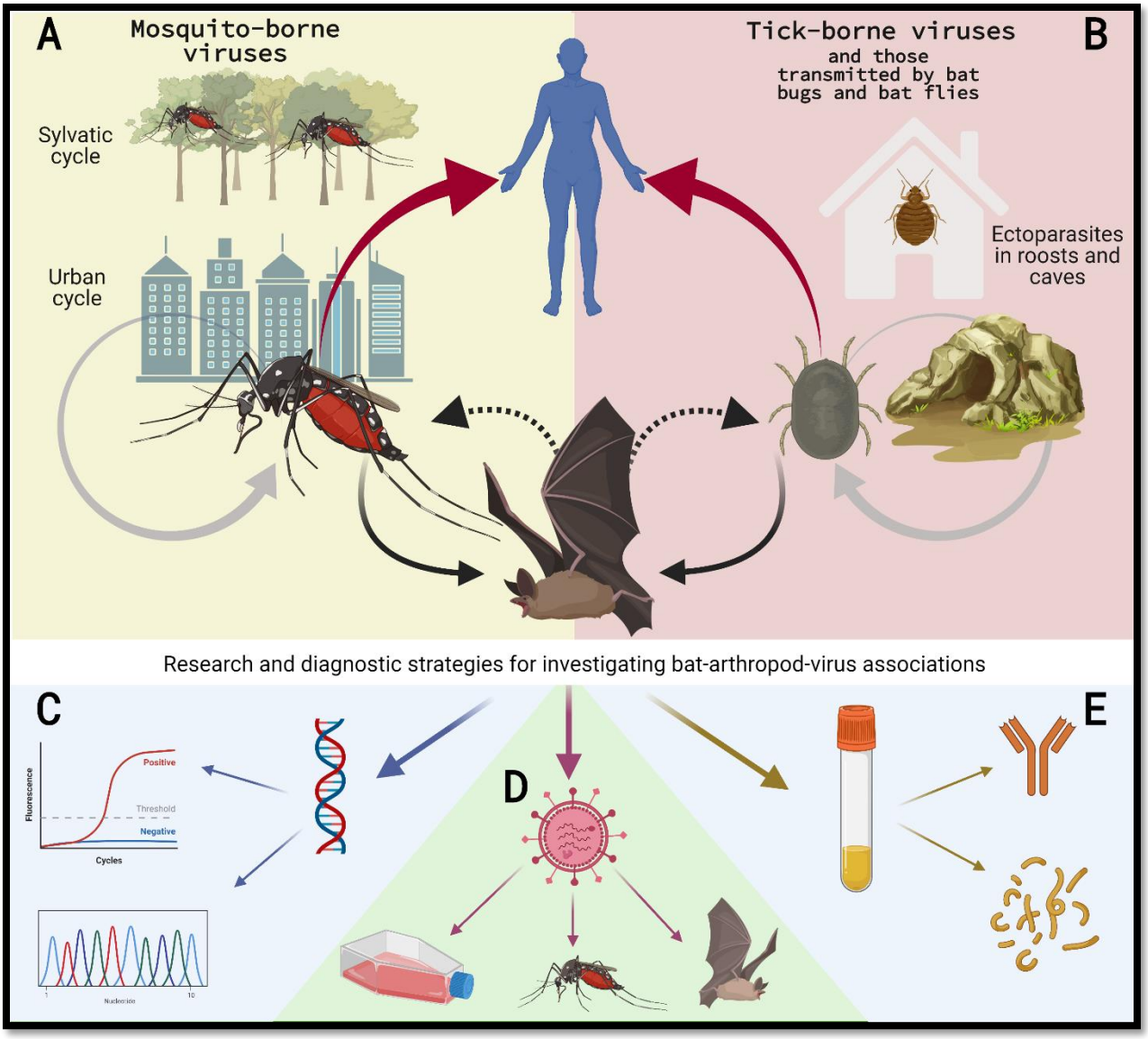

Figure 6.1: Schematic diagram outlining the study of arboviruses in bats. Bats are exposed to arboviruses by the bite of mosquitoes (A) and other ectoparasites that co-habit with bats (e.g. ticks, bat bugs, and bat flies) (B). Research and diagnostic strategies for investigating bat-arthropod-virus associations with various field-obtained sample types are also pictorially described: molecular detection (e.g. PCR) and pathogen discovery with nucleic acid (C), *in vitro* or *in vivo* analysis of growth kinetics of transmission dynamics (arthropod and vertebrate host) studies with live virus (D), and serology or proteomics with serum (E). Image prepared in BioRender.

To fulfill the vertebrate reservoir host paradigm, *in vivo* infections must support findings in the field. The isolation of Marburg virus from Egyptian rousette bats in Uganda, in addition to experimental infections demonstrating viremia and shedding in the absence of overt pathology, support the role of this

bat species as the reservoir for Marburg virus (Towner et al., 2009; Amman et al., 2015; Jones et al., 2015). For arboviruses, the combination of fieldwork and *in vivo* pathogenesis studies are lacking. Still, several examples have emerged from this review that point to bats as potentially competent amplifying hosts for arboviruses. Kaeng Khoi virus was isolated from both bats and cimicid bugs in Thailand, and was also shown to be the causative agent behind sick mine workers (Williams et al., 1976). Some bat species experimentally inoculated with CHIKV developed a viremia at a level that would be infectious to mosquitoes, and bats have been exposed to CHIKV during field investigations (Bosco-Lauth et al., 2018; Stone et al., 2018). Experimental evidence supporting transmission of RRV from *Pteropus poliocephalus* to recipient mosquitoes, in addition to identification of RRV-seropositive bats trapped in Australia and Indonesia, highlights the need for further investigation into the role of bats in the ecology of this disease (Gard et al., 1973; Olson et al., 1983; Ryan et al., 1997). A mosquito-bat-mosquito transmission cycle was established in the lab for JBEV (La Motte, 1958). Serological evidence from the field documenting bat exposure to JBEV, the sustained viremia in bats during hibernation, and demonstration of mosquito transmission from and to bats in the laboratory collectively demonstrate the capability of bats to function as reservoir hosts for this virus. Circumstantial evidence from field-sampled mosquitoes and bats supports the cycling of Babanki virus among bats and mosquitoes in Uganda (Kading et al., 2018), but experimental data are still lacking. For RVFV, bats evaluated in the lab have supported virus replication, and multiple populations of bats in the field have been found with neutralizing antibodies or natural infection with RVFV (Calisher et al., 2006; Balkema-Buschmann et al., 2018; Kading et al., 2018).

6.3: Eyes Ahead: Balancing the Interests of Bat Conservation and Public Health

To more fully characterize the true potential for bats to serve as reservoirs for arboviruses in nature, additional studies are warranted which collate the results of surveillance in free-ranging populations, experimental infection studies, and genomic analysis to infer higher resolution insights into viral evolution and host switching. However, the order *Chiroptera* faces many challenges, including anthropogenic land use change, infectious disease threats (e.g. white nose syndrome), and increased

villanization due to the pandemic and fear owing to misinformation. To enhance our characterization of the tripartite relationship between bats, arthropods, and the viruses they may transmit, research groups should carefully consider the criteria necessary to fulfill the definition of an arbovirus' vertebrate reservoir (Table 6.1) (Kuno et al., 2017). Bats are not a monolith, but rather an order of mammals with a rich and diverse taxonomy, reflected by differential susceptibility to infection with various pathogens (Agnarsson et al., 2011; Seifert et al., 2020). As such, prioritization of species sampling using the results of previously conducted field and laboratory studies will reduce unnecessary stress and euthanasia in free-ranging bat populations (Becker et al., 2020). Finally, optimization of non-lethal diagnostic strategies is warranted to improve pathogen detection in free-ranging bat populations while minimizing impact on overall population dynamics (Giles et al., 2018).

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