DNA VIRUSES OF BIG BROWN BATS: LESIONS, PATHOGENESIS, AND INNATE IMMUNE RESPONSE

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ABSTRACT

There have been numerous pandemics and epidemics throughout human history with varied origins but presently bats are being singled out as the harbingers of current and future plagues. Is there something special about the relationship bats have with their viruses that merits this claim or are they just like other mammals? Bats are unique among mammals in their ability to fly and differences found in their immune system are proposed to be secondary to evolutionary adaptations to flight. These adaptations may allow them to tolerate viruses without developing associated disease. The large numbers of viruses found across bat species could reflect this tolerance, but it may also reflect the sheer number of bat species and therefore viruses of this mammalian order. The perceived tolerance could also reflect our limited understanding of infectious diseases in bats. To address this question, we reviewed the literature on viral diseases in bats, looked for pathological changes that were potentially associated with viral infection in routine post-mortem evaluations of Western Canadian bats, and examined the innate response to DNA in big brown bat cells. We isolated two DNA viruses from naturally infected big brown bats. One, a herpesvirus, was not associated with any disease and the other, a poxvirus, was associated with oral ulcerations and joint swelling. Novel features were noted with both viruses: an atypical cell type supporting replication for the herpesvirus and an abnormal cellular site of replication for the poxvirus. Big brown bat cells expressed an array of DNA sensors and used multiple transcription factors to generate an innate response to a DNA surrogate. These bat cells produced a comparable innate response when they were infected with inactivated forms of these viruses. Whereas the innate of response of human cells stimulated with this surrogate was markedly proinflammatory. This proinflammatory induction was not observed in bat cells. One of these proinflammatory products, interleukin 8, was expressed at high levels in bat cells independent of treatment, including viral infections. A different proinflammatory product, interleukin 6, was inhibited in response to the DNA surrogate, but the inhibition was overcome upon infection with live poxvirus. The herpesvirus, which was not associated with any pathological changes, was able to control the innate response, whereas the poxvirus, which was associated with disease, was not. This work suggests bats are susceptible to disease caused by viruses, they are not generally tolerant to viruses, and the development of disease is dependent on the type of virus, which is similar to other mammalian species.

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DEDICATION

This thesis is dedicated to Ian and Maeve for encouraging me to take the leap and being there as I fell.

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LIST OF ABBREVIATIONS

AIM2	absent in melanoma 2
BALT	bronchus-associated lymphoid tissue
cDNA	complementary DNA
CNS	central nervous system
CWHC	Canadian Wildlife Health Cooperative
D-MEM	Dulbecco's modified eagle medium
dsDNA	double stranded DNA
EfHV	Eptesicus fuscus gammaherpesvirus
EfK3b	Eptesicus fuscus kidney cell line 3b
EMS4A1	Eptesicus fuscus membrane spanning 4 domains A1
EfPV	Eptesipox virus
EfPV/SK	Eptesipox virus/Saskatoon/01/2020
EfPV/WA	Eptesipox virus Washington strain
h.p.i.	hours post infection
H&E	hematoxylin and eosin
IFN	interferon
IHC	immunohistochemistry
IL	interleukin
IRF	interferon regulatory factor
ISH	*in-situ* hybridization
LANA	latency-associated nuclear antigen
MALT	mucosa-associated lymphoid tissue
MERS	Middle East respiratory syndrome
NFκB1	nuclear factor kappa B1
PCR	polymerase chain reaction
PIM	pulmonary intravascular macrophage
poly(dA:dT)	poly(deoxyadenylic-deoxythymidylic)
poly(I:C)	polyinosinic:polycytidylic
PYHIN	pyrin and hematopoietic interferon-inducible nuclear domain-containing protein
qPCR	quantitative polymerase chain reaction
SARS-CoV	severe acute respiratory syndrome coronavirus
siRNA	small interfering RNA
STING	stimulator of interferon genes
TEM	transmission electron microscopy
TNFα	tumor necrosis factor alpha
UV	ultraviolet
W/N CWHC	Western/Northern Regional Centre of the Canadian Wildlife Health Cooperative
γHV	gammaherpesvirus

CHAPTER 1 INTRODUCTION

1.1 Introduction

There are currently two narratives regarding bats as viral hosts. The first most reiterated view is that bats have a unique relationship whereby they tolerate viral pathogens(1) the other view is bats are the similar to other mammalian orders(2). The basis for the first theory is the reported number of viruses detected in bats is more than what has been found for other mammalian orders and their detection is made in the absence of disease. The second theory posits that the viral richness of a mammalian order reflects the species diversity of that order. To address this argument this thesis reviews the evidence for bats as hosts for viruses and whether disease is associated with viral infection.

1.2 Chiroptera

Bats belong to the order Chiroptera and are incredibly diverse with over 1400 species(3). This order is unified by a common feature: fleshy membranes that are stretched over the bones of the appendicular skeleton and used for flight. The meaning of the name Chiroptera is derived from this feature and translates into "hand" (Χέρι, cheri) "wing" (πτέρυγα, pteryga). Their diversity is reflected in numerous adaptations that allow them to subsist on insects, fruit, nectar, pollen, leaves, seeds, small mammals, fish, crabs, reptiles, amphibians, and blood(4). As the only mammals capable of true flight, they have distinct physiology. Compared to other mammals of similar size they have larger lung volumes, larger hearts, more red blood cells, and their body temperatures are often outside of the homeothermic range(5). These are all adaptations to meet the challenging demands of flight. These adaptations to flight are possibly responsible for the alterations in the immune system of bats(6).

1.2.1 Innate immune adaptations

The innate immune system is a non-specific mechanism of defence from non-self things. The first line of defence of the innate response is barriers that block non-self things like pathogens. For example, the intestinal mucosa in combination with chemical modifications like acid, mucous, and enzyme production prevents translocation of gut bacteria into host tissues. Once pathogens breach a barrier host chemical factors actively destroy pathogens. Examples of chemical factors include complement cascade products and the enzymes of white blood cells that destroy engulfed pathogens(7). Once engaged these chemical factors transmit signals recruiting more white blood cells and altering blood flow resulting in inflammation. Inflammation can be activated by various

stimuli: tissue death (necrosis), foreign material, aberrant activation against self (autoimmune disorder) or environmental substances (allergies), and pathogens(8). Since inflammation is triggered by multiple stimuli and mediated by numerous chemical factors and cells, alteration or inhibition of individual components may not prevent the development of inflammation in the host.

Bats have several recognized alterations in their innate immune response with potentially many more yet to be discovered(9). They are missing a whole family of innate immunity genes responsible for sensing DNA and triggering inflammation, the pyrin and hematopoietic interferon-inducible nuclear domain-containing protein (PYHIN) family(10). Additionally an effector molecule for these and other DNA sensors stimulator of interferon genes (STING) has an altered amino acid residue that reduces its activation(6). These are two examples of many characterized alterations in the innate response of bats. The innate response alterations are theorized to have evolved to prevent inflammation associated with DNA damage caused by intense metabolic activity, flight(6). A proposed by-product of this decreased inflammation in response to DNA damage is decreased inflammation in response to viral infection. Since viruses are packaged nucleic acids, they are recognized by many of the same sensors as damaged cellular DNA.

1.2.2 PYHIN family

The PYHIN family of genes has many functions. More recently they have been implicated as sensors for DNA but they also are involved in cell growth, cell differentiation, tumor suppression, apoptosis, senescence, and autoimmunity(11). This family is restricted to mammals, and based on the detection of this gene in mammalian genomes it is thought to have been acquired sometime after the divergence of monotremes but before that of marsupials(12). There is large variability in the number of PYHIN genes among mammalian species; for example, mice have 14, humans have 4, and cows have 1. There is only 1 gene in this family that is conserved across mammalian species: absent in melanoma 2 (AIM2)(12). Bats are the only mammals to have no full length PYHIN genes except for an AIM2 pseudogene which has been detected in one species of bat(10). The AIM2 gene is dispensable because it is also reduced to a pseudogene in cows, sheep, llamas, dolphins, dogs, and elephants(12). AIM2 is the only member that we have enough experimental data to conclude it acts as a DNA sensor; we cannot say the same for the other PYHIN proteins whose roles remain unclear and are different enough to be expressed in different parts of the cell(13). AIM2 has been shown to be critical for inflammasome activation in mice, a similar role is not observed for the other mouse PYHIN genes(14). Activators of the inflammasome in

mammals with AIM2 pseudogenes still require characterization. Comparisons of PYHIN genes among mammals are currently made based on sequence data as the structure and function of these genes are not well studied outside those of humans and mice. AIM2 is the only PYHIN gene that can be reliably compared among species as it is conserved across mammals. Bats are not unique in their loss of AIM2 as a pseudogene has been found in a bat species and several other mammalian species . Therefore, any tolerance to viruses conferred by the loss of AIM2 in bats would also be expected in these other species. Caution is warranted in discussing the meaning of genomic changes when we do not fully understand the roles of these genes, or if there are other genes that could serve a similar function.

1.2.3 Disease surveillance

Bats are not easy animals to observe, a feature that makes detecting disease in these species difficult. As part of a national wildlife disease surveillance program bats are often submitted to the Canadian Wildlife Health Cooperative (CWHC). The CWHC is a network of veterinary diagnostic laboratories based at the Canadian veterinary colleges and specific provincial veterinary diagnostic laboratories. These laboratories perform diagnostic work on wildlife species. The core activities are performing necropsies on wildlife carcasses and actively surveilling for specific diseases of wildlife. Diagnostic work involves detection of diseases of concern to wildlife, domestic animals, and humans. Common reasons for diagnostic submission of bats are predation, blunt force trauma and emaciation(15). Major pathogens of bats that are routinely diagnosed are the fungal cause of white nose syndrome and rabies virus. The case material presented in this thesis are from submissions of bats to the Western/Northern (W/N) Regional Centre of the CWHC.

1.3 Viruses

Viruses are quasi-living organisms composed of packaged nucleic acids, the instructions for replication. They require host cellular machinery to form progeny virions. The viral families are characterized by the arrangement, size, and type of genome as well as the packaging. There are RNA and DNA viruses with different requirements for host cellular machinery. The packaging is composed of a protein coat with distinct morphology depending on virus family with certain families having an outer membrane derived from host cells.

DNA viruses that are common pathogens of mammals belong to the Poxviridae, Iridoviridae, Asfarviridae, Herpesviridae, Papillomaviridae, Polyomaviridae, Parvoviridae, and Circoviridae families. These viruses are recognized by cellular DNA sensors of the host. In bats

several DNA sensors are missing, a feature that has been posited to result in an impaired ability to sense and respond to viral infections.

Viruses can infect many lifeforms from bacteria, parasites, plants, animals, and even other viruses. All viruses an ecosystem or an organism carry are called a virome. Newer techniques to detect viruses employ looking for the nucleic acid sequences of viruses within all the nucleic acid sequences from a sample. Even though all organisms tolerate a virome this sequencing analysis has been robustly employed on bat samples and the findings are then used as evidence for the large number of viruses bats carry. There are several issues with this methodology. First, outside of humans, there are no other mammalian orders for which comparable sequencing has been performed. Secondly, detection of a viral sequence in a sample from bats does not mean that the bat is the definitive host. Clouding this issue further is the fact that many of the sequences have been derived from fecal samples(16–18). These sequences could also come from the food, and they may not reflect infectious virus.

1.3.1 Viral pathogenesis

There are several mechanisms by which viral infections result in disease and can be divided into viral and host factors. Viruses can induce disease by impairing cellular function or destroying cells. Hosts can produce disease through an overactive immune response. This response induces fever, inflammation, lymphoid proliferation, destroys cells, and can result in antigen-antibody deposition(19). Disease is a complex interplay of numerous virus and host factors where most viral exposures do not result in disease(20). Since disease pathogenesis is such a complex process whether the alterations in bat immune systems are enough to protect them from disease is less clear.

1.3.2 Pandemics

During the last two decades bats have become synonymous with pandemics and emerging infectious disease, with less attention being given to other animal orders. However, since the recording of modern human history most pandemics have been associated with pathogens originating in the environment, humans, rodents, primates, pigs, and birds(21). In 2002, bats were first implicated as the origin of severe acute respiratory syndrome coronavirus 1, SARS-CoV-1. Although this virus originated in bats an intermediate host was required to initiate the pandemic. A similar pattern is seen with other coronaviruses that have also originated in bats such as Middle East respiratory syndrome (MERS) virus and possibly SARS-CoV-2 (22).

1.4 Rationale and objectives

Unique alterations in the immune system of bats has been posed as evidence that they are not as susceptible to viral disease, but mammalian immune systems are highly variable and hard data is needed to establish whether that variability is linked to susceptibility to viruses. There is a paucity of data recording natural infections and their outcomes in bats. This evidence is reviewed in the following chapter. Lack of evidence for virus induced disease in bats could reflect limited surveillance effort or could indeed be a unique virus-host relationship in chiropterans. A portion of this thesis reviews bat submissions to the W/N CWHC looking for virus-associated pathological changes to address the question of whether bats develop virus-associated disease. Rabies virus was excluded from this assessment because lesions are not a reliable feature of infection in bats and non-bat species(23–25).

A key element of the argument that bats are unique viral reservoirs relies on decreased cellular DNA sensing due to the absence of a PYHIN gene and decreased STING activity. However, most of the research on the innate immune system of bats used an RNA surrogate(6,26,27) as well as *in vitro* infections with RNA viruses(28). There have been few studies on the bat innate response to DNA viruses(6) and none using DNA surrogates in bat cells. We examine the cellular response of big brown bat cells to their DNA viruses and a DNA surrogate to address this deficit in experimental studies. Understanding how the bat response controls viruses or prevents development of disease could have applications for human or animal health and therapeutics.

The question of bats being immune to disease associated with their viruses is important to resolve. Bats are crucial for the health of ecosystems. They provide pest control, control of viral vectors, pollination, seed dispersal, and fertilizer(29). Bats are vulnerable to climate change, alteration or destruction of habitat, and pathogens introduced by man(30). To understand bat health, the effects of viruses on their hosts needs to be clarified. To date, most information has been provided through genomic comparisons, cell culture techniques, and experimental infections but only surveillance for disease in the wild populations can definitively provide an answer on whether these viruses cause disease in their hosts under natural conditions.

To address deficiencies in previous research the following are the primary objectives of this thesis.

1. Review the published literature to determine if the dogma that the relationship between bats and viruses is unique among mammals.
2. Examine submission of bats to the CWHC for pathological changes potentially associated with viral infections.
3. Isolate and characterize viruses from suspected cases.
4. Quantify the *in vitro* innate response of big brown bat cells to DNA.

These objectives will help to determine whether bats develop virus-associated disease.

CHAPTER 2 REVIEW OF VIRUS-ASSOCIATED DISEASE IN BATS

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2.1 Abstract

Viruses that have caused recent epidemics and pandemics have predominately originated in bats. These include severe acute respiratory syndrome coronavirus 1 and 2, Middle East respiratory syndrome coronavirus, Nipah virus, and Hendra virus. This has generated an intense search for the next pandemic causing virus in bats and has yielded evidence of numerous viruses from apparently healthy, clinically normal bats. Virus-associated disease is only one of several potential outcomes from exposure to a virus. One reason bats may be able to host such an array of viruses without becoming ill is because of differences in their immune systems, compared to more commonly studied species. These changes may have allowed bats to tolerate viral infection without developing disease. To summarize the evidence for presence or absence of disease associated with viral infections in bats we have reviewed the available literature. Currently there is insufficient evidence to draw conclusions about whether bats are tolerant to their viruses. Further studies on the effects of these viruses in natural infections are required.

2.2 Introduction

Bats have been implicated as the reservoir hosts for many viruses that have caused severe or widespread disease in humans(31). This has prompted the question: do bats host more viruses than other mammals and, if so, why do we not observe more disease in bats? Some researchers have even proposed that bats have an innate tolerance to viral infections(1,9,32–34). The following review outlines the evidence we have to date of viral infections and viral associated disease in bats.

Bats comprise more than 20% of living mammals making Chiroptera the second most diverse mammalian order(33,35). This diversity makes drawing generalizations about bats challenging. Bats have features in common: they are capable of flight, live longer than mammals of comparable size, and have few neoplasms(9,36,37). Bats diverged from other mammals more than 64 million years ago and potentially they have co-evolved with their viruses ever since(38). Bats are described as rich viral reservoirs due to several factors: interactions with other mammalian species, longevity, population genetics, and sampling effort(33,35,39). However, viral richness of bats may be a reflection of the species richness of their order and not a unique feature of their hosts(2).

Many articles describe bats as being able to host viruses without developing disease. The evidence cited in support of this unique relationship are the number of viruses that have been detected in bats and the alterations in their immune systems(1,32,40). There is still a lot we do not

know about the role bats play as hosts for many of these viruses(36). Since their roles are unknown it is impossible to draw conclusions from experimental studies regarding the significance of their findings and the implications for the host. Most of the hypotheses generated from these experimental studies on the immune response of bats still need to be tested at a functional level(37).

There are only a handful of articles detailing diseases in bats. Case reviews of wild bats submitted to veterinary centres have demonstrated the following diseases: pneumonia, diarrhea/enteritis, dermatitis, septicemia, uterine infections, and neurologic disease(15,41). Various pathogens were associated with disease: bacteria, parasites, and infrequently viruses(42–44). Despite bats being described as resistant to neoplasia various neoplasms have been chronicled in several species including sarcomas, carcinomas, and lymphoma(45–50). Some of these neoplasms were also associated with viral infections(49,50). The description of neoplasia in bats when they are portrayed as resistant suggests that there is a lack of evidence rather than an absence of disease. Lack of evidence rather than evidence of absence of viral associated disease in bats is also likely.

There are excellent reviews on viruses detected in or isolated from bats(36,51–54). Our goal is not to recapitulate these articles but to examine the evidence for pathological changes associated with natural or experimental infections in bats and asks the question do bats develop disease associated with viral infections?

2.3 Natural cases of viral infection in bats and their associated disease

The viral family for which there is the most evidence of disease associated with naturally acquired infections is Poxviridae. Poxvirus infections can either be localized to the site of inoculation of the epidermal keratinocytes or can be spread systemically throughout the body. Typically, there is hyperplasia of the keratinocytes with associated inflammation(55). Poxviruses have been isolated from the genus *Eptesicus* with lesions reported to vary from none to oral ulcerations, intracytoplasmic inclusion bodies, and/or marked joint swellings(56–58). In Egyptian fruit bats multiple skin nodules were present characterized by a thickened epidermis occasionally with ulceration and intracytoplasmic inclusions(59,60). One skin nodule from a survey in Southern bent wing bats was characterized by thickening of the epidermis by hyperplastic and hypertrophied epithelial cells with intracytoplasmic inclusion bodies; the presence of virions was confirmed by electron microscopy(61). The remainder of the evidence of infection of bats with poxviruses is based on genomic sequencing data(62,63).

Rhabdoviridae is the viral family most synonymous with bats, which are the primary hosts for many of these viruses. Rhabdoviruses cause fatal neurologic disease in mammals. Viral inoculation results in local replication followed by spread to the central nervous system (CNS) by peripheral nerves(64). Depending on the site of inoculation the route of spread to and within the CNS may be different. This may account for differences in clinical signs in humans infected with bat rabies variants(65). Infection is characterized by the following changes which may be subtle: inflammation within the CNS, Negri bodies within neuronal cell bodies, and gliosis(64–66). The reports on natural infections of bats from both subfamilies, Yinpteropchioptera and Yangochiroptera, with rhabdoviruses demonstrate the outcome is often fatal(67–73). When histopathological assessment of these cases is performed the characteristic brain inflammation and viral inclusion bodies are found(74–79). The clinical signs are consistent with neurologic disease and present as abnormal behaviour, depression, and paralysis(74,80–88). Additional findings on experimental infection of bats with rhabdoviruses are presented in section 2.4.

The Herpesviridae family is divided into 3 distinct subfamilies alpha, beta, and gamma herpesviruses. The all have a two-stage life cycle of quiescent asymptomatic infection for the life of the host with periodic productive infections during stressful events. The productive or lytic stage of infection is associated with pathological changes but can also be asymptomatic. The cells and organs that support the quiescent or latent state are different for each of these subfamilies(89). Detection of members of Herpesviridae is common in bats but reports of isolation and disease associated with infection are sparse(90–94). Gammaherpesviruses (γHVs) establish latency in lymphoid tissues(89). Pathological changes associated with γHV infection in natural hosts are lymphadenopathy in the acute phase and neoplasia, proliferative and immune mediated disorders with chronicity(95). Infection in big brown bats with a γHV was not associated with any disease but productive infection occurred in an unusual location implicating pulmonary intravascular macrophages in the biology of this virus (96,97). Other γHVs of bats have been associated with inflammation of eyelids and an interscapular mass(98,99). Additional γHVs have been isolated from bats but no further information is available on the biology of these viruses(100,101).

Pathogenesis of alphaherpesvirus begins with local replication in the epithelium followed by spread to the dorsal root ganglia via sensory nerves and establishment of latency. Reactivation from latency results in the production of vesicles and ulcers in the epithelium(89). There are several isolates of alphaherpesviruses from fruit bats but we have no information on the

pathogenesis of these viruses in their natural hosts(102–105). We have observed inclusions in keratinocyte nuclei of the oral epithelium adjacent to an ulcer in big brown bats that is consistent with an alphaherpesvirus infection (unpublished data). A betaherpesvirus has also been identified using electron microscopy of the salivary gland in little brown bats(106). This is consistent with the pathogenesis of betaherpesviruses which establish latency in secretory glands among other tissues(89).

Infection with viruses in the family Papillomaviridae are often asymptomatic or they may cause proliferative lesions of the skin and mucosa and in some species also infect cells of the underlying connective tissue forming fibropapillomas. Occasionally papillomaviruses can induce neoplasms at these locations(107). The first papillomavirus isolated from bats was associated with a neoplasm of the skin, a basosquamous carcinoma in an Egyptian fruit bat(49,108). The remaining evidence for papillomavirus infection of bats comes from sequencing data(109–112).

Filoviruses cause hemorrhage and necrosis in multiple organs in humans and non-human primates. Pathological changes caused by these viruses in their maintenance hosts is unknown because the reservoir has yet to be identified(113). Lloviu virus in Schreiber's bats is associated with high mortality, inflammation in the lungs, and depletion of lymphoid follicles in the spleen from chronic antigen stimulation(114). There is evidence of widespread exposure to Lloviu virus in Schreiber's bats as detected by antibodies(115). Additional mortalities were observed however the preservation of dead bats precluded any pathological assessment(116). The virus has been isolated but no further conclusions have been reached as to the pathogenicity of this virus in its natural host(117). Marburg and Marburg-like viruses have been isolated from the Egyptian rousette fruit bat, in the sole natural infection recorded there were no pathological changes(118–120). There are further studies involving experimental infection with Marburg and Ebola virus in bats, see proceeding section.

Coronavirus infection of mammals causes upper and lower respiratory and gastrointestinal disease or inflammation and necrosis in various organs(121). Disease in humans associated with host adapted coronavirus infection is restricted to the upper respiratory tract(122). These viruses replicate within the nasal epithelium with mucus production and a large influx of polymorphonuclear cells in the absence of necrosis(123). Cases of naturally acquired Myotis lucifugus coronavirus were associated with minimal lesions characterized by vacuolation, attenuation, and sloughing of the bronchiolar epithelium. These cells stained positive for the

Myotis lucifugus coronavirus nucleocapsid using immunohistochemistry. The upper respiratory tract was not assessed in these bats(124). Occasionally neuroinvasion is present in patients with comorbidities and these viruses can cause persistent infection in neuronal cells lines(122). Persistent infection with coronavirus is also observed in little brown bats and a big brown bat cell line(124,125).

Members of Adenoviridae cause asymptomatic infections. When infections progress to clinical disease they manifest as respiratory disease. Less commonly they can present as inflammation of eyelids or intestines, and with rare virulent types liver or hemorrhagic disease(126). Adenoviruses have been isolated from bats with lung lesions but the viral nucleic acids localized to other organs: kidney, intestine, and liver(127). Many adenoviruses have been isolated from bats, but disease associated with infection or pathogenesis of these viruses have not been assessed in their natural hosts. Most isolations are from apparently healthy bats involving 9 different bat species(62,128–135) and some from deceased bats, 2 additional species(136,137).

Viruses of the Hepadnaviridae family are associated with inflammation of the liver with scarring and neoplastic transformation in some cases(138). Round leaf bat, horseshoe bat, and tent-making horseshoe bat viruses were isolated from their namesakes and viral DNA was demonstrated in liver. These infections were associated with mild liver inflammation(139). Other hepadnaviruses have been isolated but no further investigation has been performed(140).

Flaviviruses have been associated with several diseases in bats. Hendra virus was isolated from aborted fetuses however the cause of abortion was thought to be related to trauma(141). Dengue virus has also been isolated from bats and associated with gliosis, alveolar septal thickening, with liver necrosis and lymphocytic infiltrate within the portal areas(142). Although a more extensive study found Dengue RNA in 6 bat species with no histopathological changes(143). Lastly molecular detection of Usutu virus was found in dead bats(144). For both Usutu virus and dengue virus there is additional experimental data, see next section. The diseases in bats are similar to what is observed in mammals infected with other members of Flaviviridae including abortion and encephalitis.

Mammalian orthoreoviruses have been isolated from bats associated with mortalities with interstitial pneumonia and a case of enteritis(145,146). Neurologic signs were also present in the Broome virus infected bats but they were attributed to a lyssavirus infection(145). In domestic

mammals orthoreovirus infections are primarily subclinical and cause respiratory or enteric disease(147).

Rodents have been identified as the primary reservoir hosts for numerous hantaviruses. Infection of the vampire bat by a hantavirus has been demonstrated with viral antigen present in the liver and heart but there is no description of histopathological changes(148).

Numerous viruses have been isolated from dead bats. Other than their association with these mortalities no additional information is available. The Kaeng Khoi virus has been isolated from wrinkle-lipped bats without any associated disease(149). However material from dead bats was infectious and caused encephalitis in mice, this was later identified as the Kaeng Khoi virus(150) Zwiesel bat banyangvirus and Issyk-Kul virus were isolated from moribund or dead bats(151,152).

2.4 Experimental infection in bats with viruses from various sources that have independently been isolated from bats

This next section reviews the experimental evidence of inoculation trials in bats. These viruses have all been isolated from bats, but the viruses used in these experiments have originated from multiple sources. These sources could include humans, other animals, and vectors. Very rarely have these viruses been isolated from bats and then used in experimental infections on bats. The following viruses have been isolated from bats and used in experimental infections in other bat species: rabies virus, Aravan virus, Irkut virus, Australian bat lyssavirus, West Caucasian bat virus, Yokose virus, Entebbe bat virus, Venezuelan equine encephalitis virus, Hendra virus and bat coronavirus field strain. The bat species from which the following viruses were isolated were the same as those used in experimental infections: rabies virus, European bat lyssavirus 1 and 2, Lagos bat virus, Montana myotis leukoencephalitis virus, Marburg virus, Influenza A H9N2 and Tacaribe virus.

Most experimental infections have been performed with rabies virus variants and it is beyond the scope of this paper to review them all. As previously discussed the pathogenesis of rabies virus may be different depending on the site of inoculation(65). This is supported by shedding of virus in saliva when bats are inoculated in the dermis but not in muscle(153). However inoculation of bats with other lyssaviruses via these routes resulted in outcomes that were not reproducible(81,154,155). The earliest work demonstrated that big and little brown bats were susceptible to dog and fox rabies through various routes of inoculation which resulted in clinical

disease with Negri bodies in all bats(156). More recent inoculation studies have not resulted in 100% mortality and they have concluded that homologous strains of bat rabies virus are more virulent than heterologous strains(153,157–161). Potentially this may be related to the ability of these viruses to disseminate within the CNS with more virulent strains infecting more neurons and regions of the brain in bats than their less virulent counterparts(162). The time for bats to develop clinical signs with rabies infection can be long up to 365 days and hibernation also prolongs this latency period(87,153).

Big brown bats inoculated with other rhabdoviruses European bat lyssavirus 1, Aravan, Khujand, and Irkut viruses have comparable mortality rates to rabies virus inoculations, 55-75%. All of these viruses elicited clinical disease in a subset of these bats with virus detected in the brain but no pathological examination was performed(154,163). European bat lyssavirus 1 in serotine bats and 2 in Daubenton's bats display variable mortality dependent on the inoculation route(155,164). When Australian bat lyssavirus and big brown bat rabies virus are inoculated into grey-headed flying foxes clinical signs and brain inflammation are observed in 3/10 and 2/4 bats respectively(165). Different strains of Lagos bat virus when inoculated intracerebrally or intramuscularly into straw-coloured fruit bats have variable mortality and induce inflammatory changes without Negri bodies(166,167). West Caucasian bat virus isolated from a Schreiber's bat resulted in mortality in 3 out of 15 big brown bats inoculated intramuscularly. These bats were not examined for lesions(168). There are many additional rhabdoviruses that have been isolated or detected with sequencing from bats but no further information is available on infection of their natural hosts(68,71,73,83,169–179).

The characteristics of long latent periods, variable mortality, and rabies virus neutralising antibodies are given as evidence of bats' unique relationship with these encephalitic viruses. However, these features are observed in other hosts. Variable mortality is seen with rabies virus inoculation in foxes mortality and is dependent on dose(180). A rabies isolate from skunks administered to racoons is avirulent but subsequently inoculating them with a fox variant resulted in high mortality(181). Mount Elgon bat virus, Bokeloh bat lyssavirus, rabies virus, European bat lyssavirus 1, and Lagos bat virus inoculated into mice resulted in mortality ranging from 20-80% similar to what is reported from these bat studies(182–185). Longer latency periods have been recorded in humans(186). Hibernation extends the latency period of other viruses in bats like Japanese encephalitis virus(153,187). Neutralising antibodies are not exclusive to bats and have

been recorded in other species(188). The meaning of these antibodies is also less clear as experimentally inoculated individuals may not develop detectable antibodies or they can produce high levels before succumbing to disease(153,189,190). Experimentation has also identified the possibility of antibody mediated enhancement of disease in inoculated bats(158).

In humans, infections with flaviviruses often cause subclinical viremic infections. When clinical signs are observed the symptoms are varied the most severe disease is hemorrhagic or neurologic in nature(191). These viruses are usually vector borne but there are also species with no identifiable vector(192). Japanese encephalitis virus has been isolated from various bat species(193,194). Inoculation of big brown, tricolored, little brown, and Mexican free-tailed bats with Japanese encephalitis virus resulted in viremia with no histological changes or disease(195–198). In a separate experiment with big brown and tricolored bats a similar outcome was observed but the duration of viremia was prolonged by hibernation(187). When a frugivorous bat, the black flying fox, was inoculated with Japanese encephalitis virus no clinical signs or viremia were observed but these bats could transmit virus to a mosquito vector(199). Pregnant Mexican free-tailed bats can transmit Japanese encephalitis virus to their fetuses and only rarely do they transmit another flavivirus St. Louis encephalitis(200). Mexican free-tailed bats are the only host from which St. Louis encephalitis virus has also been isolated(201,202). In the same studies as Japanese encephalitis virus, St. Louis encephalitis virus was also inoculated into little brown bats and Mexican free-tailed bats. Bats were never symptomatic but infectious virus was recovered and the possible natural host, Mexican free-tailed bats, were more susceptible to infection than little brown bats(195,197,198).

Dengue virus infection of *Artibeus* species was minimally pathogenic. Two species of bat that belong to the *Artibeus* genus were inoculated with dengue virus, the Jamaican fruit bat could not sustain viral replication and there were multiple hematomas in great fruit eating bats. Although tissue sections of skin were taken there was no microscopic description of the hematomas or comments on the presence or absence of vascular changes(203,204). In humans severe dengue virus is caused by increased vascular permeability and altered coagulation primarily driven by the immune response(205). Mortalities were observed with intracerebral dengue virus inoculation in little brown bats(206). Intracerebral inoculation of the Montana Myotis leukoencephalitis virus isolated from little brown bats resulted in mortalities in big and little brown bats(207).

West Nile virus has also been isolated from Leschenault's rousette, a fruit bat(208). Inoculation of the straw-coloured fruit bat with a human isolate resulted in no detectable disease(209). When this isolate was given to little brown bats neurologic signs were observed by all routes of administration(210). Two other insectivorous North American bat species the big brown and Mexican free-tailed bat inoculated with a crow isolate did not develop clinical signs and viremia was present only in big brown bats(211). The following experimental infections with flaviviruses did not result in any detectable disease: Yokose virus in Leschenault's rousette bat(212); Entebbe bat and Usutu viruses in the Egyptian rousette(209). Yokose virus has been isolated from Daubenton's bat(213) and Entebbe bat virus from the little free-tailed bat(214,215). The results from experimental infections with Kyasanur Forest virus were variable. Leschenault's rousette and the rufous horseshoe bat were viremic, this virus has been isolated from the latter bat(216–218); whereas, greater short-nosed fruit bats became moribund and died(219).

Venezuelan equine encephalitis and Sindbis virus have been isolated from bats but these viruses are maintained in rodents or birds respectively and are transmitted by mosquitoes(220–223). Infection is characterized by inflammation of the CNS in mice and joint inflammation in humans(223,224). Inoculation of 3 bat species with 4 different strains of Venezuelan equine encephalitis virus resulted in variable viremia and two abortions in late term pregnancies. The variability in the response between bat species led the authors to conclude that generalizations about the host response to viral infection at the genus level were not possible(225,226). When Sindbis virus was inoculated into little brown bats by varying routes of infection they all displayed nervous clinical signs but there was no further examination of tissues(227).

Illness caused by Paramyxoviruses in mammals can be restricted to the respiratory tract like bovine parainfluenza 3 or illness can be severe and systemic sometimes involving the CNS like canine distemper virus, New Castle disease or avian paramyxovirus type 1, and rinderpest(228). In humans paramyxoviruses cause additional disease glandular swelling-mumps, and rashes-measles(229). Henipavirus belong to this family and the search for these viruses in bats has gained in intensity because these viruses have caused several epidemics in humans and domestic animals(230). Pathological changes associated with henipavirus infections in mammals is characterized by inflammation of blood vessels and cellular damage in multiple organs(231). Nipah viruses have been isolated from the urine of various fruit bats, however the only information we have on lesions in bats is through experimental infection(141,232). In experimental infections

of the black-flying fox and the large flying fox with Nipah virus there was either no observed or minimal lesions were potentially associated with infection. The lesions were characterized by inflammation of a vessel wall in the submucosa of the intestine and trigeminal ganglia in separate bats(233).

Hendra virus inoculation resulted in minimal pathological changes with necrosis of the adrenal gland in one of 22 grey-headed flying foxes(234) or no pathological changes in juvenile black flying foxes(235). In separate experiments with grey-headed flying foxes infections were asymptomatic with degenerative changes in the vasculature and virus was isolated from fetal tissue(236,237). Additional paramyxoviruses have been isolated with bats for which we have no information on the pathogenesis(238,239,239–248).

Alpha and betacoronaviruses have primarily been studied in bats through experimental infection. Inoculation experiments in fruit bats were performed with field strains from intestinal homogenates because no bat coronaviruses were isolated. Infection resulted in no clinically apparent disease and transmission failed in several of the bats. Failure of transmission is likely due to species specificity of coronavirus replication where natural host-virus systems are required for viral replication in bats(249,250). A Middle East respiratory syndrome related virus was isolated from the lesser bamboo bat but no further information on the pathogenesis of this virus is available(251). Additional experimental infections in bats with coronavirus strains from other mammalian orders is found in the next section.

Filoviridae is a family of viruses that cause hemorrhagic fevers in humans which are often fatal(113). Other than the Lloviu virus the reservoir hosts for these viruses have not been definitively determined. There is evidence that bats and primates could be reservoir hosts however, primates develop disease similar to humans suggesting they are not the maintenance hosts (252–256). In non-human primate models filovirus exposure occurs through breaks in the skin and mucosa, although many cell types can be infected macrophages and dendritic cells initially produce virus and disseminate it to other organs. There is necrosis in the liver and adrenal gland which could predispose to hemorrhage with impaired hormone and clotting factor production. Additional cytokines produced by macrophages as well as tissue factor expression likely also contribute to activation and consumption of clotting factors. This culminates in altered blood pressure, vascular permeability, and impaired clotting resulting in hemorrhage and death(257). Experimental inoculation of Marburg virus into the Egyptian rousette bat did not result in

significant disease but there were inflammatory and necrotic areas scattered throughout the livers(258,259). Microscopic evaluation of tissues was not performed in other experimental infections(260). Infectious virus could be recovered from the infected bats and they are refractory to viral shedding with second viral challenges demonstrating an adaptive immune response(259,261,262).

Orthomyxoviridae encompasses the influenza viruses which cause respiratory illness in humans(263). Very few have been isolated from bats and there is sparse information on their pathogenesis in this mammalian order(264). Egyptian fruit bats inoculated with H9N2 influenza A virus isolated from the same species demonstrated minimal to mild inflammation in the nose but similar changes were observed in co-housed controls(265). Seba's short-tailed bats inoculated with the recombinant H18N11 virus developed cell death within the nose 4 days after infection and diarrhea was observed 9-21 days after infection without any histological changes in the intestines(266). This recombinant virus was made from bat influenza genomic sequencing (267–269). Infection of Jamaican fruit bats with this virus resulted in no pathological changes in the tissues examined but naïve co-housed bats developed nasal and ocular discharge and virus was isolated from rectal swabs(270). Overall bats develop mild respiratory and gastrointestinal signs associated with infection of influenza A viruses.

Viruses in the Arenaviridae family have rodents as reservoir hosts and cause hemorrhagic fevers in humans. There has been some suggestion that bats have been infected with Tacaribe virus(271). Experimental inoculation of Jamaican fruit bats with a viral isolate from the great fruit-eating bat resulted in viral clearance at the low dose and morbidity and mortality with high doses(272,273). The lesions consisted of cell death in multiple organs and an inflammatory infiltrate in the lungs or brain. Transmission to uninfected animals did not occur suggesting these bats are dead end hosts(273).

Peribunyaviruses are usually maintained in a vertebrate arthropod cycle(274). Experimental infection with Nepuyo virus in Jamaican fruit bats did not result in any apparent disease(275). Several members of the Phenuiviridae family are associated with fevers and encephalitis. Rift valley fever experimental inoculation of Schreiber's bat and the cape serotine bat resulted in no clinical disease(276).

2.5 Experimental infection of bats with viruses sourced from non-bat species

Infection of the Egyptian rousette bat with recombinant paramyxovirus Sosuga isolated originally isolated from a bat biologist did not result in any clinical disease however there were microscopic changes in multiple organs. There were mild changes in the intestines in 83% and in salivary glands in 50% of challenged bats. In 1 of the 12 bats there was severe inflammation in the salivary gland(277). A separate study had consistent findings with erosions in the small intestine and inflammation within the salivary glands with epithelial changes. Given the lack of inflammation associated with erosive changes the intestine the authors posited that this was evidence of tolerance to the paramyxovirus in this location(278). Similar but more severe lesions, ulcers of the oropharynx, were recorded in the human case(279). When bats were experimentally inoculated with New Castle disease virus a non-bat paramyxovirus they exhibited illness with 1 of 5 strains administered. Little brown bats developed characteristic neurologic signs as seen in other species; whereas, big brown became obtunded(280–282).

Lesions from Jamaican fruit bats inoculated with MERS were infrequent and mild characterized by rhinitis and multifocal interstitial pneumonia(283). SARS-CoV-2 challenge in fruit bats resulted in rhinitis with viral antigen staining in the debris and a slight increase of alveolar macrophages(284). Big brown bats and Mexican free-tailed bats challenged with SARS-CoV-2 were not infected or could be infected but shed variable amounts of virus without disease. There were no histopathological findings or results were inconclusive in the Mexican free-tailed studies(285–287). When bats are infected with an avian coronavirus they exhibited respiratory signs and mortality was observed(288). Localization of SARS-CoV-2 is similar to humans involving the upper and lower respiratory tracts(289,290). Severe outcomes in people infected with these highly pathogenic coronaviruses are associated with additional comorbidities. Acutely in the lungs there is pneumocyte death with syncytia formation, inflammation, vascular thrombosis, fibrin, and edema in the alveoli with progression to fibrosis. Numerous extrapulmonary organs may also be affected(291). More extensive experimental evidence or examination of naturally acquired cases is required in bats to exclude the possibility of severe disease in this order.

In humans, infections with viruses of Togaviridae results in encephalitis, fever, rashes, or joint pain and swelling(292). Semliki Forest virus has no defined host but is transmitted by mosquitoes and affects mammals and humans(293). Little brown bats displayed neurologic signs associated with inoculation of Semliki Forest virus through various routes(294). The pathogenesis

of Chikungunya virus is similar to Sindbis virus although reservoir host(s) have not been identified(295). No symptoms were observed with chikungunya inoculation of the straw-colored fruit bat, big brown bat, and Leschenault's rousette but the latter two developed variable viremias(296,297). Inoculation of big brown, little brown, Keen's myotis, and tricolored bats with Eastern equine encephalitis virus resulted in no detectable disease(298).

There is experimental evidence for infection of bats with viruses from Picornaviridae which encompasses viruses that infect the digestive system, liver, and upper respiratory tract(299). Although many have been sequenced from bats there is nothing in the literature regarding isolation from or infection in bats(16,17,300–305). The human Coxsackie B-3 and Lansing strain poliomyelitis viruses were inoculated into bats. Coxsackie virus was recovered from tissues and mortality was observed in big brown bats with inflammation of the meninges and brain(306). Poliomyelitis virus when inoculated into little brown bats resulted in death in a route dependent manner and infectious virus could be passaged into mice whereas passage of contagious material from inoculated big brown bats was not possible(307,308).

Alphaherpesviruses are associated with vesicles, ulcers, and erosions of the epithelium, rashes, and encephalitis. When little brown bats were inoculated with the human herpesvirus simplex virus 5/5 developed neurologic signs for intranasal and intracerebral inoculation and 3/5 for intratesticular(309). A similar alphaherpesvirus B virus now known as *Cercopithecine herpesvirus* 1 produced neurologic symptoms in little brown bats when inoculated intracerebrally(310). Suid alphaherpesvirus 1 inoculated into big brown bats through multiple routes resulted in neurologic signs in all bats(311). Most herpesviruses are host restricted but severe disease arises with cross species transmission as was seen in these bats.

Filoviruses cause hemorrhagic fevers in humans and non-human primates. Ebola virus is produced at high levels in the blood following inoculation in little free-tailed bats with no pathological changes(312). Various species of *Ebolavirus* were found to produce small areas of inflammation throughout the livers of Egyptian rousette bats but no infectious virus was recovered(259,313).

Orthobunyaviruses cause abortion, congenital malformations, encephalitis, and hemorrhagic fever in humans and domestic animals(314). Bwamba and Bunyamwera viruses inoculated into little brown bats produced neurologic signs by multiple routes of inoculation which was only observed in a few bats for the latter(294,315).

Five flaviviruses from non-bat sources have been experimentally inoculated into bats: Zika, yellow fever, Ntaya, and tick-borne encephalitis. Zika virus has been inoculated experimentally in multiple different bat species from the following genera *Artibeus, Mops, Rousettus, Lissonycteris*, and *Myotis* with outcomes varying from no clinical disease with little infectious virus to fatal neurologic disease(315,316). Unfortunately, the pathological changes in the clinical cases were not recorded. Viremia without clinical disease was detected with yellow fever inoculation of *Eidolon, Rousettus, Mops, Carollia, Phyllostomus, Eumops, Molossus* species and Ntaya virus inoculation of *Eidolon* and *Mops*(209,317). A separate experiment using mosquitoes to inoculate yellow fever virus in vampire, black mastiff, and velvety free-tailed bats failed to detect any viral infection(318). Infection with tick-borne encephalitis resulted in mortalities for *Myotis, Barabstella,* and *Plecotus* species but the cause of the mortalities was unclear and possibly related to husbandry (319).

2.6 Virus isolation or genomic sequencing of viruses in bats

This section outlines the evidence for viruses that have been isolated from bats without examination of bats for disease. Additionally, we include evidence of viral infections in bats from genomic sequencing studies.

Additional RNA viruses have been isolated from bats. These viruses belong to the following families or orders: Paramyxoviridae, Rhabdoviridae, Arenaviridae, Peribunyaviridae, Phenuiviridae, Nairoviridae, Coronaviridae, Flaviviridae, Togaviridae, and Reovirales, see Table 2.1 for details. While there is only genomic sequencing evidence for Bornaviridae and Kolmioviridae(300,320). Several DNA viruses have also been isolated from bats without further characterization belonging to the following families: Adenoviridae, Herpesviridae, and Circoviridae, see Table 2.1. Using metagenomic tools additional viruses have been identified by sequencing in bats. We have no isolates from the following viral families despite their identification by sequence in bats: Astroviridae, Parvoviridae, Picornaviridae, Polyomaviridae, Retroviridae, Genomoviridae, Smacoviridae, Asfarviridae, Anelloviridae, Picobirnaviridae, Birnaviridae, Hepeviridae, Matonaviridae, Tobaniviridae, and Caliciviridae(17,18,94,110,172,300–302,321–331). The following viruses have been reported as isolated from bats but the primary literature source was not available for review to determine if the bats were examined or if there were any pathological changes associated with infection: Duvenhage, Carey Island, Central European encephalitis 1, Jugra, Saboya, Uganda S, Gossas,

Yogue, Catu, Guama, Juruaca, Agua Preta, and Parixa(36). Additional information on members of this list that are possibly arboviruses can be found in the CDC's arbovirus catalog(332).

2.7 Discussion

Bats develop disease associated with natural and experimental viral infections. For infections with poxviruses, rhabdoviruses, and a papillomavirus disease is typical of that found in other species. For other viruses like the flaviviruses the association with the reported lesion or mortality is less clear. Virus isolation was more common from dead or moribund bats; however the cause of mortality may be unrelated to viral infection. We speculate that viral isolation is more successful from these cases due to greater viral production in ill or stressed bats. Since many of the natural infections only demonstrate an association with disease additional experimentation is required to determine the role of these viruses in the disease. However, this is challenging for wildlife species with low reproductive potential like bats.

The experimental inoculations of bats are usually with viruses from other mammalian hosts or vectors. Even though there are a number of viruses that have been isolated from bats isolates from non-bat hosts are used. Additionally, the bat host used in these challenges are not usually of the same species or genus as the suspected maintenance host. By using non-host adapted isolates the outcomes of these experiments may not represent actual disease in their natural hosts. Rabies virus is an excellent example of this where hosts are most susceptible to their respective strains and there is marked difference in the virulence of isolates as well. The opposite pattern is sometimes observed with cross species transmission where profound disease can be caused by a virus which was asymptomatic in the maintenance host. The severe outcomes from cross species experimental infections were reported from the experiments performed in the 1950s and 60s, see Table 2.1. More recent experimental infections do not reproduce these diseases in bats however they are using different hosts or viruses. For confirmation of bat-virus tolerance or resistance natural virus-host relationships should be used(333,334). Outcomes of experimental infections are also difficult to compare with different routes of exposure, doses used, viral isolates administered, and passaging of these viruses in cell cultures and laboratory animals. The latter is particularly true for the RNA viruses where the high rate of mutation can result in passaged virus with different properties from the wild type strain(270,335).

Detection of viral nucleic acids is not the same as detecting infectious virus and inferring bats are hosts for these viruses cannot be drawn from this evidence alone. This is an excellent

initial starting point, but it is only a first step in determining the function of bats in the maintenance and transmission of these viruses. Several of the metagenomic techniques also identify viral sequences from the ingesta of bats, which may represent viruses of the bat prey rather than the bat itself. Therefore caution is warranted in interpreting these results from hematophagous or insectivorous species(16,18).

Frequently with experimental infections no clinical signs were observed but pathological changes were present. Clinical signs are an insensitive indicator of disease in bats. Therefore, future studies should prioritize histopathological evaluation. Pathological changes with viral infections may not appear grossly and even the histopathological changes can be subtle. Health and disease are a continuum and severe disease is more often found in hosts that are stressed and this may not be easily recreated in a laboratory setting.

To circumvent the issues around experimental studies and extrapolation of their findings examining disease associated with natural infections in wild bats is a viable option. This is also not without its challenges of monitoring for diseases in wild bats: some are solitary, they are active at night, have roosts in inaccessible areas, some species are small making identification of ill or dead bats difficult, and dead or incapacitated bats are often rapidly scavenged. Furthermore, the smaller the body size the faster the rate of decomposition which usually affects the evaluation of pathological changes and virus isolation(336,337).

There is a lot to be learned about the relationship bats have with their viruses and there is not currently enough information to conclude that they are tolerant or resistant to disease associated with infection.

Table 2.1 Diseases associated with viral infection in bats.

		Natural cases	
Virus	Host	Symptoms/Lesion	Reference
Eptesipox virus	*Eptesicus fuscus*	Synovial hyperplasia, fibrinosuppurative and necrotizing tenosynovitis and osteomyelitis, oropharyngeal ulcers	(56,57)
Hypsugopox virus	*Hypsugo savii*	ND[a]	(58)
Israeli Rousettus aegyptiacus pox virus	*Egyptian fruit bat*	Moribund bats with skin nodules, lingual and epidermal ulcers	(59,60)
Unnamed poxvirus	*Miniopterus schreibersii bassanii*	Skin nodule	(61)
Australian bat lyssavirus[c]	*Pteropus alecto*	Abnormal behaviour and non-suppurative meningoencephalitis with inclusion bodies	(74,338)
Taiwan bat lyssavirus 1	*Pipistrellus abramus*	Mortality, Negri bodies, non-suppurative sialoadenitis	(73,75)
Taiwan bat lyssavirus 2	*Nyctalus plancyi velutinus*	Mortality, non-suppurative encephalitis, sialoadenitis, Negri bodies	(75)
Gannowura bat lyssavirus	*Pteropus medius*	Mortality and neurologic signs, mild non-suppurative meningoencephalomyelitis	(76)
Rabies virus[c]	*Eptesicus fuscus, furinalis Artibeus lituratus, Myotis nigricans, austroripaurius, grisescens Lasiurus blossevillii, ega, borealis, seminolus, and intermedius Desmodus rotundus, Tadarida brasiliensis, Antrozous pallidus, Molossus molossus, Pipistrellus subflavus, Nycticeius humerali*	Abnormal behaviour, mortality, no lesions to variable degree of non-suppurative meningoencephalitis with Negri bodies	(77–79,86–88), unpublished data
Lleida bat lyssavirus	*Miniopterus schreibersii*	Mortality	(67)
Kotalahti bat lyssavirus	*Myotis brandtii*	Mortality	(68)
Lagos bat virus[c]	*Eidolon helvum Epomorphous wahlbergi*	Mortality, abnormal behaviour	(69,72)
Shimoni bat virus	*Hipposideros commersoni*	Mortality	(71)
Irkut virus [c]	*Murina leucogaster*	Mortality	(70)
European bat lyssavirus 1[c]	*Eptesicus serotinus*	Neurologic signs	(80)

European bat lyssavirus 2 [c]	*Myotis daubentonii*	Abnormal behaviour	(81,82)
Bokeloh bat virus	*Myotis nattereri*	Moribund, paralysis	(83,84)
Eptesicus fuscus gammaherpesvirus	*Eptesicus fuscus*	ND	(97)
Unnamed gammaherpesvirus	*Pteropus vampyrus*	Lymphoplasmacytic blepharitis, meibomianitis, and myositis	(98)
Bat gammaherpesvirus 8	*Myotis velifer incautus* cell line	Intrascapular mass	(99)
Unnamed betaherpesvirus	*Myotis lucifugus*	Salivary gland virions, cytomegaly	(106)
Rousettus aegyptiacus papillomavirus 1	*Rousettus aegyptiacus*	Basosquamous carcinoma	(49)
Lloviu virus	*Miniopterus schreibersii*	Mortality, interstitial pneumonia, hemoptysis/epistaxis	(116,117,339)
Myotis lucifugus coronavirus	*Myotis lucifugus*	Mild attenuation sloughing bronchiolar epithelium	(124)
Bat adenovirus 2	*Pipistrellus pipistrellus*	Moribund or dead, mild inflammation in lung	(127,137)
Bart adenovirus 250-A	*Corynorhinus rafinesquii*	Mortality	(136)
Roundleaf bat, horseshoe bat, and tent-making bat Hepatitis B viruses	*Hipposideros cf ruber, Uroderma bilobatum, Rhinolophus alcyone*	Mild inflammation in the liver	(139)
Hendra[c]	*Pteropus poliocephalus*, *Pteropus alecto*	Trauma, isolate from aborted fetal tissue	(141)
Usutu virus[c]	*Pipistrellus pipistrellus*	Mortality	(144)
Dengue virus[c]	*Carollia perspicillata, Phyllostomus discolor*	Gliosis, BALT hyperplasia, alveolar septa thickening, lymphocytic portal hepatitis, liver necrosis	(142)
Mammalian orthoreoviruses	*Plecotus25uratuss, Myotis mystacinus, Pipistrellus pipistrellus, nathusii, and kuhlii, Nyctalus noctula*	Interstitial pneumonia, enteritis	(146)
Broome virus	*Pteropus scapulatus*	Neurologic signs Australian bat lyssavirus isolated	(145)
Hantavirus	*Desmodus rotundus*	ND	(148)
Kaeng Khoi virus	*Chaerephon plicata*	Mortality	(150)

Zwiesel bat banyangvirus	*Eptesicus nilssonii*	Moribund or mortality	(151)
Issyk-Kul virus	*Eptesicus nilssonii* *Nyctalus noctula*	Moribund or mortality	(152)
Experimental infection in bats with viruses from various sources that have independently been isolated from bats[d]			
Rabies virus	*Eptesicus fuscus and furinalis Artibeus lituratus, Myotis nigricans, austroripaurius, and grisescens Lasiurus blossevillii, ega, borealis, seminolus, and intermedius Desmodus rotundus, Tadarida brasiliensis Antrozous pallidus, Molossus molossus, Pipistrellus subflavus, Nycticeius humeralis*	Abnormal behaviour, mortality, no lesions to variable degree of non-suppurative meningoencephalitis with Negri bodies	Isolation (77–79,86,88)
	Desmodus rotundus, Eptesicus fuscus, Myotis lucifugus, Lasiurus noctivagans, Artibeus intermedius, Artibeus jamaicensis	Variable neurologic signs, mortality, Negri bodies	Experimentation M.l. fox, dog isolates (156) D.r. homologous isolate (157) L.n. homologous or Eptesicus fuscus 2 and Myotis lucifugus 1 isolates (153) M.l. homologous or Eptesicus fuscus 2 and Lasiurus noctivagans 1 isolates (158) D.r. homologous isolate (159) E.f. homologous isolate (160) A.i. heterologous Desmodus rotundus isolate (161) A.j. laboratory isolate CVS-24 (162)
Aravan virus	*Myotis blythi*	NE[b]	Isolation (340)
	Eptesicus fuscus	Abnormal behaviour, moribund, mortality	Experimentation E.f. Myotis blythi isolate (163)
Khujand virus	*Myotis mystacinus*	NE	Isolation (340)
	Eptesicus fuscus	Abnormal behaviour, moribund, mortality	Experimentation E.f. Myotis mystacinus isolate (163)
Irkut virus	*Murina leucogaster*	Mortality	Isolation (70)
	Eptesicus fuscus	Abnormal behaviour, moribund, mortality	Experimentation E.f. Murina leucogaster isolate (163)
European bat lyssavirus 1	*Eptesicus serotinus*	Neurologic signs	Isolation (80,85)
	Eptesicus fuscus *Eptesicus serotinus*	Variable mortality, paralysis	Experimentation E.f. serotine bat isolate (154) E.s. serotine bat isolate (164)
European bat lyssavirus 2	*Myotis daubentonii*	Abnormal behaviour	Isolation (81,82)
	Myotis daubentonii	Variable neurologic signs	Experimentation M.d. homologous isolate (155)

Australian bat lyssavirus	*Pteropus alecto*	Abnormal behaviour and non-suppurative meningoencephalitis with inclusion bodies	Isolation (74,338)
	Pteropus poliocephalus	Variable abnormal behaviour, moribund, paralysis, nonsuppurative meningoencephalomyelitis and ganglioneuritis	Experimentation P.p. homologous isolate and Eptesicus fuscus rabies virus (165)
Lagos bat virus	*Eidolon helvum*	Mortality, NE	Isolation (69,72,341)
	Eidolon helvum	Neurologic signs, lymphocytic meningoencephalitis	Experimentation E.h. 3 homologous (1956 Lagos, Nigeria, 1985 Dakar, Senegal, and 2015 Kumasi, Ghana) isolates (166) isolation and experimentation E.h. homologous 2015 Kumasi, Ghana isolate (167)
West Caucasian bat virus	*Miniopterus schreibersii*	NE	Isolation (70)
	Eptesicus fuscus	Moribund, mortality	Experimentation E.f. Miniopterus schreibersii isolate (168)
Japanese encephalitis virus	*Miniopterus schreibersii* and *fuliginosus*, *Rhinolophus cornutus cornutus*, and group B viruses from *Myotis macrodactylus*, *Vespertilio superans*	NE	Isolation (193)
	Rousettus leschenaulti, *Murina aurata*	NE	Isolation (194)
	Eptesicus fuscus, *Pipistrellus subflavus*, *Myotis lucifugus*, *Tadarida brasiliensis*, *Pteropus alecto*	ND, viremia	Experimentation E.f. and P.s. mosquito isolate (187) T.b. human Nakayama and mosquito OCT-541 isolates (195) T.b. and M.l. human Nakayama isolate E.f. mosquito OCT-541 isolate (197) E.f. mosquito OCT-541 isolate (196) T.b. and M.l. human Nakayama isolate (198) P.a. mosquito TS3306 isolate (199)
St. Louis encephalitis virus	*Tadarida brasiliensis*	NE	Isolation (201,202)
	Tadarida brasiliensis, *Myotis lucifugus*	ND, viremia	Experimentation T.b. and M.l. human Hubbard, mosquito 57-5MB4, flicker bird 55MB7 isolates (195) T.b. human Hubbard isolate (197) T.b. Human Hubbard isolate (198)
West Nile virus	*Rousettus leschenaulti*	NE	Isolation (208)

	Eptesicus fuscus, Tadarida brasiliensis	ND, E.F. viremia	Experimentation E.f. and T.b. crow New York 99 isolate (211)
	Myotis lucifugus	Neurologic signs	E.f. human B956 isolate (210)
	Eidolon helvum	ND	Experimentation E.h. human B956 isolate (209)
Yokose virus	*Myotis daubertonii*	NE	Isolation (213)
	Rousettus leschenaulti	ND, limited viral replication	Experimentation R.l. Myotis fuliginosus Oita-36 strain isolate (212)
Kyasanur Forest virus	*Rhinolophus rouxi*	NE	Isolation (218)
	Cynopterus sphinx	Moribund, mortality	Experimentation C.s human P9605 isolate (219)
	Rousettus leschenaulti	ND, variable viremia	Experimentation R.l. human P9605 isolate (216)
	Rhinolophus rouxi	Viremia	Experimentation R.r. tick isolate (217)
Entebbe bat virus	*Chaerephon pumilus*	NE	Isolation (214,215)
	Rousettus aegyptiacus	ND	Experimentation R.a. Chaerephon pumilus isolate (209)
Usutu virus	*Rousettus aegyptiacus*	ND	Experimentation (209)
Dengue virus	*Artibeus jamaicensis*	Cannot sustain viral replication	Experimentation A.j. type 1 human Hawaii and type 4 H241 human tissue culture adapted isolates (204)
	Artibeus intermedius	Hematomas	Experimentation A.i. type 2 human New-Guinea C isolate (203)
	Myotis lucifugus	Moribund and mortalities with intracerebral inoculation	Experimentation M.l. human Hawaii isolate (156)
Montana myotis leukoencephalitis virus	*Myotis lucifugus*	ND isolation	Isolation and experimentation M.l. and E.f. Myotis lucifugus isolate (207)
	Eptesicus fuscus, Myotis lucifugus	Mortality	
Venezuelan equine encephalitis virus	*Desmodus rotundus, Uroderma bilobatum*	NE	Isolation (221,222)
	Artibeus jamaicensis, Artibeus lituratus, Phyllostomus discolor, Sturnira lilium, Carollia subrufa	Viremia, abortion	Experimentation A.j., A.l., and P.d. mosquito 63A216, Uroderma bilobatum 71YE, human 69Z1, and horse 69T1501 isolates C.s. and S.l. mosquito 63A216 isolate (225,226)
Sindbis virus	*Hipposideridae and Rhinolophidae species*	ND	Isolation (342)

	Myotis lucifugus	Neurologic signs	Experimentation M.l. mosquito isolate (227)
	Eidolon helvum	ND, rare viremia	Experimentation E.h. human K163/61 isolate (209)
Hendra virus	*Pteropus poliocephalus Pteropus alecto*	Aborted fetal tissue	Isolation (141)
	Pteropus alecto	Rare adrenal necrosis	Experimentation P.a. Pteropus poliocephalus isolate (234)
	Pteropus alecto	Self-limiting infection	Experimentation P.a. equine GenBank HM044317 isolate (235)
	Pteropus poliocephalus	Variable lymphocytic vasculitis or fibrinoid degeneration of vessels	Experimentation P.p. source not specified (237)
	Pteropus poliocephalus	Variable fibrinoid degeneration and nonsuppurative perivascular cuffing	Experimentation P.p. source not specified (236)
Nipah virus	*Pteropus lylei*, island flying foxes, *Pteropus vampyrus*	NE	Isolation(232,343,344)
	Pteropus vampyrus	ND	Experimentation P.v. human isolate (234)
	Pteropus poliocephalus	ND	Experimentation P.p. human isolate (233)
Bat coronaviruses field strains	*Rousettus leschenaulti*	ND	Experimentation R.l. Cynopteris brachyiotis intestine (249)
SARS-like coronavirus	*Rousettus aegyptiacus*	ND	Experimentation R.a. WIV-CoV clone (250)
Marburg or Marburg like viruses	*Hipposideros species, Rousettus aegyptiacus*	NE	Isolation (118,119,259,345)
Marburg virus	*Rousettus aegyptiacus*	ND, viremia	Experimentation R.a. homologous 371bat isolate (Amman et al. 2015; Schuh, Amman, Sealy, et al. 2017; Schuh, Amman, Jones, et al. 2017) R.a. human Hogan isolate (346)
	Rousettus aegyptiacus	Liver mild multifocal lymphohistiocytic inflammation and necrosis	Experimentation R.a. human SPU 148/99/1 isolate (347)
	Rousettus aegyptiacus	Liver mild multifocal mixed inflammation	Experimentation R.a. homologous 371bat isolate (258,259)
	Rousettus aegyptiacus	ND	Experimentation R.a. homologous 371bat isolate (260)
Influenza A H9N2	*Rousettus aegyptiacus*	NE	Isolation (264)
	Rousettus aegyptiacus	Bat origin minimal to mild rhinitis, avian origin ND	Experimentation R.a. H9N2 homologous A/bat/Egypt/381OP/2017 and A/layer

			chicken/Bangladesh/VP02-plaque/2016 isolates (265)
Influenza A H18N11	*Artibeus planirostris*	NE	Recombinant construction (267,269)
	Carollia perspicillata,	Mild necrotizing rhinitis, diarrhea	Experimentation recombinant H18N11 A/flat-faced bat/Peru/033/2010 isolate (266)
	Artibeus jamaicensis	Oculonasal discharge	Experimentation A.j. recombinant H18N11 A/flat-faced bat/Peru/033/2010 isolate (270)
Tacaribe virus	*Artibeus species*	NE	Isolation (272)
	Artibeus jamaicensis	Interstitial pneumonia, meningitis, gliosis or necrosis in various organs	Experimentation A.j. Artibeus bat TRVL-11573 isolate (273)
Rift valley fever virus	*Micropterus pusillus, Hipposideros abae*	NE	Isolation (348)
	Miniopterus schreibersii, Laephotis capensis	ND	Experimentation isolate not specified (276)
Nepuyo virus	*Artibeus jamaicensis, Artibeus lituratus*	NE	Isolation (349)
	Artibeus jamaicensis	ND	Isolation and experimentation A.j. mosquito TRVL 1846 isolate (275)
Experimental infection of bats with viruses sourced from non-bat species			
Sosuga virus	*Rousettus aegyptiacus*	Mild intestinal erosions, sialoadenitis	(277,278)
New Castle disease virus	*Myotis lucifugus*	Neurologic signs	(280,280,350)
	Eptesicus fuscus	Variable mortality	(282)
MERS virus	*Artibeus jamaicensis*	Infrequent mild interstitial pneumonia, rhinitis	(283)
SARS-CoV-2	*Rousettus aegyptiacus*	Rhinitis, slightly increased alveolar macrophages	(284)
	Eptesicus fuscus	Cannot sustain viral replication	(285)
	Tadarida brasiliensis	ND	(286,287)
Infectious bronchitis virus	*Myotis lucifugus*	Variable mortality	(288)
Semliki Forest virus	*Myotis lucifugus*	Neurologic signs	(294)
	Eidolon helvum, Rousettus aegyptiacus, Mops condylurus	ND, viremia	(209)
Chikungunya virus	*Rousettus leschenaulti*	ND, infrequent viremia	(297)

	Eptesicus fuscus	ND, viremia	(296)
	Eidolon helvum	ND	(209)
Eastern equine encephalitis virus	*Myotis lucifugus*, *Eptesicus fuscus*, *Perimyotis subflavus*, *Myotis keeni*	ND, viremia	(298)
Coxsackie B-3 virus	*Myotis lucifugus*	Variable mortality, meningoencephalitis	(306)
Poliomyelitis virus	*Myotis lucifugus*	Variable mortality, neurologic signs	(307)
	Eptesicus fuscus	ND	(308)
Herpes simplex virus	*Myotis lucifugus*	Neurologic signs	(309)
Cercopithecine virus 1	*Myotis lucifugus*	Neurologic signs	(310)
Suid alphaherpesvirus 1	*Eptesicus fuscus*	Neurologic signs	(311)
Ebolaviruses	*Rousettus aegyptiacus*	ND	(259)
Zaire Ebolavirus	*Mops condylurus*, and *pumila*, *Epomorphous wahlbergi*	ND	(312)
Ebola virus	*Rousettus aegyptiacus*	ND, variable viremia, mild leukocytosis	(313)
Bwamba virus	*Myotis lucifugus*	Neurologic signs	(315)
Bunyamwera virus	*Myotis lucifugus*	Variable neurologic signs	(294)
	Eidolon helvum, Rousettus aegyptiacus, Mops condylurus	ND, viremia	(209)
Zika virus	*Artibeus jamaicensis*, *Mops condylurus*, *Rousettus aegyptiacus*, *Lissonycteris angolensis*	ND, variable viremia	(316,351)
	Myotis lucifugus	Neurologic signs	(315)
Yellow fever virus	*Eidolon helvum, Rousettus aegyptiacus, Mops condylurus*	ND, variable viremia	(209)
	Carollia perspicillata, Phyllostomus panamensis, Eumops californicus, Molossus species, *Molossus molossus* and *rufus*, *Desmodus rotundus*	ND	(317,318)
Ntaya virus	*Eidolon helvum Mops condylurus*	ND, M.c. minimal viremia	(209)

Tick-borne encephalitis	*Myotis majotis, Barbastella barbastellus, Plecotus auritus*	ND, Viremia	(319)
		Isolation only	
Mount Elgon bat virus	*Rhinolophus hildebrandtii eloguens*	NE	(352)
Matlo bat lyssavirus	*Miniopterus natalensis*	NE	(353)
Kolente virus	*Hipposideros jonesi*	NE	(176)
Fikirini rhabdovirus	*Macronycteris vittatus*	NE	(178)
Oita virus	*Rhinolophus cornutus*	NE	(354)
Kern Canyon virus	*Myotis yumanensis*	NE	(355)
Malsoor virus	*Rousettus species*	NE	(356)
Toscana virus	*Pipistrellus kuhli*	NE	(357)
Mojui dos campos	Bat	NE	(358)
Montana Myotis leukoencephalitis virus	*Myotis lucifugus*	NE	(207)
Rio bravo virus	*Molossus ater, Tadarida brasiliensis*	NE	(359,360)
Tamana bat virus	*Pteronotus parnellii*	NE	(359)
Phnom-Penh bat virus	*Cynopterus brachyotis angulatus*	NE	(361)
Dakar bat virus	*Mops condylurus*	NE	(362,363)
Bukalasa bat virus	*Chaerephon pumilus*	NE	(364)
Tonate virus	*Trachops cirrhosus*	NE	(220)
MERS-like virus	*Tylonycteris pachypus*	NE	(251)
SARS-like coronavirus WIV16	*Rhinolophus sinicus*	NE	(365)
Achimota virus 1 and 2	*Eidolon helvum*	NE	(238)
Achimota 3 virus	*Eidolon helvum*	NE	(239)
Teviot virus	Pteropid	NE	(245)
Alston virus	Pteropid	NE	(246)
Tioman virus	*Pteropus hypomelanus*	NE	(240)
Menangle virus	*Pteropus alecto*	NE	(247)
bat paramyxovirus B16-40	*Miniopterus schreibersii*	NE	(248)

Kanhgág virus	*Desmodus rotundus*	NE	(241)
Guató virus	*Carollia perspicillata*	NE	(241)
Cedar virus	Pteropid	NE	(242)
Parainfluenza type 2	*Rousettus leschenaulti*	NE	(244)
Mapuera virus	*Sturnira lilium*	NE	(366)
Taï Forest reovirus	*Chaereophon aloysiisabaudiae*	NE	(367)
Nelson Bay orthoreovirus	*Pteropus poliocephalus, Pteropus hypomelanus, Pteropus species, Acerodon celebensis*	NE	(368–370)
Pteropine orthoreoviruses	*Rousettus leschenaulti, Pteropus vampyrus, Lissonycteris angolensis, Eonycteris spelaea, Rousettus amplexicaudatus, Rousettus aegyptiacus, Cynopterus sphinx*	NE	(40,371–376)
North American mammalian orthoreoviruses	*Lasionycteris noctivagans, Eptesicus fuscus*	NE	(377)
Asian mammalian orthoreoviruses	*Hipposideros species, Myotis species*	NE	(378)
European mammalian orthoreoviruses	*Eptesicus serotinus, Myotis daubentonii, myotis, and emarginatus, Rhinolophus hipposideros*	NE	(379,380)
Rhinolophus pusillus mammalian orthoreovirus	*Rhinolophus pusillus*	NE	(381)
Chobar gorge virus	*Rousettus aegyptiacus, Nycteris nana*	NE	(382,383)
Group A rotavirus	*Rhinolophus hipposideros*	NE	(384)
Ife virus	*Eidolon helvum*	NE	(385)
Japanaut virus	*Syconycteris crassa*	NE	(386)
Heramatsu virus	*Myotis macrodactylus*	NE	(387,388)
Leopard Hills virus	*Hipposideros gigas*	NE	(389)
Kasokero	*Rousettus aegyptiacus*	NE	(390)

Ryukyu virus 1	*Pteropus dasymallus yayeyamae*	NE	(391)
Eidolon helvum adenovirus 1	*Eidolon helvum*	NE	(62)
Bat adenoviruses	*Rhinolophus pusillus, Rousettus aegyptiacus, Myotis macrodactylus, Vespertilio sinensis, Eidolon helvum, Rousettus leschenaulti, Miniopterus schreibersii, Rhinolophus sinicus, Rhinolophus cornutus*	NE	(128–131,133,134)
Circo-like virus	*Miniopterus schreibersii*	NE	(392)
Pteropus lylei alphaherpesvirus	*Pteropus lylei*	NE	(103)
Rhinolophus gammaherpesvirus 1	*Rhinolophus ferrumequinum*	NE	(100)
Alphaherpesvirus	*Eidolon dupreanum*	NE	(104)
Alphaherpesvirus	*Pteropus lylei*	ND	(104)
Fruit bat alphaherpesvirus 1	*Pteropus* species	ND	(102)
Bat betaherpesvirus 2	*Miniopterus fuliginosus*	ND	(393)
Miniopterus schreibersii betaherpesvirus	*Miniopterus schreibersii*	NE	(394)

[a]Not detected

[b]Not examined

-Information not specified

[c]Additional experimental infection, see "Experimental infection in bats with viruses from various sources that have independently been isolated from bats"

[d]These viruses have all been isolated from bats, but the viruses used in these experiments have originated from multiple sources.

SARS-CoV-2 severe acute respiratory syndrome coronavirus 2, MERS Middle East respiratory syndrome, BALT bronchus-associated lymphoid tissue

Preface to chapter 3

The literature review in Chapter 2 achieves our first objective and highlights how little information we have on the pathogenesis of these viruses in bats. To provide additional information on natural viral infections of bats we capitalized on the bat submissions to the Canadian Wildlife Health Cooperative. Bats are submitted for various reasons: human or domestic animal exposure and concern of rabies, illness, mortality, predation, or misadventure. During surveillance for virus-associated lesions in these bats changes were noted in the trachea of one of these submissions, fulfilling our second objective of detecting virus-associated disease. The ensuing investigation into a viral cause for these changes is covered in Chapter 3.

Although we could not determine the cause of the tracheal lesions, we isolated a previously described γHV from this bat. Further analysis demonstrated the virus remained dormant in lymphoid tissues consistent with other γHVs. There was also a unique feature of this virus infection in its host. We demonstrated viral nucleic acids in cells of the lung with qualities suggestive of pulmonary intravascular macrophages. This is a cell type that has not been reported to be involved with γHV infection and it is the first report of these cells in bat lungs. This fulfills our third objective of isolating and characterizing viruses in bat submissions.

CHAPTER 3 TISSUE AND CELLULAR TROPISM OF EPTESICUS FUSCUS GAMMAHERPESVIRUS IN BIG BROWN BATS, POTENTIAL ROLE OF PULMONARY INTRAVASCULAR MACROPHAGES

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3.1 Abstract

Gammaherpesviruses are recognized as important pathogens in humans but their relationship with other animal hosts, especially wildlife species, is less well characterized. Our objectives were to examine natural Eptesicus fuscus gammaherpesvirus (EfHV) infections in their host, the big brown bat (*Eptesicus fuscus*) and determine if infection is associated with disease. In tissue samples from 135 individual big brown bats, EfHV DNA was detected by polymerase chain reaction in 43 bats. Tissue slides from 59 of these cases, including 17 from bats with detectable EfHV genomes, were analyzed. An isolate of EfHV was obtained from one of the cases and electron micrographs and whole genome sequencing were used to confirm that this was a unique isolate of EfHV. While several bats exhibited various lesions, we did not establish EfHV infection as a cause. Latent infection, defined as RNAscope probe binding to viral latency-associated nuclear antigen (LANA) in the absence of viral envelope glycoprotein probe binding, was found within cells of the lymphoid tissues. These cells also had colocalization of the B cell receptor probe. Probe binding for both LANA and a viral glycoprotein, was observed within individual cells dispersed throughout the alveolar capillaries of the lung and had characteristics of pulmonary intravascular macrophages (PIMs). Cells with a similar distribution in bat lungs expressed MHC class II a marker for antigen presenting cells and the existence of PIMs in bats was confirmed with transmission electron microscopy. The importance of this cell type in gammaherpesvirus infections warrants further investigation.

3.2 Introduction

Gammaherpesviruses (γHVs) have the ability to establish life-long infections in immune cells and most host species examined are parasitized by one or more γHV(395). They are typically associated with disease when there is cross species transmission or the host is immunosuppressed(396,397). There are seven genera within this subfamily: *Rhadinovirus, Lymphocryptovirus, Percavirus, Patagivirus, Bossavirus, Manticavirus,* and *Macavirus* (398,399). Virus-host interaction for the human and mouse γHVs have been better characterized than those of other animals(397). For human and mouse members of Gammaherpesvirinae, infection is characterized by a two-stage life cycle divided into latent and lytic or productive infection. In latency the virus is found in a quiescent state where the genome is circularized, chromatinated, and tethered to the host DNA(400). During this latent state only a few genes that promote and maintain latency are expressed. One gene which is particularly important in

maintaining a latent state is the latency-associated nuclear antigen (LANA)(401). Multiple triggers can reactivate the virus from this state into lytic replication where infectious virus is produced and released from cells(402). During lytic infection of cells by herpesviruses viral gene expression occurs in an ordered manner and genes are categorized as Immediate Early (IE), Early (E), or Late (L) on their temporal order of expression. The expression of Late genes follows the initiation of viral genome replication and these genes code primarily for proteins that make up the virion(403). The different gene expression profiles in latent and lytic infection can be used to identify the state of the virus within a host.

The genomes of several γHVs have recently been detected in bats(91,92,94,98,101,321,404,405). Isolation of these viruses from bats is rare (to date only 2 γHVs have been isolated from bats species) and little is known about their pathogenesis(50,96,99,100). Diseases associated with γHV infection in humans include lymphoproliferations, autoimmune disorders, and neoplasms(95). In bats γHV infection has been associated with lesions but causation remains undetermined(50,98)**.** *Eptesicus fuscus gammaherpesvirus* (EfHV, also called *Vespertilionid gammaherpesvirus 3*), the sole member of the *Patagivirus* genus(96) can be detected in the blood of 20 - 80% of captive and free ranging bats(406) suggesting that there is a high prevalence of the virus in big brown bats. This virus can be propagated in cultured cell lines we have derived from *E. fuscus*(407).

The cellular and tissue tropism of this virus are unknown. Sites of latency of other γHVs are primarily associated with B cells and sometimes macrophages or dendritic cells of lymphoid tissues in the spleen, thymus, and bone marrow(408–410). Lung as a site of latency has also been observed in rodents(411). However pulmonary intravascular macrophages (PIMs) which are found within the lung of other species are absent in rodents(412). PIMs have been shown to support viral replication with other viruses but a role for these cells in γHV infection has not been reported(413,414).

Here we characterize the expression of Late viral genes under conditions thought to mimic lytic infections, describe the tissue and cellular distribution of EfHV nucleic acids in naturally infected bats, and isolate and sequence the genome of a new isolate and variant of EfHV.

3.3 Materials and methods

3.3.1 Virus isolation

Pooled liver, lung, and kidney frozen at -20℃ from this female bat were homogenized in 1.5 ml of Dulbecco's modified eagle medium (D-MEM, Gibco, cat. 12430112) at 30 Hz for 4 minutes in a (Retsch Mixer Mill) with a 5.5 mm stainless steel grinding bead (MP biomedicals, 116540431) and 0.1 g of silica beads (Fisher, cat. 360991112). The sample was centrifuged at 15700 *x g* for 15 minutes. One millilitre of the supernatant was filtered using 0.2 μM PES filter (Whatman, cat. 6780-2502) and added to a 75 cm^2 flask (Sarstedt, cat. 83.3911.002) of passage 8 *Eptesicus fuscus* kidney cell line 3b (EfK3b) cells in D-MEM containing 10% fetal bovine serum, penicillin, streptomycin, and amphotericin B (Antibiotic-Antimycotic, Gibco, cat. 5240062). Cytopathic effect was first observed 4 days post infection and 7 days post infection the flask had 95% cytopathic effect and was frozen at -80℃.

3.3.2 Genome sequencing

This unique virus isolate along with the isolate we use in our laboratory were sequenced. The sequencing library was prepared using the Nextera XT Library Preparation Kit (Illumina, cat. FC-131-1024). The library was diluted to 8 pM and sequenced using a V2 500 cycle Nano Kit (Illumina, cat. MS-103-1003) on a MiSeq instrument. Reads were trimmed for quality using Trimmomatic(415) sliding window 4:30 and minlen 36. Reads were assembled into contigs using SPAdes 3.12.0(416) and mapped to the reference genome Eptesicus fuscus gammaherpesvirus accession NC_040615 using Geneious Prime 2022.0.1. Gaps between contiguous sequences and several regions with multiple single nucleotide polymorphisms were confirmed using polymerase chain reaction (PCR) with Top taq DNA polymerase (Qiagen, 200205), purification of products using MinElute PCR purification kit (Qiagen, cat. 28004) or gel extraction and purification with the QIAquick gel extraction kit (Qiagen, cat. 28706), followed by Sanger sequencing (Macrogen). PROVEAN 1.1.3 with a cut-off value of -2.5 was used to predict whether the differences in the coding regions of the viral isolates and the reference sequence would change the biological function of the protein.

3.3.3 In vitro lytic gene expression

Acyclovir was used to arrest cellular DNA synthesis to identify the Late viral genes and cycloheximide was used to arrest cellular protein synthesis to identify immediate early genes. EfK3b cells were maintained in D-MEM (Gibco, cat. 12430112) supplemented with 10% fetal

bovine serum and penicillin streptomycin. They were infected with EfHV at a multiplicity of infection of 1 in the presence of acyclovir 45 μg ml^{-1} (Calbiochem, cat. 114798) or cycloheximide 100 μg ml^{-1} (Sigma, cat. 501784364) for 1 hour at 37°C. Inoculum was removed and replaced with complete media containing the same concentration of chemical. Controls were treated in the same way without the addition of chemicals to the media. Cells were incubated at 37°C for 24 hours. Experiments were performed in triplicate and gene expression was normalized to a 4°C 1 hour infection control.

RNA was extracted using the QIAGEN RNeasy plus mini kit (cat. 74134) as per manufacturer's protocol. RNA (121 ng) from the acyclovir experiment were used for complementary DNA (cDNA) synthesis with the iScript gDNA clear cDNA synthesis kit (Bio-Rad, cat. 172-5034). The cDNA was diluted to a final volume of 200 μl and 5 μl was used in the real time PCR reaction with 15 μl of SsoFast EvaGreen supermix (Bio-rad, cat 172-5204). Four micromoles of each primer was used for each of the viral open reading frames as previously described(96). Briefly the thermocycler program was enzyme activation 95°C for 30 seconds, denaturation 95°C for 5 seconds, annealing/extension variable temperature for 5 seconds, with the denaturation and annealing/extension repeated for 40 cycles. The melt curve was performed from 65-95°C in two second steps with 0.5°C increments.

3.3.4 PCR

Big brown bat cases were submitted for necropsy to the Western/Northern Regional Centre of the Canadian Wildlife Health Cooperative (W/N CWHC). Cases were screened for γHV infection by PCR on DNA extracted (Qiagen Dneasy blood and tissue kit, cat. 69504) from pooled samples of liver, spleen, and lung. A subsample of 135 out of 314 cases submitted between 2017-2021 were chosen based on the availability of these tissues. PCR was performed as previously described using primers targeting viral BGLF4 and big brown bat GAPDH(96).

3.3.5 Case description

A female big brown bat was submitted for necropsy by an animal rehabilitator for weakness. On postmortem examination the long bones were brittle (osteopenia) and pectoral muscles were pale. Histologically there were basophilic or eosinophilic intranuclear inclusions within the tracheal epithelium accompanied by cellular and nuclear enlargement (cyto and karyomegaly) and dysplasia.

3.3.6 Histopathology

Tissues were fixed in 10% neutral buffered formalin embedded in paraffin and stained with hematoxylin and eosin. After fixation sections with bone were decalcified in 20% formic acid for less than 24 hours then embedded. The spleen and lymph node were assessed for lymphocyte hyperplasia which was recorded as present or absent. Splenic lymphoid hyperplasia was defined as more than 50% of the white pulp containing secondary follicles(417,418). Hyperplasia of the lymph node could involve expansion of the paracortical region with lymphocytes or follicular hyperplasia with or without interfollicular proliferation(419).

3.3.7 In-situ hybridization

Probes were designed against viral nucleic acid sequences and can bind DNA or RNA. These targeted viral genes expressed in productive infections an envelope glycoprotein gp52 (V-Ef-gammaherpesvirus-gp52-C1 cat. 1070981-C1); in latency, latency-associated nuclear antigen LANA (V-Ef-gammaherpesvirus-gp74-C2 cat. 1070971-C2); and host B cell receptor of big brown bats, *Eptesicus fuscus* membrane spanning 4 domains A1 EMS4A1(Ef-MS4A1-C1 cat. 1070991-C1). Control probes included a positive control targeting the peptidylprolyl isomerase B variant X1 of big brown bats (Ef-PPIB-C1 cat. 1073191-C1) and a negative control dihydrodipicolinate reductase gene from bacteria (Negative control probe DapB cat. 310043). Cell culture control was used to test the specificity of the viral probes by infecting one of two 175 cm^2 flasks of EfK3b cells at a multiplicity of infection of 1 and at 24 hours post infection embedding cells in agarose prior to fixation in 10% neutral buffered formalin. Both cell pellets were embedded in a single paraffin block. Serial sections of slides made from tissue blocks of submitted cases were probed in quadruplicate with the positive control peptidylprolyl isomerase B, negative control dihydrodipicolinate reductase, gp52 and LANA, or LANA and B cell receptor.

Images were captured with an Olympus BX41, Infinity 5 camera, and Infinity Analyze version 7.0.3.1111 software. To determine if the probe binding for LANA greater than the background observed with the control probe the number of red foci indicative of probe binding within the lymphoid tissues were counted per 60x field of view 0.37 mm. These counts were performed on the serial sections probed with either LANA and B cell receptor or the negative control.

Slides from the well-preserved PCR positive cases were used for *in-situ* hybridization (ISH). Five-micron tissue sections from formalin-fixed paraffin-embedded tissues were mounted

on Superfrost Plus slides (Fisher Scientific cat. 12-550-15). They were prepared using the RNAScope 2.5 HD Duplex assay kit (ACDbio cat. 322430) following the manufacturer's protocol with pre-treatment in the target retrieval buffer for 15 minutes at 95-100℃ and 15 minutes for protease plus treatment at 40℃ for lymphoid tissues, lung section, and cell culture. For the sections of trachea and decalcified head the pre-treatment conditions are as follows: pre-treatment in the target retrieval buffer for 15 and 30 minutes respectively at 95-100℃ and 30 minutes protease plus treatment at 40℃.

3.3.8 Immunohistochemistry

Immunohistochemistry (IHC) for MHC class II alpha chain was performed by Prairie Diagnostic Services Inc using their commercially available HLA-DR-α chain clone TAL.1B5 (Agilent, cat no. M074601-2). Lung from two separate cases were submitted for IHC from an uninfected otherwise healthy bat and the case with viral probe binding in the lung (envelope glycoprotein gp52 and LANA probe colocalization).

3.3.9 Transmission electron microscopy and viral purification

Virus was purified by serial centrifuging the supernatant at 4℃ after 3 freeze thaw cycles: 1500 *x g* for 5 minutes (Sorvall Legend RT, Thermo Scientific), 10 000 x *g* for 15 minutes (Sorvall RC6 Plus, Thermo Scientific, Waltham), and 80 000 x *g* for 1 hour (Sorvall Wx Ultra, Thermo Scientific). The pellet was resuspended in 50 µl of PBS with 20 µl of 2% glutaraldehyde in 0.1 M sodium cacodylate stored at 4℃ until imaging. Transmission electron microscopy (TEM) was performed as previously described(96). Virus purified for sequencing was ultracentrifuged with 6 ml of 30% w/v sucrose cushion and resuspended in 50 µl 10 mM Tris pH 8.5.

For cellular TEM 3 flasks with 90% cytopathic effect were used. Media and trypsinized cells were centrifuged at 325 x *g* for 10 minutes (Sorvall legend, Thermo Scientific) and the pellet was resuspended in 36 ml PBS and centrifugation repeated. Pellet was fixed with 10 ml 2% glutaraldehyde in 0.1 M sodium cacodylate and incubated at 4℃ for 4 hours. Cells were pelleted with previous centrifugation step and resuspended in 1 ml of 0.1 M sodium cacodylate. Measurements were taken with Image-Pro Premier version 9.3.3.

The hematoxylin and eosin-stained slide of lung with probe binding was submitted for TEM and images were captured with the Hitachi HT7700 transmission electron microscope.

3.4 Results

3.4.1 Virus isolation and genome sequencing

In an attempt to isolate a virus from a case with large intranuclear inclusion bodies within the epithelium of the trachea of a bat submitted to the W/N CWHC, the supernatant of homogenized tissue was added to cultured EfK3b cells. Rounded cells characteristic of herpesvirus cytopathic effect were observed. TEM and whole genome sequencing were used to identify the isolate. The virus displayed intranuclear replication *in vitro* and the negative stained virus was spherical, enveloped, and measured 105 ± 5 nm which was consistent with it being a herpesvirus. A γHV (EfHV/SK/01/2016) had previously been isolated in our lab, and so to confirm our isolate was unique and not a contaminant we sequenced the genomes of both isolates for comparison. Illumina sequencing resulted in 464x coverage of the retained reads for EfHV/Saskatoon/02/2020 and 397x coverage for EfHV/Saskatoon/01/2016. The reference sequence NC_040615.1 used for comparison was created from the original virus isolate EfHV/SK/01/2016 our sequencing of this isolate used in our lab had 99.9% nucleotide identity with the reference. The difference in EfHV/SK/01/2016 nucleic acid sequence from the reference were characterized by 2 insertions, 1 transition, 2 transversions, 12 substitutions, and 2 insertions or deletions in tandem repeats (Table A 1); whereas, the nucleotide sequence identity of the isolate presented here (EfHV/SK/02/2020) was 99.7%. The variations in the nucleotide sequences between EfHV/SK/02/2020 and the reference included 3 insertions, 6 deletions, 61 transitions, 20 transversions, 10 substitutions, and 16 insertions or deletions in tandem repeats (Table A 2). The whole genome sequence for EfHV/SK/02/2020 was deposited in Genbank (accession number OM517184). We predicted the alterations these variations in the coding regions had on the amino acid sequence for both isolates (Table A 3).

3.4.2 In vitro lytic gene expression

To select gene targets that could aid in differentiating lytic infection from latency, we identified genes expressed at high levels late in the lytic cycle following the onset of viral DNA replication and which were not expressed if viral DNA replication was blocked. In addition, we identified genes that were transcribed in the absence of new protein synthesis. Earlier research on herpesviruses had suggested that these genes are required for the initiation of the viral replicative cycle and, therefore, may be markers for the transition from latency to productive viral replication. Infected cells were maintained in the absence or presence of acycloguanosine (acyclovir), which

specifically inhibits viral DNA replication or cycloheximide, which globally inhibits protein synthesis. The relative amounts of transcripts for various viral genes (96) were then determined.

Twenty-four hours after infection all known genes were expressed (Figure 3.1). Acyclovir treatment significantly decreased the expression of several genes. The gene with greatest expression and most inhibition with acyclovir treatment was envelope glycoprotein gp52 (Figure 3.1). This glycoprotein was not expressed in the presence of cycloheximide at 8 hours post infection (Figure A 1), we therefore selected gp52 as the ISH probe target to identify cells undergoing a lytic infection.

Multiple two-tailed t-tests comparing expression 24 hours post infection in the presence or absence of acyclovir were performed using Prism version 9.3.1 using the two-stage step-up method and a false discovery rate of 1.00%, 4 df.

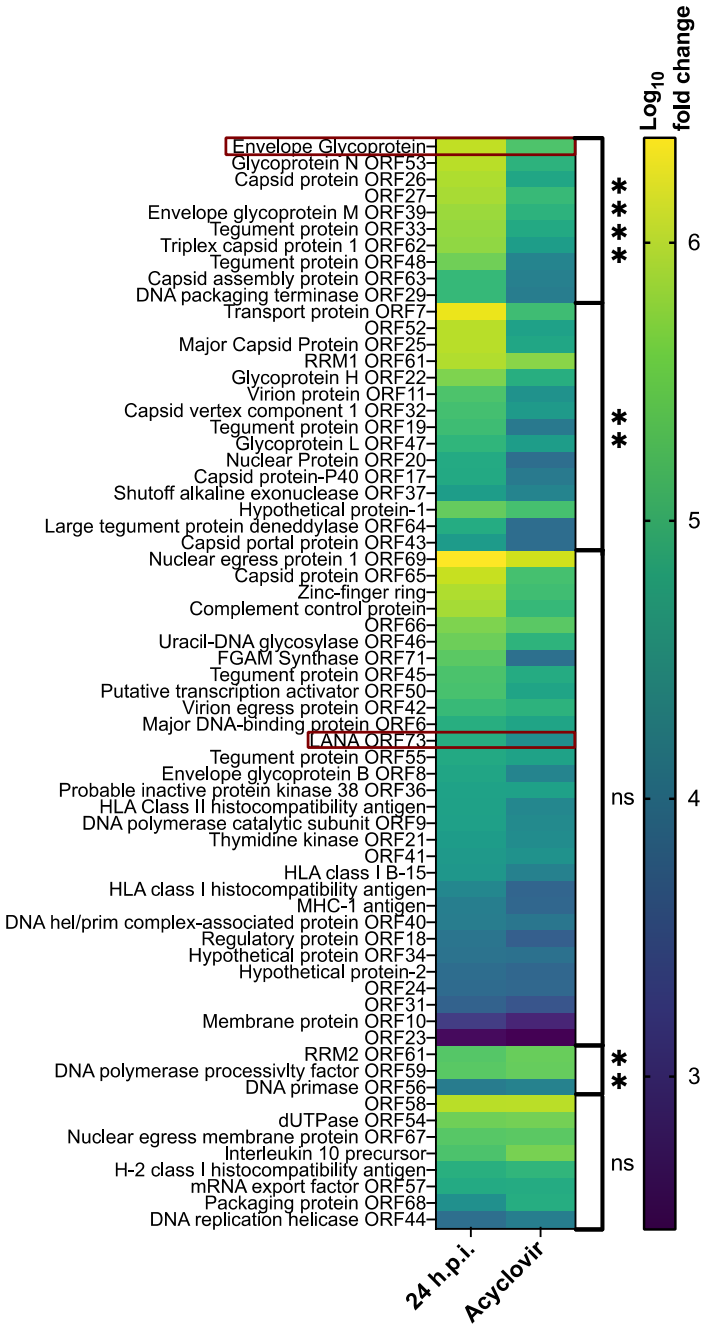

Figure 3.1 Heatmap of the log$_{10}$ fold change of EfHV genes relative to 4°C infection control. Gene expression was measured at 24 h.p.i. with or without acyclovir. They are ordered by gene expression for those that were inhibited by acyclovir at the top and those that were potentiated at the bottom, followed by p-value comparing the two groups, then by fold change at 24 h.p.i. (highest to lowest). Red boxes indicate target genes for ISH probes. Statistical significance of the comparisons between the acyclovir treated and untreated cells at 24 h.p.i. is indicated to the right of the graph (ns not significant, $p<0.0001$ **** and $p<0.01$ **).

EfHV - Eptesicus fuscus gammaherpesvirus, RRM1 - ribonucleoside-diphosphate reductase large subunit, RRM2 - ribonucleoside-diphosphate reductase small subunit, h.p.i. - hours post infection, ISH - *in-situ* hybridization, LANA - latency-associated nuclear antigen

3.4.3 Histopathology

The purpose of examining cases of big brown bats presenting for necropsy was to determine if there are any pathological changes associated with EfHV infection. Of the pooled tissues from 135 big brown bats submitted between 2017-2021, EfHV DNA was detected in 43. Of those with good preservation, 59 slides were available for review, 17 of which were from bats infected with EfHV. All slides were assessed for neoplasia/tumors and proliferation of the lymphoid tissues. Splenic white pulp n=51 and lymph nodes n=27 were examined and no association was found between infection with EfHV and hyperplastic changes. The bronchus-associated lymphoid tissue (BALT) was examined but no hyperplastic lesions were observed (n=47). Fisher's exact test was used to determine if there was an association between infections and hyperplasia of the splenic white pulp or lymph nodes $p=0.1023$ and $p>0.999$ respectively. Various neoplasms have been associated with γHV infections and none were detected in our study. Two of the EfHV positive cases had large intranuclear inclusion bodies within the tracheal epithelium with features of dysplasia. From one of these cases a unique isolate of Eptesicus fuscus gammaherpesvirus/Saskatoon/02/2020 was cultured (Figure 3.2). Since nuclear inclusion bodies are frequently associated with DNA viruses, we tested this case for adenovirus using IHC, which was negative.

3.4.4 In-situ hybridization and immunohistochemistry

In-situ hybridization (ISH) was used to determine the localization of viral nucleic acids in tissues and the subtype of lymphocyte infected. To detect productive infection we used a probe with complementary sequences to gp52 (Figure 3.1, Figure A 1). To detect latently infected cells we probed for EfHV LANA nucleic acids; in other yHVs this gene is required for the maintenance of latency. We expected latently infected cells to bind the LANA probe without binding the gp52 probe. Since LANA is also expressed during productive infection when both viral probes colocalized in cells this was interpreted as productive infection. The cellular probe targeted B lymphocytes (a site of latency for other γHVs) and was directed against the nucleic acids of the B cell receptor CD20, known as EMS4A1. Although not entirely specific for B cells EMS4A1 is expressed at high levels in lymphoid tissue (420). Latently infected B cells were defined as those in which probe colocalization for the B cell receptor and LANA occurred. Cells could not be probed with both gp52 and EMS4A1 since this was a colorimetric assay capable of detecting two colours and these probes were assigned to the same colour (Figure A 2).

To validate the viral probes, cell culture pellets from either uninfected cells or cells infected for 24 hours with EfHV were embedded in the same block and treated with gp52 and LANA probes; positive, and negative controls were similarly treated. The cell pellet section containing infected cells showed abundant probe binding for gp52 and LANA whereas there was minimal non-specific probe binding in the uninfected cell pellet. (Figure 3.2). Nine EfHV positive cases submitted for necropsy were selected for ISH based on the preservation of tissues, size of sections, and recentness of submission. The serial sections from the tissue block of each case containing the spleen were probed with the positive control, negative control, LANA and B cell receptor, and gp52 and LANA. Since RNAscope probes can bind both RNA and DNA, and the levels of LANA expression in tissues are unknown, no pre-treatment with DNase was performed which meant our probes detected both DNA and RNA. Additional lymphoid tissues were occasionally present on the same slide, lymph node n=2 and BALT n=4. One case was excluded because of weak probe binding in the positive control for a total n=8. The average number of foci per field were compared between the negative control and LANA/gp52 probes with an unpaired two-tailed t-test in Prism version 9.3.1 without corrections $p<0.05$.

The probe binding for LANA in the spleen was significantly different from the negative control in 4 out of 8 cases with probe colocalization with the B cell receptor probe in a subset of cells (Figure 3.2). No probe binding for the envelope glycoprotein was observed in the spleens of these cases indicating there was no splenic viral reactivation. For BALT all slides demonstrated probe binding to the LANA and B cell receptor but only 2 of the 4 were significantly different from the negative controls. For the lymph node 1 of 2 had significantly greater amount of LANA probe binding than its negative control.

When lungs were examined n=7 one of the cases had strong probe binding of individual cells with both gp52 and LANA. These cells were distributed uniformly within the alveolar capillaries, and they did not bind the B cell receptor probe (Figure 3.2). To identify these cells an antibody against MHC Class II antigen was applied to a serial section of the lung. This antigen is a non-specific marker for antigen presenting cells including macrophages, dendritic cells, monocytes, and B lymphocytes (420). Epitope binding in a subset of cells in the lung were observed and based on the distribution and location of these cells, PIMs were suspected as the cell type with intense probe binding(421,422). There is no information on whether bats have PIMs to provide support for this hypothesis. A similar pattern of MHC class II epitope binding was

observed in lung from an otherwise healthy EfHV PCR negative case (Figure 3.2) suggesting that these cells are not exclusive to EfHV-infected bats.

The intense probe binding observed in the lung of one bat was interpreted as possible productive infection, therefore the sagittal section of the head which included the nasal turbinates, salivary glands, and mucosa-associated lymphoid tissue (MALT) was processed for ISH. Within MALT there was probe binding for LANA and the B cell receptor but not for the envelope glycoprotein probe. Taken together, the ISH results suggest that latency is present in B cells of lymphoid tissues (spleen, lymph node, BALT, and MALT) while cells of the lung are involved in infection but whether this represents productive infection, phagocytosis of free virus, or phagocytosis of infected cells is unclear.

3.4.1 Transmission electron microscopy

To confirm that the cells from the bat lung with intense ISH probe binding were intravascular, we examined lung sections by TEM. Large cells with abundant cytoplasm and indented nuclei were adherent to the endothelial surface of alveolar capillaries consistent with PIMs(421).

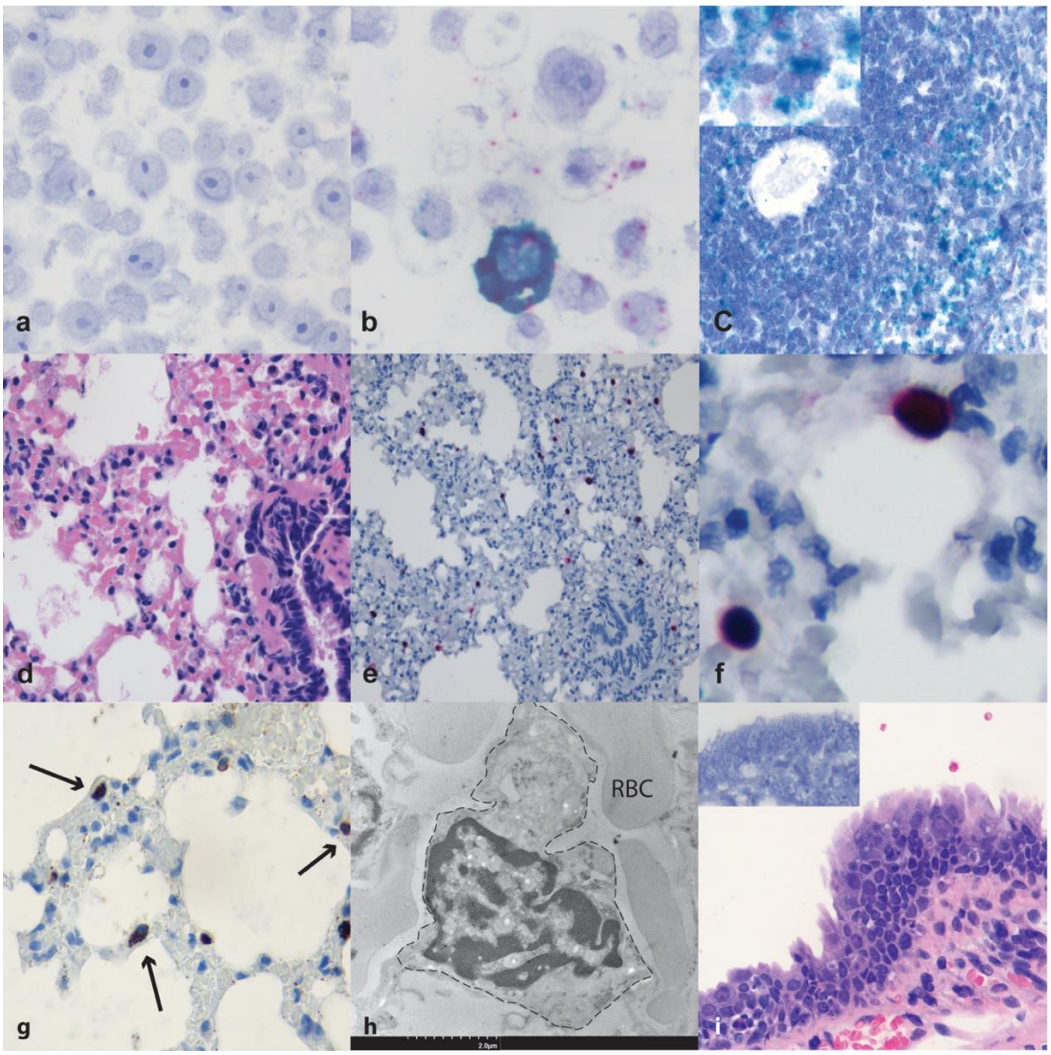

Figure 3.2 Photomicrographs of EfHV infection in cell culture and big brown bats. ISH colocalization of EfHV LANA (red) and envelope glycoprotein (teal) probes was not observed in uninfected cells (a) but was in EfK3b cells infected with EfHV (b). ISH colocalization of LANA (red) and EMS4A1 (teal) probes in splenic white pulp (c), inset with higher magnification demonstrating colocalization of markers. H&E section of lung from a lytic infection in a big brown bat (d) and serial section ISH demonstrating intense probe binding with LANA (red) and envelope glycoprotein (teal) (e). Higher magnification showing colocalization of these probes in cells within alveolar capillaries (f). Cells with MHC II IHC antibody binding (brown) as indicated by the arrows displayed similar distribution to the cells binding the ISH probes in e & f (g). TEM of lung tissue taken from slide shown in d, e, & f demonstrating a pulmonary intravascular macrophage outlined by a dashed line (h). Case from which EfHV/SK/02/2020 was isolated showing dysplasia and intranuclear inclusion bodies in the tracheal epithelium H&E section (i). Inset ISH serial section of trachea negative for EfHV envelope glycoprotein/LANA.
EfHV - Eptesicus fuscus gammaherpesvirus, EfK3b - Eptesicus fuscus kidney cell line 3b, EMS41 - *Eptesicus fuscus* membrane spanning 4 domains A1, H&E - hematoxylin and eosin, IHC - immunohistochemistry, ISH - *in-situ* hybridization, LANA - latency-associated nuclear antigen, MHC II - major histocompatibility complex II, RBC - red blood cell, TEM - transmission electron micrograph

3.5 Discussion

Observing γHVs in their hosts is a challenge because most infections are latent and asymptomatic making information about pathobiology with respect to establishment of latency, viral reactivation, and shedding scarce. Murine herpesvirus 4 infection in laboratory mice is the most common model to study γHVs but these mice are not a natural host and the virus is not easily transmitted to other mice(423). These challenges can be overcome by studying natural infection in other primary host species like bats. We determined the tissue and cellular distribution of EfHV within naturally infected big brown bats. Latent virus was found within lymphocytes of lymphoid tissues. Individual cells in alveolar capillaries of the lung are either involved in virus production or in the immune response to infection. No association could be established between viral infection and any pathological change.

By using *in vitro* gene expression and combining this with what is known about other γHVs we chose ISH probes that were able to determine cell and tissue distribution of EfHV nucleic acids with the potential to distinguish lytic and latent infection in wild bats. We regarded cells with binding of the EfHV LANA probe, a gene expressed during latency by related γHVs, in the absence of probe binding for a viral structural glycoprotein, as latently infected. The latent reservoir for EfHV was identified as B cell receptor expressing lymphocytes of lymphoid tissues the. There was intense probe binding for the B cell receptor within germinal centres of the splenic white pulp indicating that they are most likely B lymphocytes.

The lytic probe localized within individual cells that were scattered throughout the alveolar capillaries, and these cells also bound the latent probe. The distribution of these probe positive cells are most consistent with PIMs. The cells also did not bind the B cell receptor probe, supporting their identity as PIMs. We cannot confirm these cells are not B lymphocytes as reactivation of other γHVs occurs with plasma cell differentiation and these differentiated B cells have variable expression of transcripts for the B cell receptor(424) a non-specific macrophage marker and these cells would be. . As we could not differentiate DNA or RNA with our methods, this probe binding could either represent productive viral infection, or phagocytosis of infected cells or free virus if these are PIMs.

Supporting our hypothesis that these cells are PIMs we demonstrated with TEM that cells with the morphological characteristics of PIMs are present in bat lungs. PIMs have roles in several viral infections either supporting viral replication or in contributing to disease through their

activation(414,425–428). Pulmonary pathological changes are observed with several γHV infections and predominantly is characterized by fibrosis of the lungs including idiopathic pulmonary fibrosis in humans(429–434). Direct involvement of lung macrophages is seen in equine multinodular pulmonary fibrosis with viral inclusion bodies in alveolar macrophages although no involvement of these macrophages was observed in our case(435,436).

γHVs in bats are common (50,92,99,100) but rarely are there any reports on diseases associated with infection(50,98). The lack of pathological changes or association of lymphoid hyperplasia with γHV infection in our cases is not surprising because in infected but otherwise healthy individuals these viruses are maintained in a latent state. Disease is usually only seen during acute infections or in latent infections with concurrent immunosuppression and the typical lesion of lymphoid proliferation is a non-specific finding with multiple causes. No conclusions could be made about the pathologic features of dysplasia with cyto and karyomegaly of the tracheal epithelium and γHV infection given the small sample size, but we speculate that they are related to immunosuppression and a concurrent unidentified viral infection.

The unique isolate of EfHV/SK/02/2020 was distinct from the original isolate EfHV/SK/01/2016 used in our lab as confirmed by whole genome sequencing. The differences in some of the nucleic acid sequences do alter the predicted proteins of multiple open reading frames but the significance of these differences requires further investigation. Since viruses are not a homogeneous population and can mutate in cell culture it is expected that after multiple passages there were several small differences in EfHV/SK/01/2016 to the sequence deposited in Genbank, only one of which altered the predicted amino acid sequence of MHC class I antigen.

Based on our findings we propose a model for the infectious cycle of EfHV in *E. fuscus*, its primary host (Figure 3.3). This is largely conjectural and is based on our observations as well as the literature on other γHV.

3.5.1 Transmission

Our previous work indicates that EfHV is shed from the oral cavity of juvenile bats (unpublished data) suggesting that the virus may be transmitted in saliva. For γHVs there are multiple potential modes of transmission but those that are applicable to wild bats would include contact with saliva, inhalation, or sexual transmission(408,437). Epstein-Barr virus is shed in saliva and primed for infecting B cells(438,439) and gaps within the epithelium and basement membrane of tonsillar crypts would allow direct viral access to susceptible cells(440,441) or via

transcytosis of virus through epithelial cells(442). We excluded the olfactory epithelium is the point of entry since natural transmission via this route has not been demonstrated(443,444).

3.5.2 Colonization

Based on the literature for murine herpesvirus 4 spread from the primary site of infection to regional lymph nodes occurs through serial myeloid to lymphoid transmission(445). How γHVs go on to colonize the spleen is less certain possibly through cell free viremia but more likely through cell associated transfer(408,446–450). Splenic colonization occurs through cell to cell spread from the marginal zone to germinal centre(446).

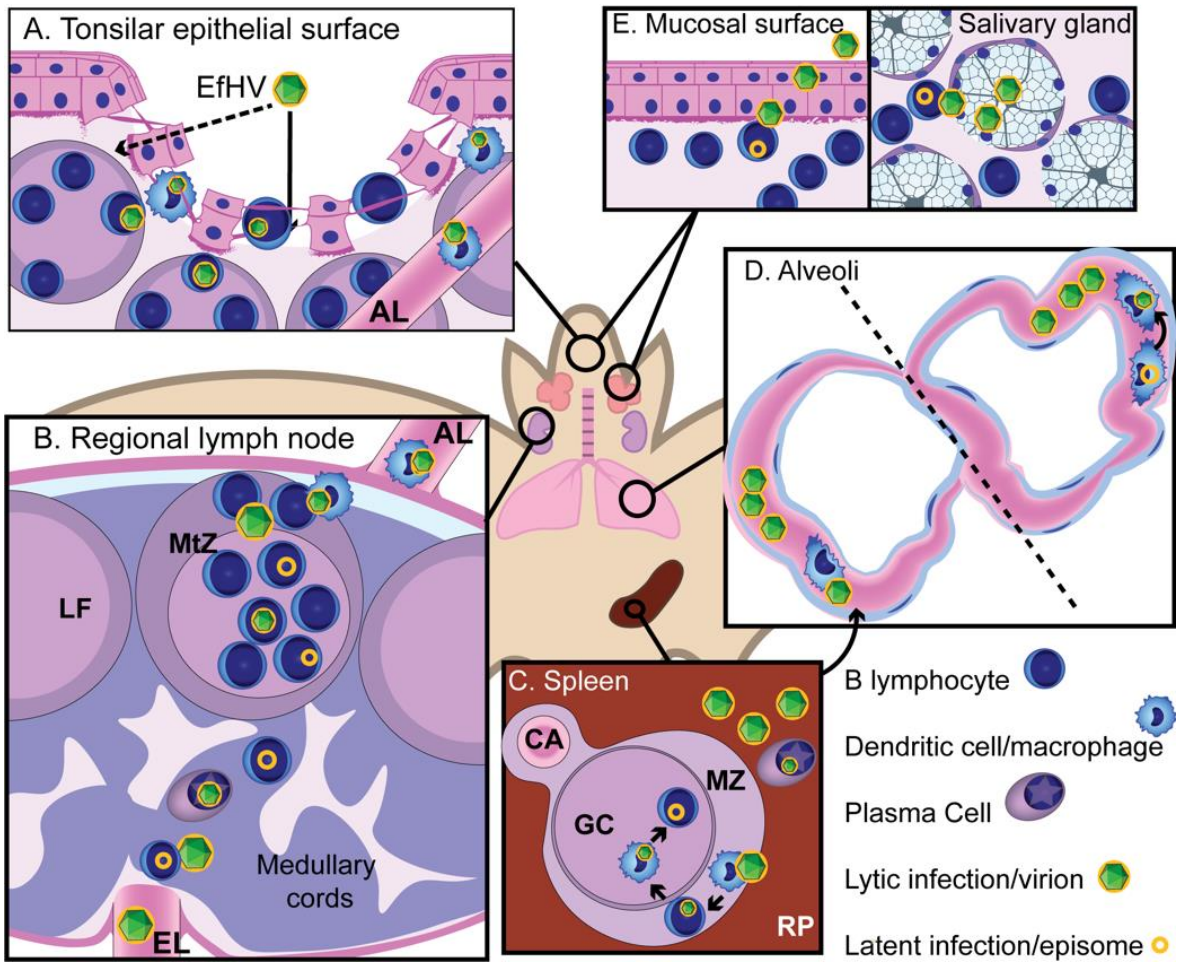

Figure 3.3 Proposed pathogenesis for EfHV. A) new host contacts saliva containing virus primed for infecting B lymphocytes and dendritic cells (DC). Exposure to virions could occur through gaps in the epithelium of the tonsillar crypts (arrow) or transcytosis (dashed arrow). Infection of B lymphocytes and DCs occur. B) DCs migrate to regional lymph node via the afferent lymphatics and infect follicular B cells and eventually memory B cells in the germinal centre. Further systemic spread occurs by reactivation of latently infected B cells during differentiation into plasma cells. Dissemination is possibly by infected B cells or cell free viremia. C) The primary site of latency the splenic white pulp is colonized by infection of marginal zone macrophages then marginal zone B cells, follicular dendritic cells, and finally germinal centre B cells through cell to cell spread. Differentiation of splenic B cells into plasma cells results in reactivation of gammaherpesvirus infection. D) PIMs could be involved in the viral cycle in a number of ways: phagocytosing virus or virus infected cells following splenic reactivation, becoming infected during phagocytosis or being directly involved in productive infection, either via latently infected migratory monocytes which then differentiate into PIMs or resident PIMs that reactivate virus. E) Virus is shed from epithelial or glandular cells which are infected by lytic infection in B cells. B cells were infected by migrating macrophages, cell free virus produced from a myeloid cell, or they reactivate virus following latent infection.
AL - afferent lymphatic, CA - central arteriole, EfHV - Eptesicus fuscus gammaherpesvirus, EL - efferent lymphatic, GC - germinal centre, LF - lymphoid follicle, MtZ - mantle zone, MZ - marginal zone, RP - red pulp

3.5.3 Reactivation

The final steps of reactivation and viral shedding are not as well characterized. Experimentally, reactivation occurs at the primary site of infection(451). We did not observe reactivation at the most likely primary site of infection the tissues of the head or in the lymphoid tissues that harbour latent infection in big brown bats. We only demonstrated the possibility of lytic infection in what are likely PIMs but lung is only reported as a primary site of infection under experimental conditions(411,452). Probe binding in what we believe are PIMs could represent: 1. phagocytosis of virus or virus infected cells following reactivation at a different site possibly leading to infection and virus replication in PIMs, 2. synchronous reactivation from latency in PIMs infected *in-situ* or in monocytes that then migrate and differentiate into PIMs, or 3. acute infection at the dissemination phase. The first explanation is favoured because no latently infected macrophages were identified in the lung in other cases, the spleen had no staining for latent infection, and we speculate that reactivation occurred here followed by infection of the PIMs. This bat had significant co-morbidities and was likely immunocompromised favouring reactivation. The role of these cells in γHV infection would be overlooked solely studying rodents and humans because they have not been reported in the former and occur at low numbers in the latter(412,421).

3.5.4 Shedding

Since no lytic viral probe binding was observed in the oral cavity, the following discussion is based on peer-reviewed literature. Virus is transferred from lymphocytes to the epithelium(453,454). *In vivo* viral shedding has been demonstrated from salivary glands(455) and not from the oral mucosa(456) but either or both sites are possible. We favour reactivation at secondary sites either directly in PIMs or with PIMs as an intermediate step resulting in the production of virus that could reinfect lymphocytes in MALT maintaining the serial lymphoid-myeloid transmission. Cells in the MALT would then in turn infect epithelial cells.

There were many limitations to our study. The cell culture experiments need to be interpreted with care as γHV gene expression is not always conserved between cell lines, within a cell culture, and in the host. Additionally, RNA expression was measured and protein expression should be used to support the findings. We were restricted to bats submitted to the W/N CWHC with no possibility of experimental infection. As they were natural infections the timing of infection relative to sampling is unknown. We could not control for the effects of concurrent illnesses on lymphoid tissues possibly obscuring an association with lymphoid hyperplasia. The

variations between samples and individual characteristics of the spleen led to an inconsistent number of fields examined between cases for ISH of latent infection. With ISH we cannot differentiate between viral nucleic acids in the cytoplasm or in endolysosomes of macrophages. Although pre-treating our slides with DNase would discriminate between the RNA and DNA of EfHV we would not be able to rule out the possibility of the RNA being from a phagocytosed cell. However, porcine PIMs are more cytolytic and have limited phagocytic capability relative to alveolar macrophages(457). As our results are observational, we cannot conclude with certainty on the significance of our findings to the pathogenesis of the γHV.

Future directions for research include investigating the role of PIMs in γHV dissemination, transmission, lung disease, and immunity. Examining the pathogenesis of γHVs following viral reactivation and how primary or secondary sites contribute to viral transmission. Gammaherpesviruses can also be used as a proxy measure for stress and to understand the role between stress and viral shedding. To study virus host interactions in bats and how stress contributes to viral transmission using this γHV, development of bat cell lines (B lymphocytes and PIMs) that supports latent infection is crucial.

Preface to chapter 4

In Chapter 3 surveillance for virus-associated disease in bats identified lesions from which a causative virus could not be detected; however, we isolated a γHV from this submission. While fulfilling our second objective of evaluating diagnostic cases for virus-associated disease another case with lesions consistent with a viral infection was identified. Chapter 4 covers the isolation of this poxvirus and examination of the association of the virus with lesions involving the oral cavity and joints. This poxvirus was previously reported in big brown bats with lesions restricted to the joints. We describe additional ulcerations of the oral cavity and cell culture experiments suggesting the ability of this virus to replicate in the nucleus, the first for a poxvirus. This chapter fulfills the third objective by isolating and characterizing a virus from these bat submissions.

CHAPTER 4 EPTESIPOX VIRUS ASSOCIATED LESIONS IN NATURALLY INFECTED BIG BROWN BATS

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4.1 Abstract

Bats have many unique qualities amongst mammals; one of particular importance is their reported tolerance to viruses without developing disease. Here we present evidence to the contrary by describing and demonstrating viral nucleic acids within lesions from Eptesipox virus (EfPV) infection in big brown bats. One hundred and thirty-five bats submitted for necropsy to the Western/Northern Regional Centre of the Canadian Wildlife Health Cooperative (W/N CWHC) between 2017 and 2021 were screened for EfPV by polymerase chain reaction (PCR); two had amplifiable poxvirus DNA. The lesions associated with infection were ulcerations and joint swelling in 2/2 and 1/2 cases, respectively. These changes are not specific for poxvirus infection, although intracytoplasmic viral inclusion bodies within the epithelium are diagnostic when present. Viral nucleic acids, detected by ISH, were observed in the epithelium adjacent to ulcerative lesions from both cases and within the joint proliferation of one case. A new isolate of EfPV was obtained from one of the cases and its identity was confirmed with electron microscopy and whole genome sequencing. Juxtanuclear replication factories were observed in most cells; however, rare intranuclear virus particles were also observed. The significance of the presence of virus particles within the nucleus is uncertain. Whole genome assembly indicated that the nucleotide sequence of the genome of this EfPV isolate was 99.7% identical to a previous isolate from big brown bats in Lynnwood, WA, USA between 2009-2011. This work demonstrates that bats are not resistant to the development of disease with viral infections and raises questions about the dogma of poxvirus intracytoplasmic replication.

4.2 Introduction

Bats have been thought of as having a special relationship with viruses where they are more resistant to infection associated disease(458). This relationship is thought to have arisen as a consequence of adaptations to flight and allowed them to be reservoirs for numerous different viruses(10). As hosts of a diverse array of viruses, bats are often implicated as the source of multiple epidemics and pandemics(34,459). This has resulted in many studies trying to identify viruses of bats, primarily through next generation sequencing, but there are very few studies examining the pathological effects of infection in the host and only a few reports from experimental inoculations(51).

Poxviruses are double stranded DNA (dsDNA) viruses that replicate in the cytoplasm and encode many proteins that modulate the host response to infection. Poxviruses appear to have

acquired some of these modulating genes from their hosts by horizontal gene transfer(460,461). Ulceration and proliferation of the epithelium are clinical manifestations common to many poxviruses(462). Infections are sometimes more extensive and affect multiple organs, such as the wet form of avipox infections in birds or myxoma virus infections in domestic rabbits(463). This is due to the variability in cellular tropism of poxviruses and the variability in viral replication of infected cells within host tissues (464).

Poxviruses have been found in bats from around the world, most often discovered by sequencing data, but occasionally associated with lesions. Evidence in Yinpterochiroptera (megabats) for poxvirus infection include: partial poxviral sequences from swabs in Ghanian *Eidolon helvum*(62), whole genome sequencing for pteropox virus associated with multiple crusting lesions on the wings of *Pteropus scapulatus* in Australia(63), and a virus isolated from nodular wing lesions in *Rousettus aegyptiacus* from Israel named Israeli Rousettus aegyptiacus pox virus(60). Similar evidence is also available for Yangochiroptera (microbats): hypsugopoxvirus was sequenced from *Hypsugo savii* in Italy(58), nodular skin lesions with viral inclusion bodies and virions visible on electron micrograph from *Miniopterus schreibersii bassanii* in Australia(61), and isolation and sequencing of EfPV from the big brown bat *Eptesicus fuscus* in the United States of America with fibrinosuppurative, necrotizing tenosynovitis and osteoarthritis(56,465).

The objectives of this research were to examine wild bats submitted for necropsy to the W/N CWHC to look for evidence of lesions associated with viral infection, to isolate and identify virus(es), and to determine if there is an association of observed pathological changes with viral replication.

4.3 Materials and methods

4.3.1 Case description

The first diagnosed case of EfPV from which virus was isolated was an adult male big brown bat submitted July 8, 2020. This bat had been relinquished to a wildlife rehabilitation unit for inability to fly and had multiple swollen joints that did not improve with 10 days of supportive care. The second case, also an adult male big brown bat, had been submitted May 13, 2019, after being cared for by wildlife rehabilitators for 74 days with a chronic non-healing ulcer over the nose. Intracytoplasmic inclusions were noted in the pharynx with histopathological examination and a poxvirus infection was suspected but no further diagnostics were pursued.

4.3.2 Screening cases for EfPV

Following the submission of the index case of EfPV to the W/N CWHC, tissues from previous and ongoing submissions for gammaherpesvirus surveillance were also screened for poxvirus infection using conventional PCR. There were 294 big brown bats submitted for necropsy during this period and of these we received 135 pooled tissue samples. Of the remaining cases pooled tissues were not received for a variety of reasons: necropsies were performed but tissues were not collected, tissue samples were not suitable due to advanced decomposition or scavenging, or no necropsy was performed. DNA was extracted from the liver, lung, and spleen using the Qiagen DNeasy blood and tissue kit (cat. 69504). HotStarTaq (Qiagen, cat. 203205) was used following manufacturer's protocol which briefly involved: denaturation at 95°C x 15 min, 40 cycles of denaturation at 94°C x 30 sec, annealing at 62°C x 39 sec, and extension at 72°C x 1 min with a final extension at 72°C x 10 min. The following primers were used: forward 5'-GACGAACACGATGCATCACG-3' and reverse 5'-TAGTGGAGGTAGCGGTGGA-3', that targeted the type A inclusion protein and generated a 765 bp product.

4.3.3 Histopathology

Tissues were fixed in 10% neutral buffered formalin, paraffin embedded, sectioned at 5 µm, mounted on glass slides, and stained with hematoxylin and eosin using standard protocols. Sections containing bone, which included sections of joints and oral mucosa, were decalcified after fixation by immersion in 20% formic acid for 24 hours, prior to embedding. Slides were reviewed using an Olympus BX41 microscope and images were captured using an Olympus BX41, Infinity 5 camera, and Infinity Analyze version 7.0.3.1111 software.

4.3.4 In-situ hybridization

Unstained 5 µm tissue sections from formalin-fixed paraffin-embedded tissue blocks were mounted on Superfrost Plus slides (Fisher Scientific cat. 12-550-15). They were probed using the RNAScope 2.5 HD Assay Brown kit (ACDbio cat. 322300) following the manufacturer's protocol with pre-treatment in the target retrieval buffer for 30 minutes at 95-100°C and 15 minutes for protease plus treatment at 40°C for all tissues. Probes were designed to target viral nucleic acids encoding the p39 putative membrane-associated core protein and the p4b precursor protein, genes gp99 and gp100 respectively (ACDbio cat. 107961-C1). Two viral gene targets were used because of the lack of diversity in the EfPV genome. Serial sections of slides were probed with a positive control targeting the peptidylprolyl isomerase B variant X1 gene of big brown bats (Ef-PPIB-C1

cat. 1073191-C1) and a negative control probe dihydrodipicolinate reductase gene from bacteria (Negative control probe DapB cat. 310043).

4.3.5 Virus isolation

Pooled liver, lung, and spleen frozen at -20°C were homogenized in 1.5 ml of Dulbecco's modified medium (D-MEM, Gibco, cat. 12430112) at 30 Hz for 4 minutes in a Retsch Mixer Mill with a 5.5 mm stainless steel grinding bead (MP biomedicals, 116540431) and 0.1 g of silica beads (Fisher, cat. 360991112). The sample was centrifuged at 15700 × *g* x 15 minutes. 1 ml of the supernatant was added to a 75 cm^2 flask (Sarstedt, cat. 83.3911.002) of passage 12 Eptesicus fuscus kidney cell line 3b (EfK3b) cells in 4 mL of D-MEM and incubated for 1 hour at 37°C. After incubation D-MEM containing 10% fetal bovine serum, penicillin, streptomycin, and amphotericin B (Antibiotic-Antimycotic, Gibco, cat. 5240062) was added to the flask. Seven days post infection 95% CPE was observed, and flasks were frozen at -80°C. This isolate was named Eptesipox virus/Saskatoon/01/2020 (EfPV/SK).

4.3.6 Electron microscopy and viral purification

Virus was purified from four 175 cm^2 flasks of EfK3b cells inoculated at a multiplicity of infection of 0.001 with EfPV. When 90% cytopathic effect was observed the supernatant was frozen and thawed 3 times followed by serial centrifugation at 4°C: 1500 g x 5 minutes (Sorvall Legend RT, Thermo Scientific) 10 000 × *g* x 15 minutes (Sorvall RC6 Plus, Thermo Scientific, Waltham), and 80 000 × *g* x 1 hour (Sorvall Wx Ultra, Thermo Scientific). Pellet was resuspended in 40 µl of PBS with 20 µl of 2% glutaraldehyde in 0.1 M sodium cacodylate stored at 4°C until imaging. Electron microscopy was performed as previously described(96). Virus was purified in a similar manner for genome sequencing by ultracentrifugation with 6 ml of 30% w/v sucrose cushion and resuspended in 50 µl 10 mM Tris pH 8.5.

For cellular electron microscopy one 175 cm^2 flask of EfK3b cells was inoculated with 10 µl of purified virus diluted in 5 ml of D-MEM and incubated for 1 hour at 37°C then complete media was added. After 18-, 24-, or 36-hours media and trypsinized cells were centrifuged at 325 g x 10 minutes (Sorvall legend, Thermo Scientific) the pellet was resuspended in 36 ml PBS and centrifugation repeated. Cells were fixed with 10 ml of 2% glutaraldehyde in 0.1 M sodium cacodylate and incubated at 4°C for 4 hours. They were pelleted with previous centrifugation step and resuspended in 1 ml of 0.1 M sodium cacodylate. Images were captured with the Hitachi

HT7700 transmission electron microscope and measurements taken with Image-Pro Premier version 9.3.3.

4.3.7 Genome sequencing

DNA was extracted from purified virus using the Qiagen DNeasy blood and tissue kit (cat. 69504). The sequencing library was constructed using Nexetra XT Library Preparation kit (Illumina, cat. FC-131-1024) according to the manufacturer's protocol. Eight picomolar library was sequenced using a Miseq platform in 2 X 250 cycles using a Miseq V2 500 cycle Nano kit (Illumina, cat. MS-103-1003). Reads were trimmed using Trimmomatic(415) sliding window 4:30 and minlen 36 resulting in 2501x coverage of the retained reads for EfPV/SK. Quality filtered reads were assembled into contigs using SPAdes 3.12.0(416) and mapped to the reference genome eptesipox virus strain Washington (EfPV/WA) NC_035460 using Geneious Prime 2022.0.1. Gaps between contiguous sequences were resolved using HotStartaq PCR (Table A 4), purification of products using MinElute PCR purification kit (Qiagen, cat. 28004) or gel extraction and purification with the QIAquick gel extraction kit (Qiagen, cat. 28706), followed by Sanger sequencing (Macrogen). The TOPO™ TA Cloning™ (Invitrogen, cat. K45000-40) was used to clone the PCR product from the largest gap into the topo vector and submitted for sequencing by Plasmidsaurus (Eugene, OR). A prediction of whether the amino acid variations in the coding portions of the genome between our isolate and the reference sequence would change their biological function was performed using PROVEAN 1.1.3 with a cut-off value of -2.5.

4.3.8 Phylogenetic tree

The concatenated amino acid sequences from the following viral proteins were aligned using MUSCLE in Geneious Prime 2022.0.1: RPO132, RPO147, VETF-L, RAP94, mRNA capping enzyme large subunit, P4a precursor, P4b, DNA topoisomerase I, VLTF-2, NPH-II, Holliday junction resolvase, DNA packaging ATPase, and DNA primase. The poxviruses that were used included: EfPV/SK OM638613, eptesipox WA NC_035460, hypsugopox 251170-23/2017 MK860688, pteropox NC_030656, cowpox Germany 1980 EP4 HQ420895, vaccinia WR AY243312, variola NC_001611, camelpox NC_003391, taterapox NC_008291, raccoonpox NC_027213, orf NC_005336, Yaba monkey tumor NC_005179, rabbit fibroma NC_001266, deerpox W-848-83 NC_006966, swinepox NC_003389, sheeppox 17077-99 NC_004002, cotia SPAn232 NC_016924, canarypox NC_005309, and salmon gillpox NC_027707. Choristoneura biennis entomopoxvirus 'L' was used as the outgroup, NC_021248.1. The maximum likelihood

method and Jones-Taylor-Thornton model was used to construct a phylogenetic tree on the concatenated amino acid sequence alignments using PhyML 3.3.20180621 with 1000 bootstrap replicates(466).

4.4 Results

4.4.1 Screening cases for EfPV

In July 2020 a big brown bat was submitted for multiple joint swellings from which EfPV was isolated, hereafter referred to as the index case. A retrospective search of the CWHC database for similar reports of viral infection identified one other case of suspected poxviral infection from 2019. PCR screening of DNA extracted from bat tissues from a random subsample of 135 submissions from 2017-2021 from big brown bats were tested and poxvirus DNA was detected by PCR in 2/135, the cases previously identified. Only three samples were tested from 2021. These cases were selected on the availability of tissue samples from 294 total submissions. No previous record of this virus was found within the CWHC database.

4.4.2 Histopathology

To characterize the lesions associated with infection, the slides from both PCR positive cases were reviewed in addition to other cases which had previously tested negative for EfPV by PCR. The 13 controls were selected based on the presence of similar lesions, types of tissue on slides, recentness of submission, and preservation of tissue. The oral and/or nasal mucosa of all cases and controls were examined and ulcers were present in the 2 positive cases and 4 of 13 controls. The oral and pharyngeal ulcers in the 2 cases in which EfPV was detected contained large pink intracytoplasmic inclusion bodies typical of poxvirus infections (Figure 4.1). Oral ulcerations in the control cases did not have this distinguishing feature. Ulcerations and proliferations of the epidermis of the wing were present in 1 of 2 cases but no inclusion bodies were observed and wing ulcerations were found in 3 of 14 controls. In the case of poxviral infection with multiple swollen joints all were affected by a moderate to severe neutrophilic infiltrate within the surrounding tissues and joint space sometimes mixed with fibrin. Thickening of the joint capsule was present in several joints characterized by hyperplasia of the synovium with spindle morphology (fibroblast-like synoviocytes). In only one joint was there necrosis within the surrounding tendons and muscle with mineralization and regeneration similar to the previous case report but was not the primary lesion in our case(56). Joints in 3 of 4 controls had neutrophilic infiltrates as well as thickening of the synovium. Multifocal erosions or ulcerations of the stomach mucosa were present

in 1/2 infected cases and 3/6 controls. An additional lesion present in 4/13 controls but not EfPV cases was focal to multifocal liver necrosis, a lesion that has been reported in cases of poxvirus infection(467). Gross lesions encompassing joint swelling and epidermal or mucosal ulcerations were non-specific. The only diagnostic feature of infection was intracytoplasmic inclusion bodies when present.

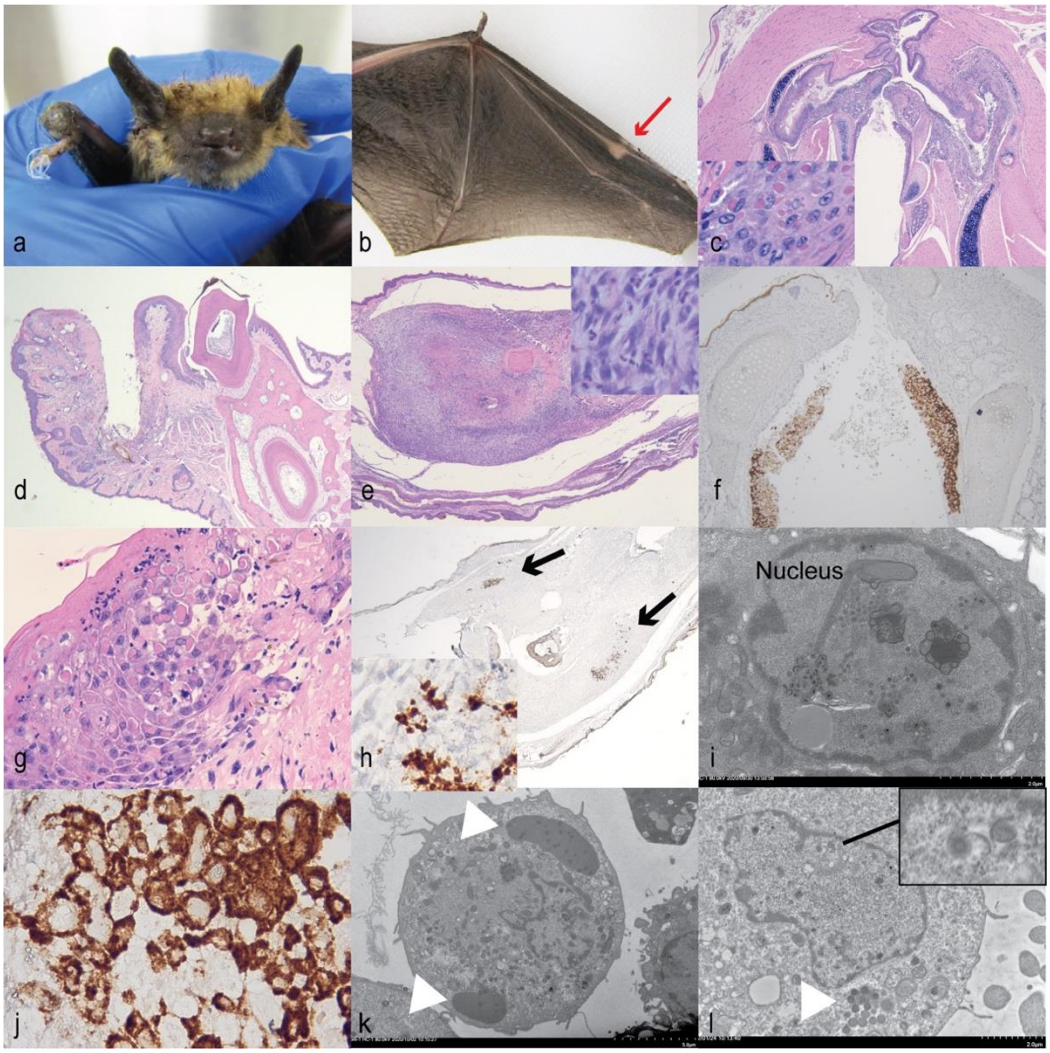

Figure 4.1 Imaging of poxviral lesions and viral cell culture. Index case from which virus was isolated a, b, d, e, g, h, j, k. Gross image of swelling and ulceration left lower lip and swelling of right carpus (a) with corresponding H&E-stained cross section of the mandible with extensive ulceration (d). Higher magnification image demonstrating large pink intracytoplasmic viral inclusions within the gingival mucosa (g) and ISH poxviral staining of a serial section (j). Multifocal swelling of the digital joints of the left wing (b). H&E staining of the metacarpophalangeal joint of the 2nd digit (red arrow b) with higher magnification inset demonstrating the spindle cell proliferation and neutrophilic infiltrate (e). ISH of serial section of joint with multifocal staining of eptesipox viral RNA (black arrows h) higher magnification inset. Microscopic images of the second case with extensive pharyngeal ulceration (c) and intracytoplasmic pink viral inclusion bodies in the adjacent epithelium (inset). Poxviral ISH staining of a serial section of the pharyngeal epithelium (f). Electron micrographs of EfK3b cells infected with EfPV (i, k, l). Large juxtanuclear replication factories in the cytoplasm (white arrow heads) k characteristic of poxviral infections (k), intranuclear viral replication (i), and intranuclear virus core and capsids and intracytoplasmic immature virions (white arrowhead) inset with higher magnification of virus core and capsids (l).
EfK3b - Eptesicus fuscus kidney cell line 3b, EfPV - Eptesipox virus, H&E - hematoxylin and eosin, ISH - in-situ hybridization

4.4.3 In-situ hybridization

To determine the association of viral nucleic acids with lesions, the same 15 cases reviewed for pathological changes were subjected to *in-situ* hybridization (ISH). Probe binding for the nucleic acids encoding the following structural proteins membrane-associated core protein and p4b precursor was only present in the mucosal and joint lesions from the PCR positive cases (Figure 4.1). There was no probe binding in the controls with or without similar lesions. Probe binding within the joints was too intense and obscured the nucleus and cellular features preventing the identification of the cell type. The ISH for EfPV was specific for EfPV nucleic acids and virus was associated with synovial proliferations and mucosal ulcerations.

4.4.4 Virus isolation

Transmission electron microscopy (TEM) was used to confirm the type of virus isolated from the bat tissues of the index case. Infected cells displayed juxtanuclear replication factories in the cytoplasm characteristic of poxvirus infection (Figure 4.1). The negatively stained purified virus had the characteristic size and shape of poxvirus, having a brick shape and measuring 222 nm long by 195 nm wide. At 36 hours post infection intranuclear viral replication was observed in one cell (Figure 4.1). Repeating the TEM at different time points demonstrated no intranuclear virus at 18 hours post infection but at 24 hours post infection two in approximately 15 cells had both intranuclear immature virions, viral capsid with core as well as evidence of intracytoplasmic replication (Figure 4.1). The diameters of the capsids in the nucleus were 118 ± 36 nm whereas the intracytoplasmic immature virions were 248 ± 16 nm.

4.4.5 Genome sequencing

A *de novo* assembly of short read data was performed to determine whether this was a new species or matched the reference sequence for EfPV (NC_035460). The isolate named Eptesipox virus/Saskatoon/01/2020 shared 99.7% nucleotide identity to the EfPV Washington strain NC_035460 indicating the isolate was EfPV(465). The sequence was deposited in Genbank accession number OM638613. Between the two isolates there were 5 deletions, 3 insertions, 89 transitions, 17 transversions, 5 substitutions, and 16 insertions or deletions in tandem repeats (Table A 5, Table A 6). These variations were less frequently present in the centre of the genome between 60 000-120 000 bp.

4.4.6 Phylogenetic analysis

A phylogenetic tree was constructed because of genetic differences in the new isolate of EfPV and to include another poxvirus from the same host genus *Eptesicus*, which led us to confirm the relationship of EfPV to other bat pox viruses (Figure 4.2)(56,63,465). Proteins were chosen based on previous phylogenies of other members of the Poxviridae family(63,465,468,469). Of the 13 protein sequences examined only 2 differed in their amino acid sequence from the reference sequence NC_035460: the mRNA capping enzyme large subunit (1/844 amino acids) and P4a (1/908 amino acids). Although the difference in the mRNA capping enzyme may not represent a true difference because a nucleic acid could not be resolved.

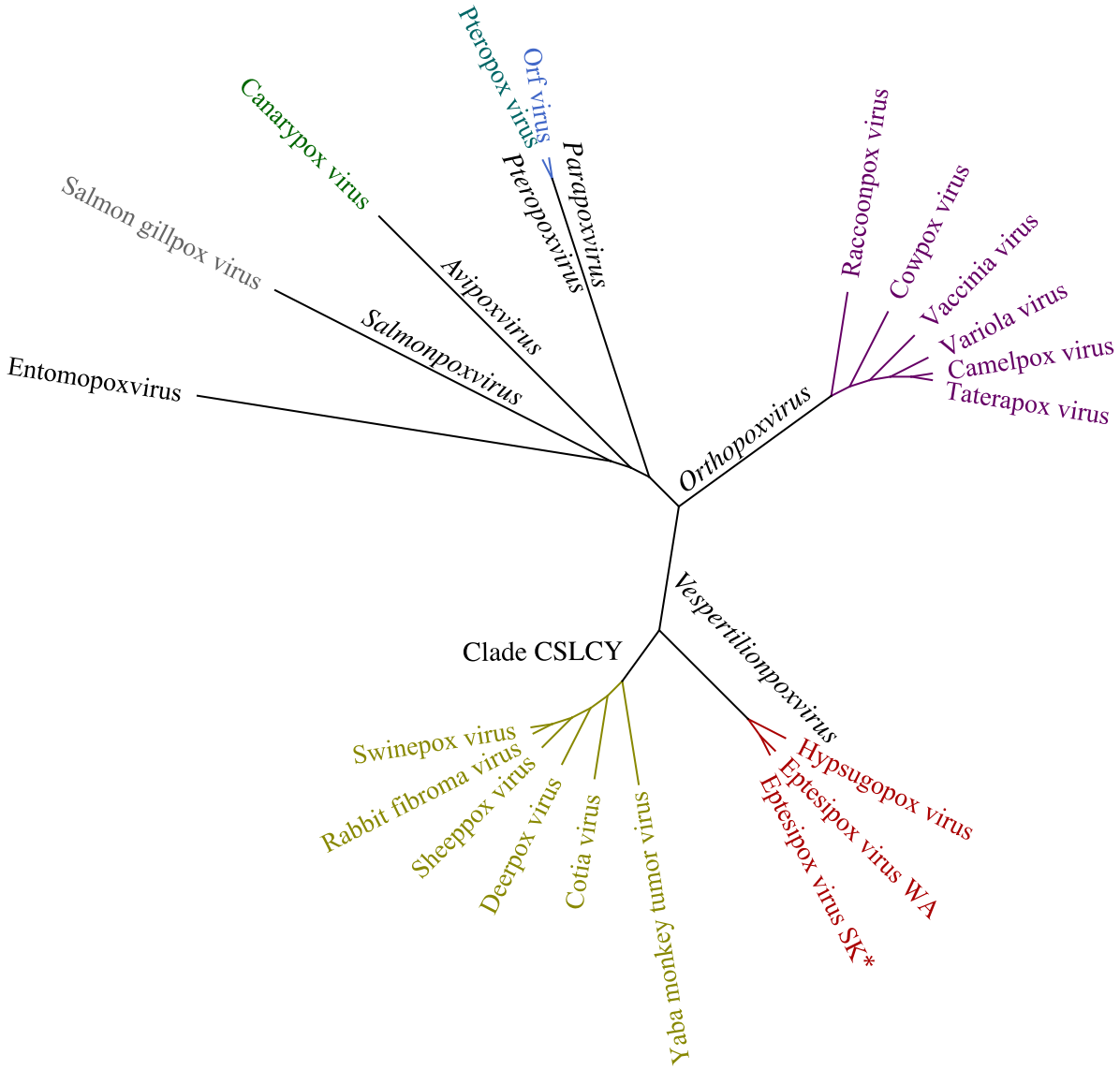

Figure 4.2 Maximum likelihood phylogenetic tree analysis of poxviruses using concatenated amino acid alignments of 13 genes rooted on an Entomopoxvirus. All other viruses belong to the *Chordopoxvirinae* subfamily and genera of the branches are in black. The new isolate Eptesipox virus/Saskatoon/01/2020 is indicated by an *.
CSCLY: Capripoxvirus, Suipoxvirus, Leporipoxvirus, Cervidpoxvirus, and Yatapoxvirus genera.
WA - Washington, SK - Saskatoon

4.5 Discussion

Our study illustrates that bats can develop significant disease associated with EfPV infections and, although not severe enough to directly result in death, ultimately these lesions could be fatal due to impaired flight, impaired feeding and/or secondary infections. Some of the lesions displayed in these cases are typical for poxviruses, such as proliferation and ulceration of the epithelium, but there are also atypical lesions in the joints. Infection with certain species of poxviruses in specific hosts can result in a systemic infection with variable necrotizing or proliferative lesions in multiple internal organs, but this was not observed in these two cases(470,471). Joint lesions, as seen in one of these cases, have not been described in poxviral infections, except for variola virus infections where it is an uncommon presentation(472). Detailed histopathological descriptions of osteomyelitis variolosa are lacking some have identified epiphyseal involvement and this manifestation of disease usually affects children(472,473). The joint lesions in bats with EfPV were primarily synovial and periarticular and detailed description of the parts of the bone affected in the original isolate is missing(56). There appears to be systemic involvement with EfPV infection (joint lesions and isolation of infectious virus from internal organs) and there is potentially a large spectrum of lesions and clinical manifestations that are unrecognized.

Several of the affected joints showed limited proliferative change and were negative with ISH, the pathogenesis may not be identical amongst the affected joints. Alternatively, this lack of probe binding could be sectioning artefact where the multifocal distribution of viral staining in the affected joint may have been missed. We also cannot definitively exclude co-infection as a possibility for the observed pathological changes.

Despite previous *in vitro* demonstration of multiple cellular mechanisms in bats that dampen the innate and inflammatory response to DNA we observed a marked inflammatory response in affected joints(1). Neutrophils display protective effects against viruses but can also contribute to disease primarily through tissue destruction by the release of granule contents(474). Experimental poxviral infections have induced strong neutrophil responses(475,476). To explain the selective nature of the inflammation poxviral chemokine binding proteins that inhibit leukocyte migration are likely to be involved. These chemokine binding proteins have been shown to either selectively inhibit monocyte chemokines but not those of neutrophils or both interfering migration of these leukocytes(477,478). The function of the EfPV chemokine binding protein is proposed to

be selective inhibition of monocyte migration based on the lesion. We have proposed several potential mechanisms of pathogenesis (Figure A 3).

Our unexpected observation of intranuclear viral replication (Figure 4.1) is not unusual for the nucleocytoplasmic large DNA viruses to which poxviruses belong(479,480). Of this group the only other viruses to replicate exclusively in the cytoplasm are mimiviruses(481,482). The lack of nuclear involvement in poxvirus replication has made for a conundrum in how poxviruses acquire host genes through horizontal gene transfer(461,483). The majority of horizontal gene transfer events between viruses and eukaryotes have been identified in the nucleocytoplasmic large DNA viruses, including poxviridae, supporting some type of nuclear involvement in their replication(484). An alternative method of horizontal gene transfer is the inclusion of host genes via reverse transcription and this method is supported by the fact that poxviral genes lack introns(485). However, these introns could be lost through different mechanisms (486,487). This observation requires confirmation and may be an artefact of the cell line or cell culture. Electron micrographs of penguin pox virus infections has also demonstrated intranuclear immature viral particles(488). These findings could be artefacts secondary to the breakdown of the nuclear envelope. However, light microscopy has provided evidence for nuclear involvement in poxvirus infections by identifying intranuclear inclusions(489–492), although inclusions do not always reflect active viral replication as in the electron micrograph of the skin nevus (freckle) associated with molluscum contagiosum virus(491). Since the virus particles in the nucleus were smaller than what we observed in cytoplasmic replication we cannot rule out the possibilities of co-isolation of a virus that replicates in the nucleus like a herpesvirus, or contamination of the viral culture. The bat from which the EfPV was isolated tested negative via PCR for Eptesicus fuscus gammaherpesvirus.

Whole genome sequencing identified our viral isolate as a previously described Eptesipox virus(56,465). There were differences between the sequences and some of these changes altered the amino acids of predicted proteins, which could result in functional changes between these isolates. The international committee on taxonomy of viruses recently introduced the genus *Vespertilionpoxvirus* to the Poxviridae family, which includes EfPV as the sole species(493). Hypsugopox virus is closely related to EfPV and currently remains unclassified. It likely belongs in the same genus as similarities in the aligned amino acid sequences range from 76.7 to 95.1 % for individual proteins and 88.7% for the concatenated sequences (Figure 4.2). The hosts of these

viruses, *Eptesicus fuscus* and *Hypsugo savii*, belong to the same Vespertilioninae subfamily despite living on different continents(494).

The limitations of this study are a small sample size and a design that does not allow us to determine a causal relationship between viral infection and the lesions observed. There are several clinical forms of variola recognized which if they also develop in bats with poxvirus could be easily missed due to their reclusive and nocturnal nature(495). Although joint involvement seems a prominent feature of infection this may be because these bats were cared for by rehabilitators and survived for a prolonged period. The spectrum of disease associated with Eptesipox infection may be broader than what is currently described. Although it would be ideal to fulfill Koch's postulates with this virus there are many impracticalities to working with wildlife like additional ethical considerations, availability of captive bred bats, or the ability to maintain wild bats in a low stress environment.

Studying the host virus interaction between bats and their viruses has opened the door to many unanswered questions. By examining the biology of wildlife viruses, we can learn much about virus-host interactions that may be overlooked when using traditional models. The applications of this research could be for a model to understand virally induced arthritides and other chronic inflammatory joint conditions. Most importantly, the results of our current study suggest that bats do not have a special, tolerant relationship with EfPV and display significant disease associated with infection. Big brown bats have an extensive host range throughout North America and have been relatively unaffected by white nose syndrome; however, these lesions raise concerns of co-infections and the effects this may have at the population level and the referred effects on their ecosystem services(496,497).

Preface to chapter 5

In Chapters 3 and 4 we isolated two DNA viruses from big brown bats. The poxvirus was associated with obvious lesions whereas no lesions were associated with the γHV. For the poxvirus we demonstrated that bats do develop disease associated with viral infection which is inconsistent with the hypothesis that bats are tolerant to their viruses and resistant to disease. To fulfill our last and fourth objective in Chapter 5, we examine whether the innate response to DNA of big brown bat cells is different from human cells and whether there is a difference between our two isolated DNA viruses. Big brown bat cells had high basal levels of expression of a proinflammatory gene and actively suppressed the expression of another, which are novel findings. Infection with both viruses generated an innate response which was controlled by the γHV. Therefore, it does not appear as though bats are tolerant to their viruses and the innate response is dependent on the individual virus.

CHAPTER 5 INNATE RESPONSE OF EPTESICUS FUSCUS KIDNEY CELLS TO DOUBLE STRANDED DNA AND E. FUSCUS DNA VIRUSES

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5.1 Abstract

Bats are reservoirs or probable reservoirs for numerous viruses that have caused multiple epidemics and pandemics in other species including humans. A proposed key feature of this role as a reservoir is their tolerance to viruses without developing disease. An overactive inflammatory response leads to disease associated with many viral diseases. Several studies suggest that bats have evolved various mechanisms for suppressing inflammation following infection with RNA viruses, while maintaining a robust antiviral interferon (IFN) response. Interplay between bats and DNA viruses has been less studied. Mechanisms that sense DNA viruses in other mammals are eliminated or repressed in bats, thereby muting the response to DNA viruses. Most of the studies examining bat-virus interactions have examined relatively few bat species, often involving viruses that are not autochthonous to the species studied. Chiroptera are a diverse order and mechanisms for modulating innate anti-viral responses may vary between species. Here we describe the ability of cells derived from big brown bats (*Eptesicus fuscus*) to respond to poly deoxyadenylic-deoxythymidylic (poly(dA:dT)), a surrogate of viral DNA. We also examined the response of *E. fuscus* cells (EfK3b) to infection by Eptesicus fuscus gammaherpesvirus (EfHV) and Eptesipox virus (EfPV) isolated from *E. fuscus* bats. Similar to human cells, EfK3b cells responded to poly(dA:dT) with a robust increase in the expression of genes for IFNβ and IFNλ, and the pro-inflammatory cytokine, tumour necrosis factor alpha (TNFα). In contrast to human cells EfK3b cells did not increase expression of the multifunctional interleukins (IL) 6 and 8, although EfK3b cells possessed constitutively high levels of IL8. Specific suppression of individual transcription factors suggested that the reaction of EfK3b cells to poly(dA:dT) was transduced by the interferon regulatory factors (IRF) 1 and 3 and nuclear factor kappa B (NFkB). The response of EfK3b cells to EfPV, which is associated with disease and EfHV, which results in long-term asymptomatic infections, differed markedly. Infection with EfPV stimulated an increase in the expression of the IFN and TNFα genes and viral gene expression was required for this response. In contrast, EfHV actively suppressed the expression of the interferons and TNFα.

5.2 Introduction

Bats are often described as having a special relationship with their viruses by having evolved to tolerate viruses in the absence of disease(1,498). These evolutionary adaptations to flight are hypothesized to suppress overt inflammation and enhance antiviral mediators following infection by RNA viruses, although the mechanisms for this vary between species of bats(37,458). Several

DNA viruses, including the herpesvirus and poxvirus described elsewhere in this thesis (Chapters 3 and 4), are known to parasitize bat species but the innate immune mechanisms that control these infections have not been examined. Bats have lost an entire family of DNA sensors the pyrin and hematopoietic interferon-inducible nuclear domain-containing protein (PYHIN) family and have decreased activation of a signal transducer from many DNA sensors, stimulator of interferon genes, STING(6,10). Although there is contraction of some innate immune genes in bat genomes there is also the expansion of others like apolipoprotein B mRNA-editing enzyme, NOD-like receptor protein 3, DNA protein kinase, and protein kinase R(499–501). The effects of these evolutionary changes on the ability of bats to resist or recover from diseases caused by DNA viruses is not known.

Activation of pattern recognition receptors results in a cascade of effects and is often measured by the production of signalling molecules, cytokines(502). Transcription factors IRF 3, 7, and nuclear factor kappa B (NFkB) are considered regulators of this response(503). Some studies have demonstrated a role for the interferon regulators factors (IRF) 1, 3, and 7 in the infection of *P. alecto* cells by the human herpes simplex virus(504). However, there are few studies on how particular species of bats respond to their own viruses. The result of cytokine signalling is often inflammation or induction of an antipathogen state. IFNs are a major class of cytokines and are subdivided into three types I, II, and III. Type I IFNs are produced by a wide array of cells and have many immune modulating effects but are primarily antiviral. In bats the type I IFN gene locus is under selection with both contraction and expansion of genes in bats(505). Expression patterns of type I IFNs are different with constitutive expression of IFNα and higher induction of IFNβ(27,506). Like Type I IFNs, Type III IFNs have broad cellular expression and induce an antiviral state but the expression of their high affinity receptor is restricted. With the restriction of this receptor in humans IFNλ is thought to primarily effect an antiviral response at the mucosal surface(507) although a larger role in immune regulation is becoming clear(508). Bats have a broader tissue expression profile of this receptor but it is unknown how this affects the innate response(509,510).

ILs are another class of cytokines involved in the innate response. They have varied functions but primarily are involved with: inflammation, leukocyte recruitment and activation, and cellular growth and differentiation(511). Less is known about the differences in bat ILs but IL-10, which has an anti-inflammatory function, is expressed at sustained high levels in *Myotis*

myotis(512). TNFα another cytokine commonly produced in response to viral infections is proinflammatory. In *E fuscus* cells its transcription is inhibited in response to stimulation with an RNA surrogate polyinosinic:polycytidylic, poly(I:C)(27).

Most of the knowledge we have regarding alterations in bat immune systems have come from in silico analysis of genomes, RNA surrogate experiments, or *in vitro* infections of a non-host species. There is little published information on the response of bats to infection by DNA viruses.

We have previously isolated DNA viruses from big brown bats; one causes a lifelong usually quiescent infection of the host, EfHV; the other induces ulcerations and joint swellings with neutrophilic infiltrate, EfPV. Our objectives were to determine: 1) if cells derived from big brown bats (EfK3b) could respond to cytoplasmic double stranded DNA (dsDNA), a molecular pattern of DNA virus infection, 2) if the response was comparable to that of human cells (MRC5), and 3) whether the cellular response of EfK3b cells was the same to these DNA viruses.

5.3 Materials and methods

5.3.1 DNA sensor detection

To determine which DNA sensors were expressed in EfK3b cells RNA was extracted using the QIAGEN RNeasy plus mini kit (cat. 74134) as per manufacturer's protocol. Complementary DNA (cDNA) was synthesized with the iScript gDNA clear cDNA synthesis kit (Bio-Rad, cat. 172-5034) using 350 ng of RNA, a control without reverse transcriptase was included. The cDNA was diluted to a final volume of 200 µl and 5 µl was used in the polymerase chain reaction (PCR) reaction. PCR was performed as per the manufacturer's protocol using HotStarTaq (Qiagen, cat. 203205) with a final reaction volume of 50 µl and 5 µm of each primer. The thermocycler protocol was initial enzyme activation at 95°C for 15 minutes seconds, 35 cycles of denaturation at 94°C for 30 seconds, annealing variable temperature for 30 seconds, and extension at 72°C for 1 minute, with a final extension at 72°C for 10 minutes. Primers were designed using Primer-BLAST(513) see Table A 7 for primer sequences and annealing temperature. The no reverse transcriptase cDNA was used as a control to ensure amplification was specific to the RNA template and not genomic DNA. Products were separated on a 1% agarose gel at 110 V for 45 minutes.

5.3.2 Measuring innate response

MRC5 or EfK3b cells were maintained in Dulbecco's modified medium (D-MEM, Gibco, cat. 12430112) supplemented with 10% fetal bovine serum and penicillin streptomycin incubated

at 37°C in 5% CO_2. Cells were seeded into 6 well plates at a density of $3.3x10^5$ cells/well and 24 hours later media was replaced with D-MEM without antibiotics. The experiment was performed using three biological replicates. Cells were then transfected with 1.5 μg/well poly(dA:dT) (InvivoGen, cat. tlrl-patn) with 12 μl/well lipofectamine 2000 (Invitrogen, cat. 52887) following manufacturer's protocol and diluted in opti-MEM (Gibco, cat. 31985070). RNA was extracted as described following incubation for 0, 1, 3, 6, 12, or 18 hours after transfection. cDNA was synthesized as described using 640 ng of RNA and was diluted to a final volume of 200 μl.

Quantitative PCR (qPCR) was performed on each of the samples measuring the innate response (Table A 8). For each qPCR reaction 15 μl of SsoFast EvaGreen supermix (Bio-rad, cat 172-5204), 5 μl of template, and 4 μm of each primer was used. The thermocycler program was enzyme activation 95°C for 30 seconds, denaturation 95°C for 5 seconds, and 40 cycles of annealing/extension at variable temperatures for 5 seconds. Increments of 0.5°C were used to perform the melt curve from 65-95°C in two second steps. Primers were designed using Primer-BLAST(513) annealing temperatures were optimized using a temperature gradient. Primer dimers were avoided by visualizing products with gel electrophoresis and only those with a single melt peak were chosen. Once optimized, primer efficiency within the range of 95-105% was confirmed with standard curve. Newly designed primer products were purified using MinElute PCR purification kit (Qiagen, cat. 28004), followed by Sanger sequencing (Macrogen). BLAST was used to confirm the correct target was amplified. See Table A 8 for primer details. Small interfering RNA knockdown of dsDNA sensor mediators EfK3b cells were seeded and cultured as previously described and small interfering RNAs (siRNA) targeting *Eptesicus fuscus* IRF1, IRF3, IRF7, and NFκB1 were used. These siRNAs were designed using RNAi Design Tool from Integrated DNA Technologies and 3 different siRNAs were chosen per target except for IRF7 where there was only a single option(514). The concentration of each siRNA was optimized by performing a dose response curve and only those with a minimum acceptable inhibition of 80% were used for further study, these can be found in Table A 9. Three biological replicates were used for each optimized siRNA. Twenty hours later cells in 6 well plates were transfected with 7.5 ul/well Lipofectamine RNAiMAX (Invitrogen, cat. 13778-030), 20 pmol/well of siRNA IRF1, IRF3, IRF7, and negative control nontargeting (IDT, cat. 51-01-14-03) or 2 pmol/well of NFκB1 and negative control nontargeting siRNAs. All reagents were diluted in opti-MEM. Cells were incubated for 42 hours

then transfected with poly(dA:dT) and RNA was extracted 6 hours post transfection, 1 μg of RNA was used in cDNA synthesis followed by qPCR as previously described.

5.3.3 Cellular response to DNA viruses

EfK3b cells were seeded and incubated in 6 well plates as previously described and 24 hours later cells were mock infected or infected at a multiplicity of infection of 5. Cells were mock infected using D-MEM, infected with live or ultraviolet (UV) inactivated EfPV or EfHV using 500 μl of inoculum and incubated for 1 hour at 37°C with rocking every 5 minutes. Viruses were inactivated by UV treating virus stock diluted in D-MEM to a final volume of 1.5 ml in a 35 mm dish for 10 minutes 8 cm from a 254 nm UV light. Efficacy of inactivation was previously confirmed using median tissue culture infectious dose with a decrease from 63000 and 35400 focus forming units per ml for herpesvirus and poxvirus respectively to 35.4 focus forming units per ml. Six hours post infection RNA was extracted and qPCR performed as described previously.

5.4 Results

5.4.1 Eptesicus fuscus cellular DNA sensors

To determine which cellular dsDNA sensors EfK3b cells express, we used PCR to detect transcripts for various genes known to sense DNA in other mammalian species (Figure 5.1). The cells expressed all DNA sensors tested, the PYHIN family was excluded because they are missing in bats. This demonstrates the potential for a varied cellular response to dsDNA in bat cells.

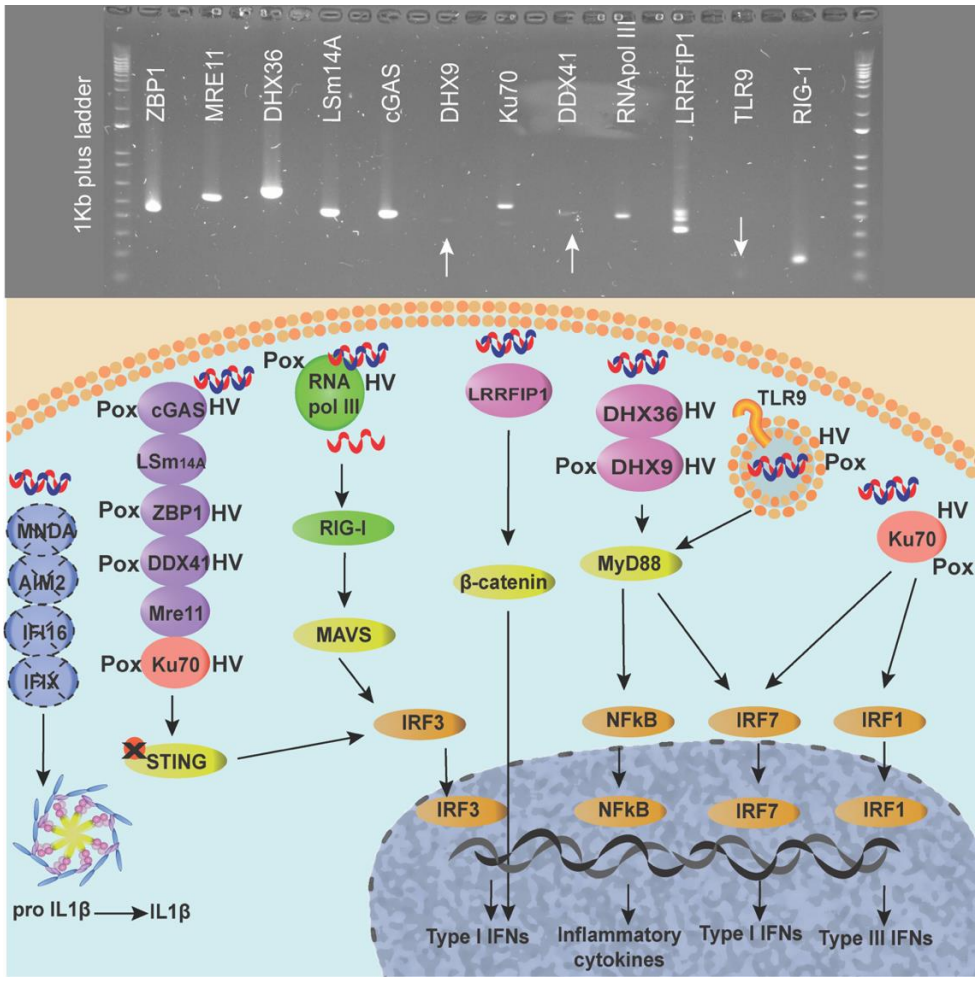

Figure 5.1 DNA sensors expressed in EfK3b cells with schematic of sensor signalling involved in innate response. Gel electrophoreogram of DNA sensors in EfK3b cells, product amplified from cDNA (top) loaded in the respective labelled lane with the no reverse transcriptase cDNA negative control in the consecutive lane. Arrows indicate faint bands. The various dsDNA sensors are illustrated in the adapted schematic(515,516), the PYHIN family (dashed lines) is missing in bats. STING signalling is decreased by the alteration of an amino acid residue that activates the protein when it is phosphorylated in other species. The sensors implicated in poxviral or herpesviral recognition are designated by Pox or HV, respectively.

AIM2 - absent in melanoma 2, cDNA - complementary DNA, cGAS - cyclic GMP-AMP synthase, DDX41 - DEAD-box helicase 41, DHX - DexH-box helicase, dsDNA - double stranded DNA, HV - herpesvirus, IFI16 - interferon-gamma inducible protein 16, IFIX - interferon-inducible protein X, IRF - interferon regulatory factor, Ku70 - Ku autoantigen 70 kDa, LRRFIP1 - leucine-rich repeat flightless-interacting protein 1, LSm14A - like Sm14A, MAVS - mitochondrial antiviral signaling protein, MNDA - myeloid cell differentiation antigen, Mre11 - meiotic recombination 11 homolog A, MyD88 - myeloid differentiation primary response 88, NFκB - nuclear factor kappa B, Pox - poxvirus, PYHIN - pyrin and hematopoietic interferon-inducible nuclear domain-containing protein, RIG-I - retinoic acid-inducible gene I, RNApol III - RNA polymerase III, STING - stimulator of interferon genes, TLR9 - toll-like receptor 9, ZBP1 - Z DNA binding protein 1

5.4.2 Bat and human innate response to a DNA surrogate

To assess the innate immune response to cytosolic dsDNA in bat cells, and compare the response to that in human cells, we stimulated EfK3b cells (bat) and MRC5 cells (human) with poly(dA:dT), a chemical surrogate for viral DNA. Cells were harvested prior to stimulation (0 hr) and at 1, 3, 6, 12 and 18 hr following stimulation. The levels of transcripts (relative to 0 hr) for interferons IFNβ and IFNλ, and the pro-inflammatory cytokines TNFα, IL6 and IL8 were determined (Figure 5.2).

In both bat and human cells, the levels of transcripts for the IFNβ, IFNλ, and TNFα increased rapidly following stimulation (Figure 5.2 A, B, C).The pattern of increase was similar in bat and human cells, except that for TNFα human cells reached the peak of stimulation before bat cells (Figure 5.2 C).

Human cells responded to poly(dA:dT) stimulation with approximately 100-fold increase in transcripts for IL6 and IL8 genes. In contrast, bat cells did not respond (Figure 5.2 D, F). To determine if bat cells constitutively expressed high levels of the IL genes and therefore did not respond, we calculated the results as ΔCt (Figure 5.2 E and G), rather than relative to 0 hr. ΔCt values are an indication of absolute levels of transcripts and have an inverse relationship with fold change. A high ΔCt value indicates a lower amount and vice versa. All values were normalized to GAPDH, a "housekeeping" gene with similar levels in all samples.

Before stimulation with poly(dA:dT) human cells contained low levels of transcripts for both IL6 and IL8 with an increase following stimulation. Bat cells contained low levels of transcripts for IL6 and these remained low following stimulation (Figure 5.2 E). In contrast, bat cells contained high levels of transcripts for IL8, which remained high following stimulation (Figure 5.2 G).

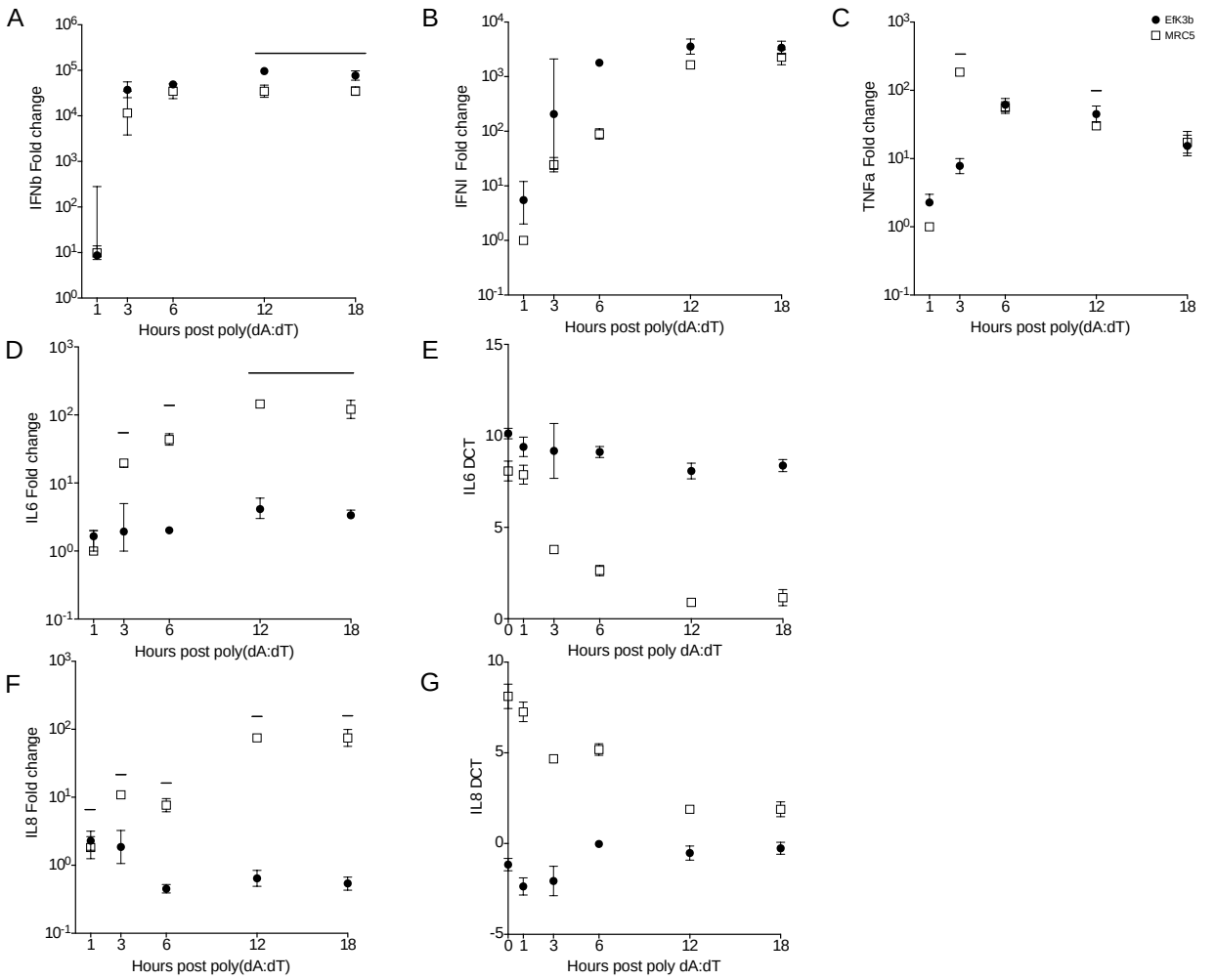

Figure 5.2 Time course of relative fold change of innate response in EfK3b and MRC5 cells with poly(dA: dT) stimulation. (A-D and F) RNA was quantified at 0, 1, 3, 6, 12, and 18 hours post-stimulation with poly(dA:dT) and normalized to an internal housekeeping gene GAPDH. Points represent fold change relative to 0 hours from 3 biological replicates with error bars indicating 2^(-ΔΔCT±sem). MRC5 and EfK3b mean ΔCT values were compared using an unpaired two tailed Holm-Sidak t-test α=0.05. P value *<0.05, ** <0.01, ***<0.001, ****<0.0001. (E and G) ΔCT for IL6 and IL8, respectively. High values indicate lower expression and low values indicate higher expression. Error bars represent standard error of the mean.

CT - cycle threshold, IFN interferon, IL - interleukin, IRF - interferon regulating factor, MRC5 - medical research council cell strain 5, NC - negative control, NFkB - nuclear factor kappa B, siRNA - small interfering RNA, TNFα - tumor necrosis factor α

5.4.3 Multiple transcription factors involved in innate response

In humans and rodents, the expression of genes for interferons and cytokines is regulated by the transcription factors IRFs 1,3 and 7, and NFkB (Fig 5.1). To determine if these factors were involved in the bat cellular innate response to poly(dA:dT), we individually and specifically reduced the mRNA for these factors in bat cells and then assessed the expression of IFN and cytokine genes.

To specifically abrogate transcripts for the transcription factors, we treated EfK3b cells with small interfering RNA (siRNAs) targeted to individual IRFs. As a control, cells were treated with siRNA with a non-targeting sequence. Compared to this control siRNA, the factor specific siRNAs eliminated detectable mRNA for the transcription factors (80-100% inhibition, Figure 5.3 A).

Specific and control siRNA-treated cells were then stimulated with poly(dA:dT) and transcripts for IFN and cytokine genes were measured. When compared with expression in the presence of control siRNA, inhibition of IRF 1 and 3 significantly reduced poly(dA:dT) enhancement of IFNλ, inhibition of IRF1 and NFκB significantly reduced the enhancement of IFNλ and the inhibition of IRF1, 3 and NFκB reduced TNFα (Figure 5.3 B,C and D).

Consistent with our observation that IL8 is constitutively expressed in bat cells, the knockdown of the transcription factors had no effect on the levels of IL8 (Figure 5.3 F). We had shown that bat cells contained low levels of IL6 transcripts, and that dsDNA did not stimulate IL6 expression. The suppression of IRF1, 3, and especially NFκB, led to increased expression of IL6 transcripts (Figure 5.3 E). These results suggest that the expression of IL6 may be actively suppressed in bat cells either directly, or indirectly by NFκB and perhaps by IRFs 1 and 3.

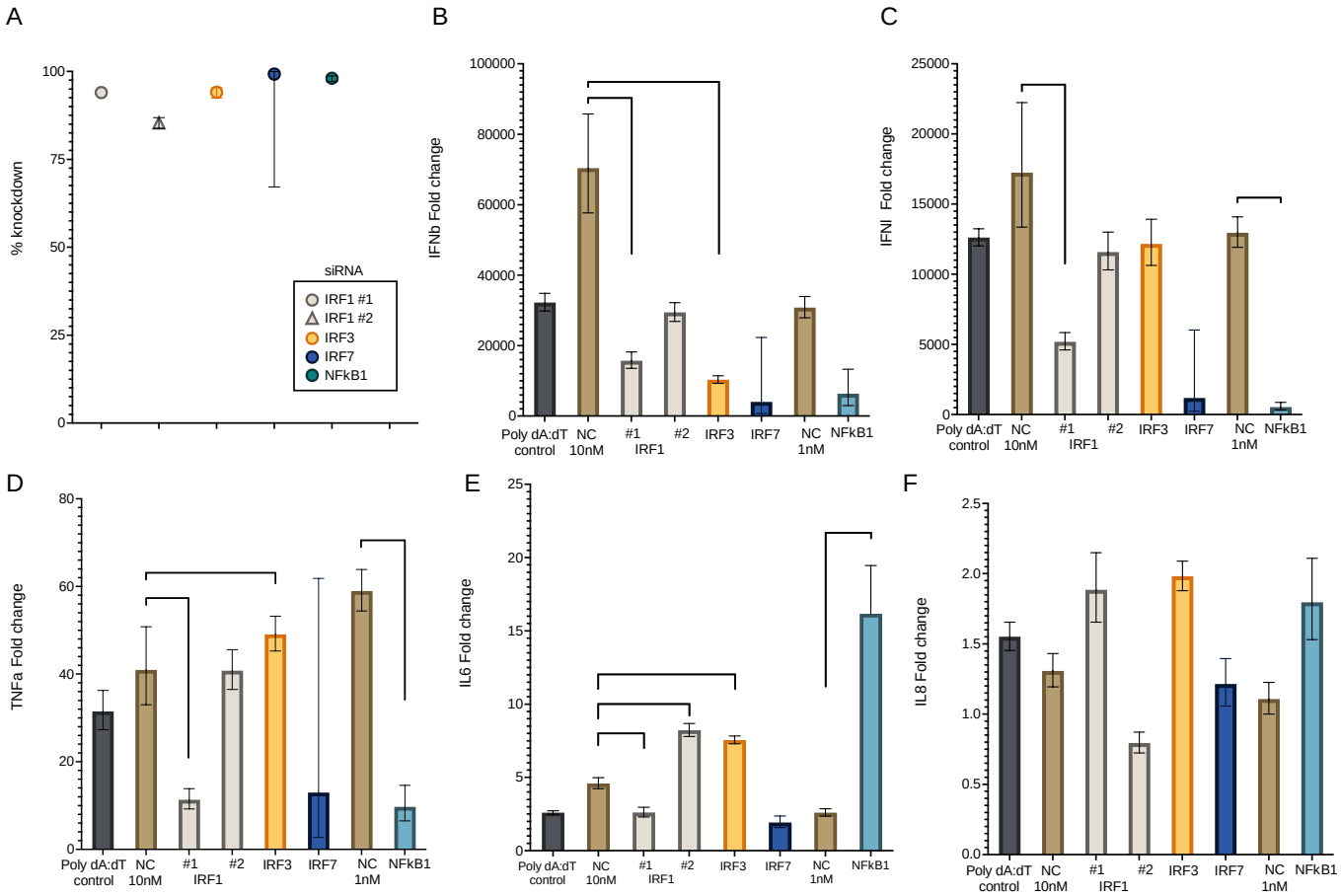

Figure 5.3 Effects of siRNAs targeting IRF1, IRF3, IRF7, and NFkB1 on innate response after challenge with poly(dA:dT) in EfK3b cells. The amount of mRNA degradation by each of the siRNA treatments is expressed as % knockdown (A). Bars represent the relative fold change compared to 10 nM negative control siRNA for IRF 1, 3, and 7 siRNAs or 1 nM for NFκB1 siRNA, values were normalized to RPS11 (B, C, D, E, and F). Error bars are 2^(-ΔΔCT±sem). Comparisons were made between the treatments and a non-coding siRNA control using an unpaired two tailed Holm-Sidak t-test α=0.05 p-values * <0.05, ** <0.01, and *** <0.001.
EfK3b - Eptesicus fuscus kidney cell 3b, IFN interferon, IL - interleukin, IRF - interferon regulating factor, NC - negative control, NFkB - nuclear factor kappa B, siRNA - small interfering RNA, TNFα - tumor necrosis factor α

5.4.4 Response of bat cells to viral dsDNA and viral infection

Our initial experiments were designed to assess the ability of bat cells to respond to cytosolic dsDNA, a hallmark of infection by DNA viruses. Since most viruses have active mechanism for suppressing the host cell's innate immune responses, we stimulated cells with poly(dA:dT) rather than virus. To assess the cellular innate response to virus infection, we infected EfK3b cells with a gammaherpesvirus (EfHV) and a poxvirus (EfPV), both isolated from naturally infected *E. fuscus* bats the same species the cell line is derived from. We have found EfPV to be associated with disease (this thesis, Chapter 4) whereas we were not able to link EfHV with deleterious effects in infected bats (this thesis, Chapter 3). To assess the ability of these viruses to actively suppress cellular innate responses we compared the effect of infecting cells with UV-inactivated virus or replication competent virus. UV-inactivated virus would infect cells, but infection would not progress to viral gene expression or replication. Figure 5.4 A shows that UV treatment reduced the infectivity of both viruses by more than 99% (reduction of $>10^3$ fold).

For IFNβ, IFNλ and TNFα the response of EfHV and EfPV differed. EfPV infection enhanced the expression of the three genes and viral gene expression was required for this enhancement as there was a significant difference when UV inactivated virus was used for infection (Figure 5.4 B, C and D, right panels). In contrast, while UV inactivated EfHV increased gene expression of the innate response genes, infection with gene expression competent EfHV caused no increase in expression (Figure 5.4 B, C and D, left panels). Infection with either EfHV or EfPV caused an increase in levels of IL6, although the increase for EfHV was modest. Viral gene expression was required for this increase as it was not seen with UV-inactivated virus (Figure 5.4 E). Consistent with our previous results from poly(dA:dT) experiments, infection had no effect on the expression of IL8 (Figure 5.4 F).

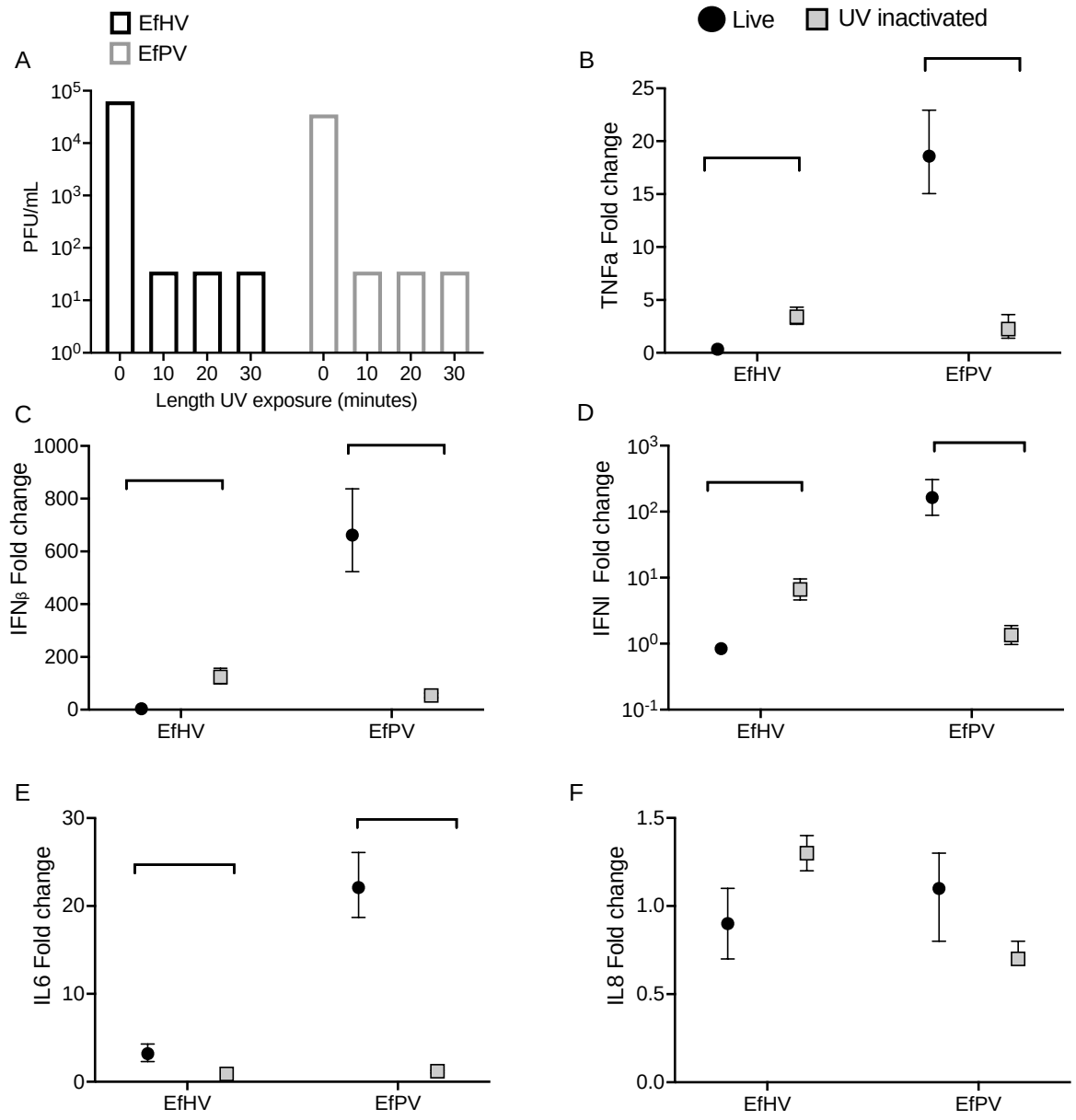

Figure 5.4 *In vitro* innate response in EfK3b cells to EfHV and EfPV infections with live or UV inactivated virus. Reduction of infective virus by UV treatment (A). RNA was quantified 6 hours post infection and normalized to an internal control RPS11 (B, C, D, E, and F). Points represent relative fold change to mock infected controls from 3 biological replicates with error bars indicating 2^(-ΔΔCT±sem). Comparisons between UV and live virus were made using an unpaired two tailed Holm-Sidak t-test α=0.05. P-values * <0.05, ** <0.01, and *** <0.001.
EfK3b - Eptesicus fuscus kidney cell 3b, EfHV - Eptesicus fuscus gammaherpesvirus, EfPV - Eptesipox virus, IFN interferon, IL - interleukin, IRF - interferon regulating factor, NC - negative control, NFkB - nuclear factor kappa B, siRNA - small interfering RNA, TNFα - tumor necrosis factor α

5.5 Discussion

Evolutionary adaptations for flight are thought to have conferred on bats unique relationships with their viruses such that bats tolerate most of their viruses without overt disease and disease. Potentially this allows bats to harbour many viruses that then spill over into other mammals causing serious and often fatal disease. This dogma is based on several studies on the relationship of bats, or cells derived from bats, with RNA viruses and surrogate molecules that mimic RNA virus infection(6,27,517–522). These studies suggest that bats respond to infection, or the presence of RNA viral surrogates, by actively suppressing excessive and destructive inflammation while enhancing antiviral interferons. There have been very few attempts to examine the relationship of bats with DNA viruses. One such study found that many species of bats lack some of the cytoplasmic DNA sensors present in other mammalian species(10). There is also a paucity of published research on the relationship of bat species with co-evolved viruses. To address this, we examined the innate response of bats to DNA and host adapted DNA viruses.

Our objective was to determine: if cells derived from *E. fuscus* could recognize and respond to a surrogate of viral DNA, how this response differed from that of human cells and whether transcription factors implicated in innate immune responses in better studied mammalian species also regulated the response in *E. fuscus* cells. We also examined how the bat cells responded to infection with viruses that are endemic in *E. fuscus* populations and may have coevolved with them.

Here we report that EfK3b cells do have the capacity to respond to poly(dA:dT), a surrogate of cytosolic DNA which is sensed by cells as a marker of DNA virus infection. For IFNβ, IFNλ, and TNFα the response of EfK3b cells was similar in magnitude and temporal dynamics to that of human cells. The increase in TNFα transcripts in the bat cells was puzzling as results from our research group had discovered that increase in the expression of the gene for TNFα is actively suppressed in cells stimulated with poly(I:C), a surrogate of RNA virus infection. This suggests that the response of these cells to surrogates of viral RNA and DNA may differ.

For the interleukins IL6 and IL8 the response of EfK3b and MRC5 cells was very different. While MRC5 cells responded to poly(dA:dT) with an increase in the expression of both interleukins, EfK3b cells did not. Even without poly(dA:dT) stimulation EfK3b cells contained high levels of IL8 transcripts and it is likely that stimulation could not increase it further. This is supported by our observation that “knocking down” the transcription factors that increase the

expression of interferon and cytokine genes had no effect on IL8 transcripts. The knocking down of transcription factors did, however, lead to an increase in the expression of IL6, suggesting that the expression of IL6 is actively suppressed in EfK3b cells.

The unresponsiveness of IL6 expression to poly(dA:dT) is consistent with the suggestion that excessive inflammation is suppressed in bats. IL6 is a pro-inflammatory cytokine that has been linked to increased pathological changes in several diseases (523–527). The expression of IL6 does appear to be suppressed in some bat species. Jayaprakash and others demonstrated that while a large increase in IL6 expression was linked to fatal human infections with Marburg and Ebola viruses, there was no increase in the livers of infected *Rousettus aegyptiacus* bats, the known reservoir for Marburg virus and suspected reservoir for Ebola virus(528).

Our studies stimulating cells with poly(dA:dT) assessed their ability to respond to cytosolic DNA, a virus molecular pattern that leads to activation of innate defenses. However, viruses have evolved mechanisms that, to varying degrees, block the ability of cells to defend themselves. To determine the innate response of EfK3b cells to viral infection we examined EfK3b cells infected with either EfHV, a γHV that does not appear to cause detectable pathological changes in big brown bats, or EfPV, a poxvirus associated with disease in these bats. To determine if viral gene expression was required to suppress the host innate response to viral infection, we compared the effects of infecting with live and UV-inactivated virus.

As demonstrated previously(27) Efk3b cells differed from human cells in not responding to poly(I:C) with induction of IL8. However, our results suggest that this lack of increase in EfK3b cells may be because of an already constitutively high levels of expression. In other mammals IL8 acts as a pro-inflammatory cytokine that attracts neutrophils to the site of infection(529). High levels of IL8 are associated with increased viral replication and pathological changes following infection of humans by several viruses(530–534). There are relatively few studies on the regulation of IL8 in response to viral infection of bat species. A transcriptome analysis of *Artibeus jamaicensis* infected with Tacaribe virus suggested an increase in IL8 expression(535) and a comparison of human and *Eonycteris spelaea* bone marrow derived mononuclear cells to lipopolysaccharide showed an increase in IL8 expression for both species(536). However, the increase in expression in bat cells was less dramatic (>100 fold in human cells vs <10 fold in bats cells).

The constitutive expression of a pro-inflammatory cytokine such as IL8 seems contrary to the suggestion that the evolution of flight led to the suppression of mechanisms that may trigger inflammation in response to damaged DNA and the activation of reactive oxygen species. It is possible that in addition to, or instead of, acting as a chemoattractant IL8 may perform functions in bats that are beneficial. In other species IL8 has been linked to DNA repair through nucleotide excision(537).

EfK3b cells reacted very differently to EfHV and EfPV. In the absence of viral gene expression, the response of UV-inactivated EfHV and EfPV-infected cells largely mimicked their response to poly(dA:dT), the expression of the interferons and TNFα was enhanced. Infection with replication competent EfHV abrogated this response. This suggested that EfHV, like other herpesviruses actively suppress cellular innate responses(538). In contrast, replication competent EfPV led to an increase in expression.

Our research is limited by the evaluation of these changes in a single cell line. Constitutive expression of IL8 could be an artefact of the cell line and expression levels in bat tissues are required for confirmation. Our measurements were performed on RNA and the expression levels of the functional form of these genes, the proteins, may not reflect these. We cannot separate the initial innate response from the positive feedback loop via interferon receptors and the Janus kinases/signal transducer and activator of transcription proteins pathway(539).

In summary the primary differences between bat and human cells response to poly(dA:dT) is in the inflammatory interleukin response. A virus associated with disease is unable to inhibit the innate response whereas a virus with asymptomatic infection can control the innate immune response. The exact contributions of the inflammatory cytokines to the observed pathological changes are yet to be determined.

CHAPTER 6 GENERAL DISCUSSION

6.1 Introduction

Western cultures have vilified bats for centuries. The bible describes bats as unclean. Dante, Stoker, and Shakespeare invoked the image of bats alongside devils, vampires, and witches(540). Therefore it is not surprising we continue to vilify bats in our current research(541). The narratives we spin are unhelpful at best and harmful at worst. The current narrative is that bats host an abundance of viruses without becoming sick, some of which are transmitted to and deadly for domestic animals and humans. Despite chimpanzees being the source of the greatest zoonotic viral pandemic, human immune deficiency virus, there is no intensive sampling and metagenomic sequencing of non-human primates to identify potentially zoonotic viruses(542).

Bats provide numerous ecological services with an estimated benefit of more than 3.7 billion dollars per year to North American agriculture in 2011(30). In North America several species face severe population losses with the introduction and spread of white nose syndrome(543). Globally there are numerous challenges facing bats with climate change, habitat loss, and hunting(544). Understanding how their viruses affect them at the individual and population level has many applications from medicine to conservation biology and zoonotic virus spill over. To determine if the current narrative about bats and their viruses has merit, we investigated wild bats submitted to a veterinary diagnostic pathology lab for signs of disease associated with viral infection. We isolated and characterized two DNA viruses, Eptesicus fuscus gammaherpesvirus (EfHV) and Eptesipox virus (EfPV), from these bats. This was complemented with *in vitro* work examining the innate response to DNA in cells derived from the same bat species as these viruses.

6.2 Major findings, strengths, and limitations

6.2.1 Discussion of Chapter 2

A review of the literature on the pathological changes associated with viral infection including experimental studies revealed how little we know about these viruses in bats. There was not enough evidence to support or refute the dogma that bats are unique because they do not develop disease associated with their viruses. However, evidence of viral associated disease in bats is primarily from 37 viruses found in natural infections associated with disease. These occurrences of disease were not considered sufficient to refute the dogma as many of these viruses were only reported as associated with disease. Additional evidence from experimental infections is also

inconclusive. Only 8 viruses were isolated from bats and then experimentally inoculated into the same species. The remainder of the inoculations were performed with: 10 viruses isolated from bats administered to a different bat species, 13 viruses from non-bat hosts administered to bat species which are suspected to be natural hosts because of independent virus isolation from them, and 22 non-bat isolates for which the role of bats as hosts is unclear. Extrapolating results from these experiments to disease in a natural host is not straightforward and may not be possible for several reasons. To resolve the issue of whether bats develop disease associated with viral infections closer examination of wild populations is required.

The literature review was limited by information available in print. Lack of findings are not often published so the reports of virus-associated disease may conflate virus infection and disease in bats.

6.2.2 Discussion of Chapter 3

Numerous bat herpesviruses have been detected in their hosts using metagenomic sequencing(300,321,323) or even isolated(100,102–104,393,394), but there are only a few reports of lesions that were associated with viral infections(98,99,106). Members of the subfamily Gammaherpesvirinae are transmitted from the oral or genital mucosa with a life-long dormant infection in cells of the immune system. These viruses cause disease during primary infection or with immune suppression of the host. This may manifest as neoplasms, proliferative and autoimmune disorders.

Chapter 3 examined a case with suspected viral induced lesions in the trachea from which a gammaherpesvirus (γHV) was isolated and determined to be a distinct isolate of the same species of γHV previously isolated in our lab(96). Natural viral infection in bats was not associated with any lesions. Given that these viruses can be dormant in the host for years this finding is not unexpected, and nothing can be inferred about bats and viral induced disease from this.

Consistent with other γHVs lymphoid tissues and B lymphocytes harboured dormant virus(545). A novel finding for this γHV was viral nucleic acids in individual cells of the lung alveolar blood vessels. These were suspected to be pulmonary intravascular macrophages (PIMs) a hypothesis supported by the identification of PIMs in bat lungs using transmission electron microscopy (TEM) and immunohistochemistry. This cell type has not previously been reported to be involved in γHV infection.

6.2.3 Discussion of Chapter 4

Poxviruses have been isolated or identified in lesions from three different bat species. Only one isolation of a poxvirus from bats has occurred without associated disease(58). In Chapter 4 we isolated a poxvirus from a case with oral ulcerations and joint swellings. Sequencing confirmed it was the same virus, EfPV, as previously isolated in Washington state in 2011. The joint lesions were similar to those described but our case was less affected by cell death and had marked cellular proliferation. Poxviral nucleic acids were demonstrated within these proliferations and adjacent to ulcers. The ulcerations in the oral cavity were a newly described feature of this viral infection in bats(56). Poxviral lesion in other mammals are characterized by cellular death and proliferative changes. This work demonstrates that bats develop typical poxviral lesions associated with infection with these viruses.

6.2.4 Strengths and limitations of Chapter 3 and 4

The strengths of these studies are that we use a natural virus-host relationship and naturally acquired infections to examine the pathogenesis of these viruses in bats. This has allowed us to identify several unique features not previously reported in human diseases and the animal models used to study them. However, this also limited our findings because sampling relative to start of infection was unknown which could lead to missing disease associated with acute infections. The sample size was also small meaning rare diseases associated with herpesvirus infection and immunocompromised states would go unrecognized. Additionally, the sampling was biased where mild or severe outcomes of viral infection in these bats would not be submitted. With mild infections, lesions, or behavioural changes would go undetected by routine examination, whereas moribund or dead bats caused by severe infection are not likely to be found by the public and submitted for testing. The design was observational so we can only hypothesize as to the role of PIMs in the pathogenesis of EfHV and cannot determine causation for the joint lesions with EfPV infection.

6.2.5 Discussion of Chapter 5

We demonstrated the plasticity of the innate response in bat cells in Chapter 5. Bat cells express several DNA sensors and multiple signalling factors that contribute to the innate response. As the immune system of bats is hypothesized to be tolerant to viruses based on experiments with RNA, we examined the innate response of bat cells to infection with these viruses as well as a chemical DNA surrogate. Several of the bat innate response effectors were expressed in time and

magnitude like their counterparts in human cells. For one of these, tumor necrosis factor α (TNFα), this is contrary to what was previously reported for RNA viruses or their surrogate molecules in big brown bat cells(27). There were marked differences in the interleukin (IL) expression between humans and big brown bat cells. Stimulation of these bat cells with DNA failed to induce high levels of bat IL6 which is actively repressed by NFκB. The other IL, IL8, was consistently expressed at high levels comparable to the housekeeping gene irrespective of any treatments.

We would expect if bats were tolerant to their viruses that *in vitro* infections would generate a minimal innate response. The innate response generated by both inactivated viral infections was comparable, however live EfHV could suppress the innate response whereas EfPV could not. These findings are supported by what we observed *in vivo* with lesions and inflammation associated with EfPV infection. The degree of innate activation appears to be a function of the virus rather than the host.

Our study was unique in that it is one of the only studies quantifying the bat cellular response to a chemical DNA surrogate(546) as opposed to RNA surrogates(27,506,547,548). The applicability of using an RNA surrogate to support the hypothesis that bat cells are tolerant to DNA viruses is questionable since the sensors that detect these molecular patterns are different(549–552). We also use a natural virus-host relationship to examine the innate response using viruses isolated from the same bat species as the cell line. Inferences drawn from other model systems where the virus is derived from a different host, or used in non-host cell lines, may not be reflective of the pathogenesis. The limitations of our research do not determine the downstream effects of high basal expression and whether this occurs *in vivo.* These findings are based on changes in RNA expression this should be confirmed with protein expression data when reagents are available or validated for this species.

6.3 Importance of research

6.3.1 Discussion of Chapter 3

The pathogenesis of γHVs is not fully understood. The biggest knowledge gaps are in the method of initial infection, dissemination from primary sites to secondary tissues, where reactivation occurs and how this contributes to viral shedding. The involvement of PIMs in the pathogenesis of EfHV means this cell type may be important in productive infections either supporting viral replication following dormancy or in viral shedding, alternatively they may be

important in controlling infection. These cell types may also be important in disease associated with other γHV infections like pulmonary fibrosis in humans and equines(431,434).

6.3.2 Discussion of Chapter 4

The gold standard for determining the causation of disease is Koch's postulates(553). These are difficult to fulfill in wildlife. The challenges include: the availability of specimens, maintaining them in a stress-free environment, limited reproductive capacity, lack of specific reagents, lack of reference data, recapitulation of a naturally occurring disease with experimental infection, and the ethical considerations. An alternative method is to demonstrate a correlation between the observed lesion and presence of virus. Although this does not prove causation of the lesions it lends support for the hypothesis that viruses, like EfPV, are the etiologic agents. Finding pathological changes associated with poxviral nucleic acids also suggests that perhaps bats are like the rest of mammals and are susceptible to disease caused by viruses.

The images of EfPV infection of cultured bat cells suggests a degree of intranuclear replication may be occurring. Although this requires further confirmation this would be the first poxvirus to demonstrate this ability. Members of the higher taxonomic class of nucleocytoplasmic large DNA viruses to which poxviruses belong have the capacity to replicate in both the cytoplasm and nucleus.

6.3.3 Discussion of Chapter 5

We have identified novel patterns of IL expression in big brown bat cells. IL8 was basally expressed at high levels and was unaffected by any treatment. Although we have yet to confirm this finding *in vivo* downstream genes are expressed at high levels in these bat cells(554). High basal expression of innate immune components is a pattern that is observed in other bat cells(506). IL6 expression was actively inhibited by NFκB. Stimulation of cells with a DNA surrogate was not sufficient to overcome this inhibition but infection with EfPV was. The implications to the host regarding overall viral tolerance or resistance with these expression patterns is unknown.

6.4 Future directions

To develop a clearer picture of whether bats develop virus-associated disease further investigation into natural morbidities and mortalities is required. Experimental infections are not the best way to ascertain this because disease is a spectrum with clinically recognizable forms comprising a small part of the total number of outcomes in exposure to a pathogen. More intensive monitoring and collection of samples from bat populations is preferrable. The issues surrounding

experimental infections of wildlife were discussed and these are best reserved for investigating viral pathobiology rather than determining whether a virus causes disease. When inoculation trials are performed, using a natural host-virus relationship is imperative to ensure the data can be extrapolated to the whole population. Bats have been implicated as reservoirs for numerous viruses but their roles as maintenance or reservoir hosts require clarification to appropriately select species for further experimental studies.

We have identified cells that resemble PIMs in bats, but further characterization and quantification is required. As they were involved in EfHV infection their roles in other γHV infections warrants further investigation. Ideally this would include how this cell type may contribute to or controls productive infections and virus shedding and examination of their contribution to the pathogenesis of fibrosing conditions of the lung that are associated with γHV infection.

To circumvent the challenges of wildlife inoculation experiments further development of cell lines and organoids from bats could fill in the knowledge gap around the pathogenesis of EfPV infection. Additional imaging such as TEM on sections of specially fixed tissue could also address questions surrounding EfPV and the observed joint proliferation. To determine the extent of nuclear replication with EfPV plaque purification and fluorescently labelling the virus for live cell imaging would be best. Alternatively repeating the cell culture infection and TEM is also possible however these results are qualitative.

There is a recurring theme across studies on the innate immune system of bats: high levels of expression of various factors. The next major step is in characterizing the downstream signalling and effects an "always on" system has. Additionally, determining the signalling cascades that allow high levels of expression and what types of stimuli that can increase these further. This pattern of expression suggests bats may be more resistant to viruses rather than tolerant to them. Recent research proposes this may be the case(501,548). Additionally, determining how IL6 is inhibited and how this affects the host in concert with the other changes has applications for therapeutics. Antiviral and autoimmune disorders are the most obvious applications. Therapeutics based on innate immunity could heighten immunity to many different pathogens. In some situations, such as pandemics caused by novel viruses, this would be superior to the current method of generating immunity through vaccination because generating a specific antibody response takes time and pathogens can evolve making vaccination less effective.

6.5 Conclusion

Of the two narratives regarding bats as viral hosts this thesis provides evidence that supports bats as having a relationship with their viruses like other mammals. We have demonstrated the lack of knowledge in the published literature concerning the pathogenesis of viruses in bats. To address this deficit, we examined diagnostic case material from Western Canadian bats for signs of viral infection. We observed multiple instances of virus-associated disease and isolated two viruses from these cases, a herpesvirus EfHV and a poxvirus EfPV. Mammals infected with similar viruses either display the same pathogenesis for EfHV or pathological changes for EfPV. For each of these viruses there was a novel feature not previously described viral nucleic acids in PIMs for EfHV and virus within the nucleus of cultured cells for EfPV. These features require further confirmation and more extensive research into related viruses to determine if they are unique.

The other narrative that bats are tolerant to their viruses is partly founded on their innate immune response. We demonstrated that the innate response in human cells and big brown bat cells was more comparable than has previously been reported. There was higher basal expression of a proinflammatory factor in these bat cells, a pattern that is recurrent in the innate response of many bat species. These cells also actively inhibited the expression of a separate proinflammatory factor. How these features are involved in the pathogenesis of virus-associated disease for big brown bats is unknown. However, cell culture infection with EfPV overcame inhibition and induced expression of this proinflammatory factor and inflammation is observed in the lesions from natural infections with EfPV. These suggest that the inflammation is a host induced in response to viral infection, possibly mediated through this proinflammatory factor.

Taken together our findings regarding these viruses and the host innate response demonstrates that big brown bats are like other mammals, susceptible to diseases caused by their viruses. These findings do not allow us to generalize about all bat species, but it implies the dogma that bats are unique hosts is incorrect.

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APPENDIX

The appendix is comprised of 3 figures and 9 tables of supplementary material. The figures include Eptesicus fuscus gammaherpesvirus (EfHV) gene expression (Chapter 3); schematic of *in-situ* hybridization (ISH) probes (Chapter 3); and potential mechanism of joint pathogenesis (Chapter 4). The tables for Chapter 3 outline the variations between the sequence of our viral isolate, the original lab isolate, and the reference sequence. Tables with primer details and variations between our viral isolate sequence and that of the reference are provided for Chapter 4. Supplemental tables for Chapter 5 include details of the primers used for DNA sensors and the innate response as well as the sequences of the small interfering RNAs (siRNAs).

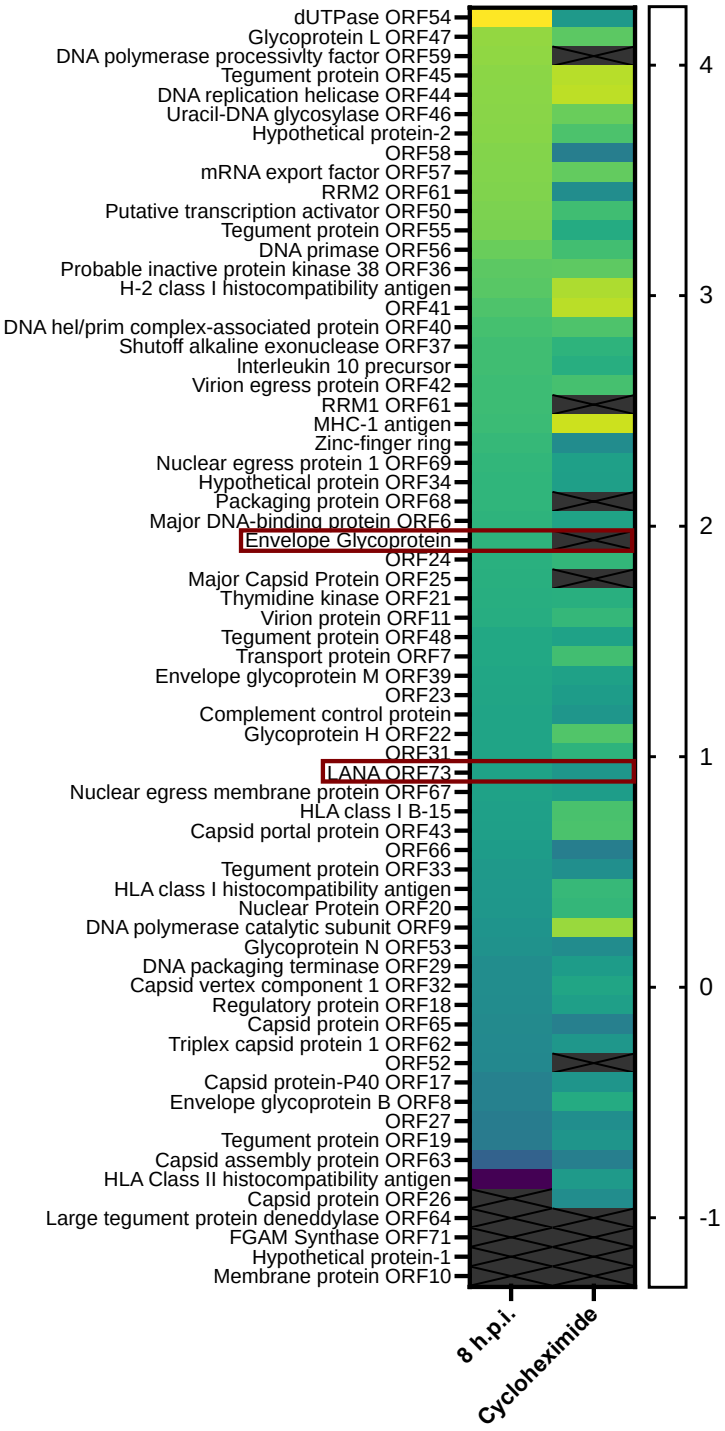

Figure A 1 Heatmap of the log10 fold change of EfHV genes relative to 4°C infection control at 8 h.p.i. with or without cycloheximide. Dark grey ells containing an x were not significantly different from the control. Red boxes indicate genes used for ISH probes.
EfHV - Eptesicus fuscus gammaherpesvirus, h.p.i. - hours post infections, ISH - *in-situ* hybridization, RRM1 - ribonucleoside-diphosphate reductase large subunit, RRM2 - ribonucleoside-diphosphate reductase small subunit

Table A 1 Variations in the nucleotide sequence of EfHV/Saskatoon/01/2016 compared to the reference sequence.

Minimum	Maximum	Length	Change	Polymorphism Type
149304	149304	1	(A)3 -> (A)2	Deletion (TR)
1713	1712	0	+GCGGGCGGGCGGGCGGCCTG CCGGCAGCCCGGATT	Insertion
164626	164625	0	+TCGCTCATCCGCCACCCCTGT CCCCCCCTCCGCCCCGCCC	Insertion
163879	163878	0	(CC)4 -> (CC)5	Insertion (TR)
2156	2156	1	Y -> C	SNP
148306	148306	1	A -> G	SNP (transition)
2091	2091	1	G -> T	SNP (transversion)
2136	2136	1	G -> T	SNP (transversion)
2095	2101	7	TTGTCCC -> GGTCGGT	Substitution
2103	2104	2	CA -> GC	Substitution
2106	2108	3	ATC -> CCG	Substitution
2110	2111	2	TT -> CG	Substitution
2114	2116	3	CAG -> GTT	Substitution
2118	2120	3	TGG -> ACC	Substitution
2123	2125	3	ATA -> CAC	Substitution
2128	2131	4	GGTT -> CCCA	Substitution
2133	2134	2	TA -> CC	Substitution
2139	2142	4	ATAC -> CCCT	Substitution
2144	2152	9	TAATATTTG -> AGCCGCCCT	Substitution
107076	107077	2	YK -> CG	Substitution

EfHV - Eptesicus fuscus gammaherpesvirus
TR - terminal repeat

Table A 2 Variations in the nucleotide sequence of EfHV/Saskatoon/02/2020 compared to the reference sequence.

Minimum	Maximum	Length	Change	Polymorphism Type
280	630	351	-GCCGCGAGCGGGCGGGCCGGG CGGCCGCGCGACGGGAGTTGGGGCCCC CTTGGCAATCATTTTTTTTTTTTTTACT GTTTCATGGTTTTGACTTCCAGAGTGTC ATGTAGTTGGAATCTTATACTCTGTAG CTGTTTCATACTGGCTTCTTTCACTTAG TGATATGTCCGCGACGGGAGTTGGGGC CCCCTTGGGCCGGAGGGGGCGGGGCTT GGGCATACCCCTTGGGCATATGTATAT GCGCCTGTTCTATTTTTAGCGCCCGTTT GGGCGCACGCGCCTCTTCTATTTTTAGC TCGGCGGCGCCAGGCCTCCCGGCGCTT CCCTTTTTTTTTGCGCGCCCGCCGCCG	Sequencing gap
992	992	1	T -> C	SNP (transition)
1551	1551	1	C -> T	SNP (transition)
2091	2091	1	G -> T	SNP (transversion)
2095	2096	2	TT -> G	Deletion
2100	2102	3	CCC -> GGT	Substitution
2104	2107	4	AGAT -> GCGC	Substitution
2109	2109	1	T -> G	SNP (transversion)
2111	2111	1	T -> CG	Insertion
2114	2116	3	CAG -> GTT	Substitution
2118	2120	3	TGG -> ACC	Substitution
2123	2125	3	ATA -> CAC	Substitution
2128	2131	4	GGTT -> CCCA	Substitution
2133	2134	2	TA -> CC	Substitution
2136	2136	1	G -> T	SNP (transversion)
2139	2142	4	ATAC -> CCCT	Substitution
2144	2152	9	TAATATTTG -> AGCCGCCCT	Substitution
2156	2156	1	Y -> C	SNP
4503	4505	3	(AAA)4 -> (AAA)3	Deletion (TR)
4731	4731	1	T -> C	SNP (transition)
5205	5205	1	G -> T	SNP (transversion)
5657	5657	1	C -> T	SNP (transition)
5717	5717	1	A -> G	SNP (transition)
5729	5729	1	A -> G	SNP (transition)
6661	6661	1	C -> T	SNP (transition)
7177	7177	1	G -> A	SNP (transition)
7639	7639	1	T -> C	SNP (transition)

7781	7781	1	T -> C	SNP (transition)
9817	9817	1	C -> T	SNP (transition)
11018	11018	1	G -> T	SNP (transversion)
11149	11149	1	A -> G	SNP (transition)
11295	11295	1	G -> A	SNP (transition)
11394	11394	1	A -> G	SNP (transition)
11460	11460	1	C -> T	SNP (transition)
11976	11976	1	C -> T	SNP (transition)
12562	12562	1	T -> A	SNP (transversion)
12688	12688	1	T -> G	SNP (transversion)
12813	12813	1	A -> G	SNP (transition)
13144	13144	1	C -> T	SNP (transition)
13246	13246	1	A -> G	SNP (transition)
13304	13304	1	A -> C	SNP (transversion)
13367	13367	1	A -> G	SNP (transition)
13445	13445	1	A -> G	SNP (transition)
13639	13639	1	G -> A	SNP (transition)
13657	13657	1	A -> C	SNP (transversion)
13772	13772	1	C -> T	SNP (transition)
13793	13793	1	T -> C	SNP (transition)
13797	13797	1	C -> T	SNP (transition)
13876	13876	1	T -> G	SNP (transversion)
13923	13923	1	A -> G	SNP (transition)
14188	14188	1	G -> A	SNP (transition)
14992	14992	1	A -> G	SNP (transition)
15394	15394	1	(T)9 -> (T)8	Deletion (TR)
19925	19925	1	G -> A	SNP (transition)
22991	22994	4	(TT)8 -> (TT)6	Deletion (TR)
24676	24676	1	G -> T	SNP (transversion)
25906	25906	1	A -> G	SNP (transition)
26689	26689	1	C -> T	SNP (transition)
27177	27177	1	A -> G	SNP (transition)
27268	27268	1	G -> C	SNP (transversion)
30921	30921	1	T -> C	SNP (transition)
31828	31828	1	C -> T	SNP (transition)
32490	32490	1	G -> A	SNP (transition)
32794	32794	1	(A)7 -> (A)6	Deletion (TR)
33186	33186	1	G -> A	SNP (transition)
33271	33273	3	(CGG)3 -> (CGG)2	Deletion (TR)
34292	34292	1	G -> A	SNP (transition)

35287	35287	1	A -> C	SNP (transversion)
37535	37555	21	-GGCGCAACGCCCGCCGCGGCG	Deletion
41159	41158	0	(C)10 -> (C)11	Insertion (TR)
42466	42465	0	+GGGGTTAGGGG	Insertion
42685	42685	1	G -> A	SNP (transition)
44159	44159	1	C -> T	SNP (transition)
46391	46393	3	(GCT)6 -> (GCT)5	Deletion (TR)
64964	64964	1	T -> C	SNP (transition)
65238	65238	1	C -> T	SNP (transition)
68540	68540	1	C -> T	SNP (transition)
68833	68833	1	T -> C	SNP (transition)
69867	69867	1	G -> A	SNP (transition)
72047	72047	1	C -> T	SNP (transition)
73192	73192	1	C -> T	SNP (transition)
73545	73545	1	T -> C	SNP (transition)
73641	73641	1	A -> G	SNP (transition)
82414	82414	1	T -> G	SNP (transversion)
89527	89527	1	T -> G	SNP (transversion)
89855	89855	1	G -> A	SNP (transition)
89946	89946	1	G -> A	SNP (transition)
92085	92085	1	G -> A	SNP (transition)
94216	94216	1	G -> A	SNP (transition)
103829	103832	4	(TCTT)8 -> (TCTT)7	Deletion (TR)
107076	107077	2	YK -> CG	Substitution
115318	115318	1	A -> C	SNP (transversion)
118323	118323	1	G -> T	SNP (transversion)
123775	123774	0	(T)8 -> (T)9	Insertion (TR)
132368	132368	1	A -> G	SNP (transition)
132620	132620	1	A -> G	SNP (transition)
133241	133241	1	A -> G	SNP (transition)
134635	134635	1	C -> A	SNP (transversion)
136577	136577	1	G -> A	SNP (transition)
137769	137769	1	G -> A	SNP (transition)
139057	139057	1	G -> A	SNP (transition)
139063	139065	3	-AAG	Deletion
139373	139373	1	G -> T	SNP (transversion)
145538	145537	0	(A)9 -> (A)10	Insertion (TR)
145727	145726	0	(G)7 -> (G)8	Insertion (TR)
147181	147181	1	C -> T	SNP (transition)
147908	147908	1	(T)10 -> (T)9	Deletion (TR)

148935	148935	1	T -> C	SNP (transition)
149227	149234	8	-AGCGGAGC	Deletion
150787	150787	1	-A	Deletion
154934	154936	3	(TGT)13 -> (TGT)12	Deletion (TR)
159864	159863	0	(T)10 -> (T)11	Insertion (TR)
162305	162305	1	(C)11 -> (C)10	Deletion (TR)
163862	163922	61	-CCACCCTCTCCCCCCCCGCACTT GCCAAGCCCCCCGGTTTTCACCATCTG ATTCACGCCCA	Deletion
164626	164625	0	+TCCGCCCCGCCC	Insertion
165189	165188	0	(GG)4 -> (GG)5	Insertion (TR)
165378	165378	1	C -> T	SNP (transition)
166183	166183	1	C -> A	SNP (transversion)

EfHV - Eptesicus fuscus gammaherpesvirus
TR - terminal repeat

Table A 3 Amino acid variations between the sequenced EfHV isolates and the reference sequence. Substitutions are written as amino acid position, reference amino acid(s), variant amino acid(s) and "." which represents a deletion.

Amino acid	Length	Direction	% NA Similarity	% AA identity	Substitution
ORF10-Membrane protein	1443	forward	98.54	98.5	297, GATPAAA, . †
ORF65-Capsid protein	453	reverse	98.9	98.7	109, S, .† 111, P, S
HLA class I histocompatibility antigen	399	reverse	99.75	99.2	45, Q, R†
HLA class II histocompatibility Antigen	459	reverse	99.78	99.3	79, D, N
ORF47-Glycoprotein L	504	reverse	99.8	99.4	27, P, S
ORF45-Tegument protein	1470	reverse	99.8	99.6	301, S, L† 441, T, P†
ORF31	777	forward	99.87	99.6	27, C, R
ORF33-Tegument protein	1056	forward	99.91	99.7	317, T, I
Complement control protein	2082	forward	99.71	99.7	320, W, L 364, K, E
MHC class I	873	reverse	99.77	99.7	252, R, G
ORF34-Hypothetical! protein	1284	forward	99.84	99.8	93, K, E
ORF32-Capsid vertex component 1	1632	forward	99.94	99.8	132, E, K
ORF9-DNA polymerase catalytic subunit	3141	forward	99.81	99.8	104, A, . 446, R, K
ORF73-LANA	3030	reverse	99.9	99.9	127 Q, QQ
ORF64-Large tegument protein deneddylase	10869	forward	99.94	99.9	1452, T, A 1536, M, V 1743, T, A 3252, R, K
ORF17-capsid protein-P40	2265	reverse	99.87	99.9	353, Q, .
ORF8-Envelope glycoprotein B	3105	forward	99.94	99.9	529, V, A
ORF7-transport protein	2310	forward	99.91	99.9	27, P, S
MHC class I*	867	reverse	97.8	97.9	2, GY, P 6, EGT, RPP 9, G, .

† Amino acid substitution predicted to alter biological function

* EfHV/SK/01/2016 original isolate amino acid variant from reference sequence

EfHV Eptesicus fuscus gammaherpesvirus

AA - amino acid, NA - nucleic acid, ORF - open reading frame

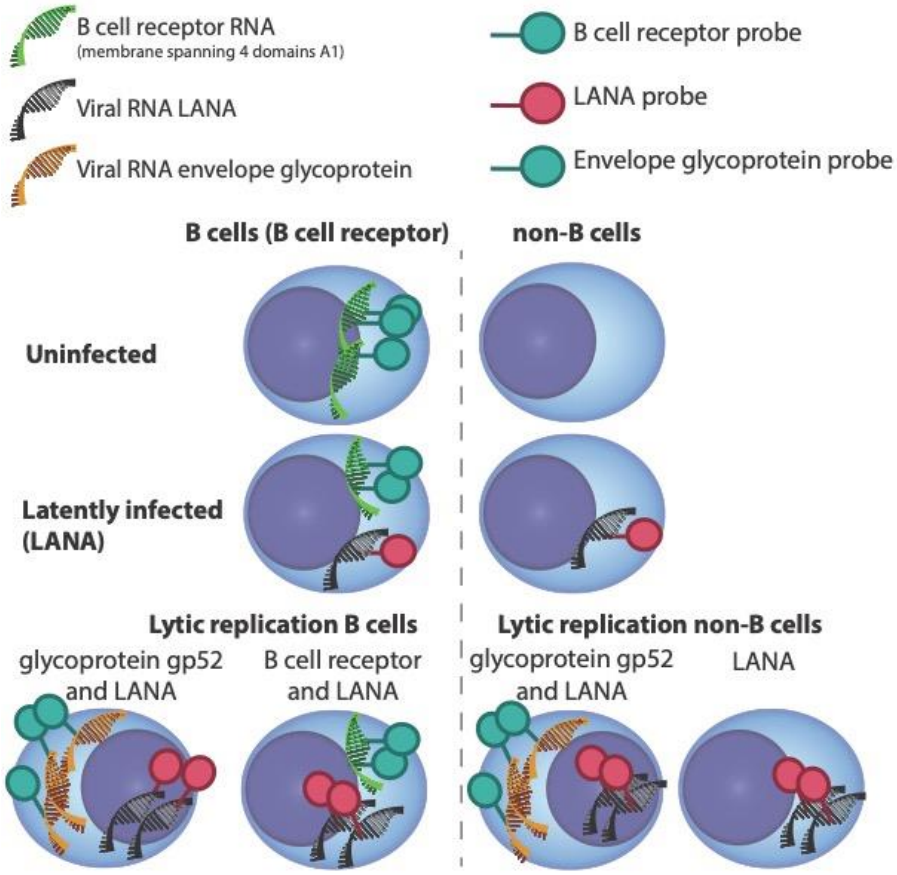

Figure A 2 Schematic representation of ISH staining. The colorimetric ISH assay only has two colours available to identify probes. Probes were used in combination to identify either latent infection in B cells (LANA and B cell receptor) or lytic replication (envelope glycoprotein gp52 and LANA). The B cell receptor and envelope glycoprotein probes developed the same colour so serial sections were used to determine if a similar distribution was observed with B cell receptor when lytic infection was identified.
ISH - *in-situ* hybridization, LANA - latency-associated nuclear antigen

Table A 4 Primers used for sequencing gaps between contiguous sequences of Eptesipox virus.

Name	Forward	Reverse	Ta° C	Special Conditions
7840 to 8180	ATACACGCGTCTGCATTTCC	TGTGATAGTGATTATAGCGATAGTGA	50	Final [Mg^{2+}] 3 mM
8671 to 9245	TACAATAACATTTCAGTCATTGCCA	AACGTGCTATTGCTAGATACAGA	50	Final [Mg^{2+}] 3 mM
96712 to 97163	CTAAATCTGAAACACATCGTATTCCA	ATCAGGTGCAGTTGTAAAATCTGT	50	Final [Mg^{2+}] 3 mM
120584 to 121037	GAGGTTTAGGAAAAACGTTCGC	GGTGCTGTTCATAAAACTACTAGCG	48	
134291 to 134837	ACGTTTGCACAAATAAAGCACA	ATCGGGATTAAAAATTCAAGGCA	48	Final [Mg^{2+}] 3 mM
142202 to 142883	ACTGTATCCGAGTTTGATGCT	AAAACTCAAATATTACCAGAACCAGT	54	Final [Mg^{2+}] 5 mM
151093 to 151459	TGGTGTGATCATACTCAAAGTGAAC	ACCATTTGCTCCTGCTGCTAT	50	PCR product used as template
152517 to 153368	TGATATTGTTACAGCAGTTGAGGAT	ACTGCATCTATGATCATATCTATACGA	50	Final [Mg^{2+}] 3 mM
165050 to 168271	CATGTGTTCACGTATGCAAACTGT	GATACAATAACATTTCAGTCATTGCC	51	Final [Mg^{2+}] 2.5 mM, extension 65°C x 1.5 minutes

Ta – annealing temperature

Table A 5 Nucleotide variation between Eptesipox virus/Saskatoon/01/2020 and the reference sequence.

Minimum	Maximum	Length	Change	Polymorphism Type
1446	1446	1	C -> T	SNP (transition)
5364	5364	1	C -> T	SNP (transition)
5480	5480	1	A -> G	SNP (transition)
6984	6984	1	G -> A	SNP (transition)
8111	8116	6	(ATCACT)3 -> (ATCACT)2	Deletion (TR)
8645	8645	1	C -> T	SNP (transition)
8744	8744	1	-T	Deletion
9113	9136	24	-GCTTTAGTTTTCTTGTTTTAAAAT	Deletion
10352	10352	1	T -> C	SNP (transition)
11082	11082	1	(T)8 -> (T)7	Deletion (TR)
11577	11577	1	G -> A	SNP (transition)
18059	18059	1	(T)7 -> (T)6	Deletion (TR)
19696	19696	1	T -> C	SNP (transition)
22759	22759	1	A -> T	SNP (transversion)
23116	23116	1	C -> T	SNP (transition)
25954	25954	1	C -> T	SNP (transition)
27112	27112	1	G -> A	SNP (transition)
29678	29678	1	A -> G	SNP (transition)
32507	32507	1	T -> C	SNP (transition)
34373	34373	1	A -> C	SNP (transversion)
34767	34767	1	G -> A	SNP (transition)
35217	35217	1	C -> T	SNP (transition)
35811	35811	1	C -> T	SNP (transition)
35847	35847	1	T -> C	SNP (transition)
37270	37270	1	A -> G	SNP (transition)
37483	37483	1	A -> G	SNP (transition)
38204	38204	1	G -> A	SNP (transition)
39051	39051	1	C -> T	SNP (transition)
39426	39426	1	A -> G	SNP (transition)
40017	40017	1	T -> C	SNP (transition)
40187	40187	1	T -> C	SNP (transition)
40382	40382	1	G -> A	SNP (transition)
40507	40507	1	C -> T	SNP (transition)
41732	41732	1	A -> G	SNP (transition)
41778	41778	1	G -> A	SNP (transition)
41792	41792	1	A -> G	SNP (transition)
42049	42049	1	C -> T	SNP (transition)

42128	42128	1	A -> G	SNP (transition)
42557	42557	1	T -> C	SNP (transition)
42663	42663	1	T -> C	SNP (transition)
48280	48280	1	A -> T	SNP (transversion)
50264	50264	1	C -> T	SNP (transition)
52637	52637	1	G -> A	SNP (transition)
52641	52641	1	T -> C	SNP (transition)
53155	53155	1	A -> G	SNP (transition)
53230	53230	1	C -> T	SNP (transition)
53279	53280	2	CG -> TA	Substitution
53297	53297	1	T -> C	SNP (transition)
53344	53344	1	G -> A	SNP (transition)
54040	54040	1	C -> T	SNP (transition)
55448	55448	1	G -> A	SNP (transition)
55493	55493	1	C -> T	SNP (transition)
57972	57972	1	G -> A	SNP (transition)
60694	60694	1	C -> T	SNP (transition)
62931	62931	1	(T)11 -> (T)10	Deletion (TR)
86027	86027	1	C -> T	SNP (transition)
88017	88017	1	C -> T	SNP (transition)
101942	101942	1	T -> C	SNP (transition)
104305	104305	1	G -> A	SNP (transition)
106143	106143	1	T -> C	SNP (transition)
119908	119908	1	A -> G	SNP (transition)
120259	120259	1	T -> C	SNP (transition)
120289	120290	2	GT -> AC	Substitution
120295	120295	1	T -> C	SNP (transition)
120300	120300	1	A -> T	SNP (transversion)
120308	120308	1	G -> C	SNP (transversion)
120310	120311	2	GT -> TC	Substitution
120315	120315	1	G -> T	SNP (transversion)
120351	120351	1	C -> T	SNP (transition)
120354	120354	1	T -> A	SNP (transversion)
120386	120386	1	C -> T	SNP (transition)
120391	120391	1	A -> G	SNP (transition)
120394	120394	1	T -> C	SNP (transition)
120402	120402	1	A -> G	SNP (transition)
120411	120411	1	G -> A	SNP (transition)
120413	120413	1	A -> T	SNP (transversion)
120446	120446	1	G -> A	SNP (transition)

120454	120454	1	G -> T	SNP (transversion)
120558	120558	1	A -> G	SNP (transition)
120564	120564	1	T -> C	SNP (transition)
120597	120600	4	AGCA -> GTTC	Substitution
120612	120612	1	C -> A	SNP (transversion)
120661	120660	0	GTT	Insertion
120663	120663	1	A -> T	SNP (transversion)
120789	120789	1	A -> G	SNP (transition)
120822	120822	1	T -> C	SNP (transition)
120875	120876	2	GG -> TA	Substitution
120906	120906	1	T -> C	SNP (transition)
120912	120912	1	A -> G	SNP (transition)
120918	120918	1	A -> G	SNP (transition)
120936	120938	3	CTT	Deletion
120986	120986	1	T -> C	SNP (transition)
121059	121059	1	A -> T	SNP (transversion)
121079	121079	1	C -> T	SNP (transition)
121585	121585	1	C -> T	SNP (transition)
123842	123841	0	(ATA)2 -> (ATA)3	Insertion (TR)
129869	129868	0	(A)7 -> (A)8	Insertion (TR)
134445	134444	0	+AAATTACCTGGACCTGTTTTTTAAAAAGTTTATTAATTGTTTTTTTAAAAAAAAAAAATTACATGGACATGTTTTTTAAAAAGTTTATTAATTGTTTTTTTAAAAAAAAAAAATTACATGGACATGTTTTTTAAAAAGTTTATTAATTGTTTTTTTAAAAAAAAAAAATTACATGGACATGTTTTTTAAAAAGTTTATTAATTGTTTTTTTAAAAAAAAAAAATTACATGGACATGTTTTTTAAAAAGTTTATTAATTGTTTTTTTAAAAAAAAAAA	Insertion
134498	134498	1	(A)11 -> (A)10	Deletion (TR)
134504	134504	1	T -> G	SNP (transversion)
134527	134527	1	A -> T	SNP (transversion)
134581	134581	1	A -> T	SNP (transversion)
134603	134602	0	(AA)3 -> (AA)4	Insertion (TR)
134653	134652	0	(AA)3 -> (AA)4	Insertion (TR)
134724	134724	1	(T)9 -> (T)8	Deletion (TR)
137740	137740	1	A -> G	SNP (transition)
141109	141109	1	C -> T	SNP (transition)
141358	141358	1	G -> A	SNP (transition)
141553	141553	1	A -> G	SNP (transition)
141689	141688	0	(T)9 -> (T)10	Insertion (TR)
142210	142210	1	T -> C	SNP (transition)
146482	146482	1	(A)8 -> (A)7	Deletion (TR)

148458	148458	1	A -> G	SNP (transition)
149895	149894	0	(ATAATACT)2 -> (ATAATACT)3	Insertion (TR)
150168	150168	1	G -> A	SNP (transition)
151144	151144	1	A -> G	SNP (transition)
151156	151156	1	G -> A	SNP (transition)
151171	151170	0	+AATAGTGATGATGTTAACAAAAGTGAT	Insertion
159356	159355	0	(T)9 -> (T)10	Insertion (TR)
162434	162434	1	A -> G	SNP (transition)
165112	165112	1	C -> T	SNP (transition)
165614	165614	1	(A)8 -> (A)7	Deletion (TR)
166337	166337	1	A -> G	SNP (transition)
166760	166760	1	G -> A	SNP (transition)
166817	166817	1	A -> T	SNP (transversion)
167105	167105	1	A -> T	SNP (transversion)
167512	167512	1	A -> G	SNP (transition)
167562	167585	24	-CAAGAAAACTAAAGCATTTTAAAA	Deletion
167945	167945	1	-A	Deletion
168044	168044	1	G -> A	SNP (transition)
168587	168592	6	(ATAGTG)3 -> (ATAGTG)2	Deletion (TR)
169705	169705	1	C -> T	SNP (transition)
171209	171209	1	T -> C	SNP (transition)
171325	171325	1	G -> A	SNP (transition)
175243	175243	1	G -> A	SNP (transition)

TR - tandem repeat

Table A 6 Amino acid variation between Eptesipox virus/Saskatoon/01/2020 and the reference sequence. Substitutions are written as amino acid position, reference residue(s), query residue(s).

Gene	Minimum	Maximum	Length	Direction	% NA identity	%AA identity	Substitution	
gp007	4931	5623	693	reverse	99.71	99.6	107,G,D	
gp010	6903	8630	1728	reverse	99.54	99.5	18,V,I 198,D,DSD	
gp011	9120	10949	1830	reverse	99.95	99.8	230,E,G	
gp012	10998	11507	510	reverse	99.8	99.4	8,T,I	
gp026**	18611	19924	1314	reverse	99.96	99.8	108,K,X	
gp028	21269	23209	1941	reverse	99.9	99.7	63,V,I 182,L,I	
gp033	25589	26269	681	reverse	99.85	99.6	137,K,E	
gp039	31979	33682	1704	forward	99.93	99.8	146,Y,H	
gp040	33706	34518	813	forward	99.94	99.82	192,Q,P	
gp041	34515	37535	3021	reverse	99.88	99.8	49,S,L 120,S,L	
gp044	38256	40331	2076	reverse	99.76	99.7	15,L,F 80,I,V	
gp045	40388	40702	315	reverse	99.68	99	97,N,D	
gp046	40817	41749	933	reverse	99.68	99.7	22,I,T	
gp047	41750	41971	222	reverse	99.55	98.6	6,A,T	
gp048	41972	42784	813	reverse	99.63	99.3	72,R,K 107,M,I	
gp054	49872	51662	1791	reverse	99.94	99.8	498,N,D	
gp056	51985	52653	669	forward	99.7	99.6	187,N,S	
gp058	53000	54316	1317	forward	99.47	99.5	63,Y,R 69,H,Y	
gp063	56946	57971	1026	forward	99.9	99.7	312,T,A	
gp093	87051	88958	1908	reverse	99.95	99.8	346,E,K	
gp106	100631	103357	2727	reverse	99.96	99.9	504,N,D	
gp123	119180	121111	1932	reverse	97.73	96.9	43,I,V 49,K,N 74,A,T 89,K,KD 99,M,I 180,K,N 182,T,. 198,N,K 203,N,A 251,H,P	265,N,Y 271,RA,KV 274,N,D 297,E,D 299,EV,TL 304,S,N 306,V,T 316,R,K 433,P,L
gp124	121167	121511	345	reverse	99.71	99.1	7,M,I	
gp128	123261	123764	504	forward	99.4	99.4	162,N,NN	

gp145*	134631	134858	228	forward	96.51	53.9	17,NYMDM,ITGTC 23,FK,LS 27,INC,LIV 31,F,L 35,NYMDM,ITWTC 41,F,L 43,K,S	45,INC,LIV 49,F,L 52,ITWTC,NYMDM 58,L,F 60,SLLIV,KFINC, 66,L,F 68,K,KK
gp162	150146	150577	432	forward	99.77	99.3	72,R,K	
gp163	150628	151503	876	forward	96.69	96.2	237,N,D 241,V,I 245,D,DNSDDVNKSD	
gp178	162465	163421	957	forward	99.9	99.7	64,N,D	
gp180	165310	165819	510	forward	99.8	99.4	8,T,I	
gp181	165868	167697	1830	forward	99.78	99.34	230,E,G 371,G,D 390,K,I † 486,H,L	
gp182	168187	169914	1728	forward	99.54	99.5	18,V,I 198,D,DSD	
gp185	171194	171886	693	forward	99.71	99.6	107,G,D	

†Amino acid substitution predicted to alter biological function
*Cannot form prediction on unknown amino acid X
**No similarity in Blast database cannot form prediction
AA - amino acid, NA - nucleic acid

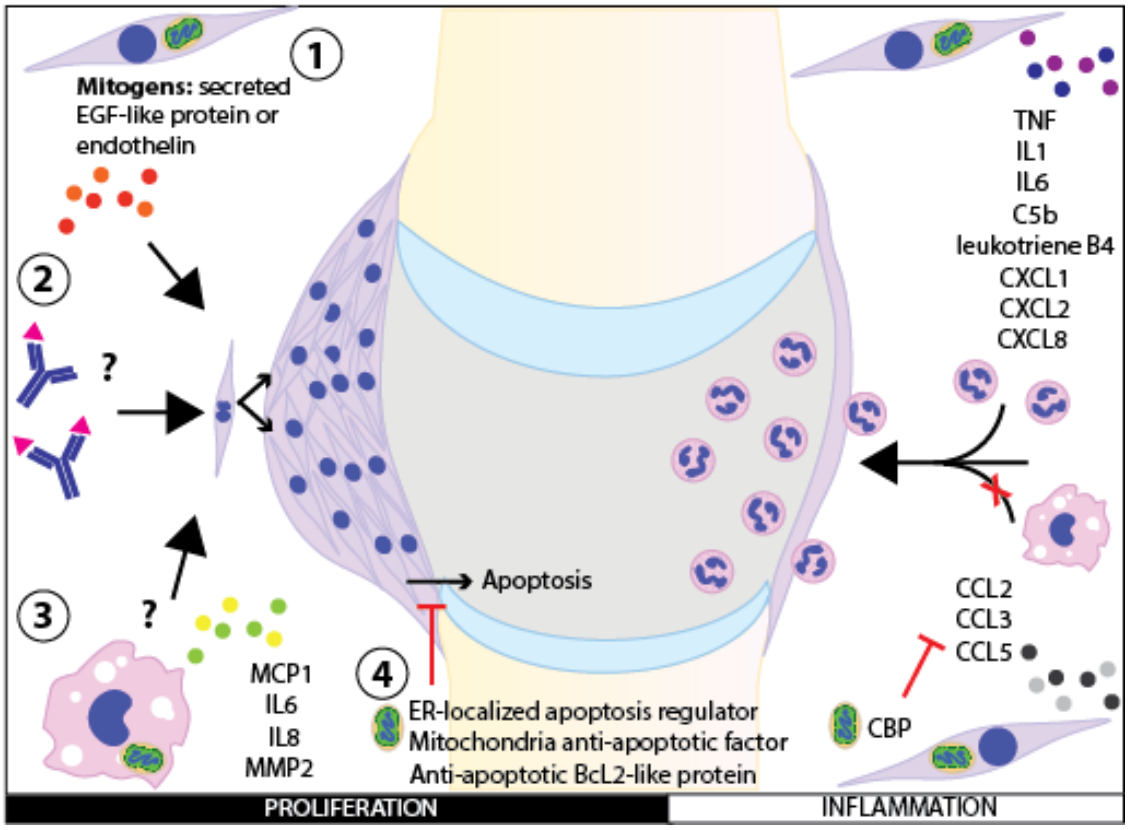

Figure A 3 Possible mechanisms of poxvirus induced joint pathogenesis. 1) The favoured explanation for the proliferative change is mitogens produced by the poxvirus. 2) Antigen-antibody complex deposition in synovial membranes induces proliferation by an unknown mechanism. 3) Infected or activated macrophages produce pro-inflammatory and proliferative cytokines which act through an unknown mechanism. 4) Inhibition of apoptosis by viral proteins in infected cells leads to their accumulation. Neutrophilic infiltration occurs through the release of neutrophil specific chemoattractants from infected cells. Viral chemokine binding protein may selectively inhibit macrophage chemotaxis as has been shown for Orf virus.
BcL2-like B cell lymphoma 2-like, CBP - chemokine binding protein, CCL - C-C motif ligand, CXCL - C-X-C motif ligand, EGF-like - epidermal growth factor-like, IL - interleukin, MCP1 - monocyte chemoattractant protein-1, MMP2 - matrix metalloproteinase 2, ER-localized - endoplasmic reticulum-localized, TNF - tumor necrosis factor

Table A 7 PCR primers to detect EfK3b DNA sensors.

Target	Size	Direction	Sequence	Annealing temperature
DHX9	448	F	ACCGTCCTAAGAGGGCTTGT	58
		R	AAGTTCCCTACCTGCCAAGAG	
DDX41	480	F	TGAAGGGCCCTATGGACTCA	61
		R	GCAAAGATGAGCACCTGTATGAC	
cGAS	498	F	CGCGCAGAAATCTCAGAAGC	56
		R	CATGCCTCTCTGGGTGCTAC	
LSm14A	498	F	GAACTCCACCGTAGCTCTCG	57
		R	GCTTGTTCCATGGTAGGGCT	
Ku70	554	F	GCAGGCGTGTATCCAGAGTG	58
		R	CTCGGACCTTCCTCAACAGG	
Mre11	579	F	ACGGGGAGTTTGAATGGTCC	55
		R	ACAGTGCCTTCCGTTGACTT	
DHX36	597	F	TGCTACCAACATTGCGGAGA	56
		R	CGTCTAGCTTCTTCCCAGCC	
LRRFIP1	356 428	F	GAAGGGGTCTCGTAACCTGC	60
		R	CTTGGGCGAACTGAAACTGC	
TLR9[10]	121	F	CTGCCACATGACCATCGAG	60
		R	GGCCAGGGTCCGGAGGGCGGGGG	
RIG-I[10]	171	F	CTGGACCCCACCTACGTCCTC	56.5
		R	AGCATCCAAAAAGCCACGA	
RNApol III	440	F	GGCATCAACGATAACGGCAC	56.5
		R	AGGGAGAGCTTGACGAGGAT	
ZBP1[a]	449	F	TGTCAGAATGGACCACACGG	62
		R	CTCAGGCTGTTGTTGTTGCC	

[a]40 cycles

[10] From: Banerjee, A., Rapin, N., Bollinger, T., and Misra, V. (2017). Lack of inflammatory gene expression in bats: a unique role for a transcription repressor. Sci Rep *7*. 10.1038/s41598-017-01513-w.

Table A 8 Real time PCR primers to measure innate response in MRC5 and EfK3b cells

Target	Size	Direction	Sequence	Annealing temperature
Ef IL8[10]	183	F	AAACATGACTTCCAAGCTGG	52
		R	TGTGGTCCACTCTCAATCAC	
Ef IL6	138	F	AGAACCAAAGCCCTGGTCAA	54
		R	AATCGTCGTGGTCTTCAGCC	
Ef GAPDH[10]	197	F	GGAGCGAGATCCCGCCAACAT	56
		R	GGGAGTTGTCATACTTGTCATGG	
Ef IFNβ[10]	166	F	GCTCCGATTCCGACAGAGAAGCA	56
		R	ATGCATGACCACCATGGCTTC	
Ef IFNλ	99	F	CTGACACTGAAGGTCCTGGG	61
		R	CTGAAGCGTGGAGTGGATGT	
Ef TNFα[10]	159	F	GCCCATGTTGTAGCAAACC	63
		R	GCCCTTGAAGAGGACCTGGG	
Ef RPS11	104	F	GCGTGGTGACCAAGATGAAG	52
		R	CATGTTCTTGTGGCGCTTCT	
Ef IRF1	118	F	GGCTGGGACATCAACAAGGA	56
		R	CACAGCGAAAGTTGGCCTTC	
Ef IRF3	112	F	CTCTTGCTGAGCCCCAACTT	56
		R	TCACCTCGAACTCCCAGTCT	
Ef IRF7[10]	202	F	CCCGCACTGCACCATCTACCT	56
		R	CAGGTCCTCGTACAGGCTGTTG	
Ef NFκB1	139	F	TATGATGGGACGACACCCCT	56
		R	CCCTTTCCCACGAGTCATCC	
GAPDH[10]	197	F	GGAGCGAGATCCCTCCAAAAT	57
		R	GGCTGTTGTCATACTTCT CATGG	
IFNβ[10]	166	F	ATAGATGGTCAATGCGGCGTC	54
		R	GCTTGGATTCCTACAAAGAAGCA	
IFNλ	122	F	TCCCAGACAGAGCTCAAAACT	51
		R	CTGTCACCCAGGGTCTGTTT	
IL6	111	F	AGTGAGGAACAAGCCAGAGC	52
		R	ATTTGTGGTTGGGTCAGGGG	
IL8[10]	112	F	ACTGAGAGTGATTGAGAGTGGAC	51
		R	AACCCTCTGCACCCAGTTTTC	
TNFα[10]	142	F	CAGCCTCTTCTCCTTCCTGA	57
		R	AGATGATCTGACTGCCTGGG	

[10] From: Banerjee, A., Rapin, N., Bollinger, T., and Misra, V. (2017). Lack of inflammatory gene expression in bats: a unique role for a transcription repressor. Sci Rep *7*. 10.1038/s41598-017-01513-w.

Table A 9 siRNAs targeting IRF1, IRF3, IRF7, and NFκB1.

Target	Direction	Sequence
Ef IRF1	Sense	5′-CrCrCrArArGrArCrArUrGrGrArArGrGrCrCrArArCrUrUTC-3'
	Antisense	5′-rGrArArArGrUrUrGrGrCrCrUrUrCrCrArUrGrUrCrUrUrGrGrGrArU-3'
Ef IRF1	Sense	5′-rCrCrArArArGrUrCrArGrUrGrArArArUrGrUrGrArArGrGAA-3′
	Antisense	5'-rUrUrCrCrUrUrCrArCrArUrUrUrCrArCrUrGrArCrUrUrUrGrGrArA-3′
Ef IRF3	Sense	5′-rGrUrCrGrArGrGrArCrArUrGrGrArUrUrUrCrUrArGrGrUCA-3′
	Antisense	5′-rUrGrArCrCrUrArGrArArArUrCrCrArUrGrUrCrCrUrCrGrArCrCrA-3′
Ef IRF7	Sense	5′-rGrUrArCrGrArGrGrArCrCrUrGrGrArGrCrArCrUrUrCrCTG-3′
	Antisense	5′-rCrArGrGrArArGrUrGrCrUrCrCrArGrGrUrCrCrUrCrGrUrArCrArG-3′
Ef NFκB1	Sense	5′-CrArArUrGrCrGrUrCrCrArArCrUrUrGrArArArArUrUrGTA-3′
	Antisense	5′-rUrArCrArArUrUrUrUrCrArArGrUrUrGrGrArCrGrCrArUrUrGrGrG-3′