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PATHOGENESIS AND TRANSMISSION OF VENEZUELAN EQUINE ENCEPHALITIS VIRUS

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PATHOGENESIS AND TRANSMISSION OF VENEZUELAN EQUINE ENCEPHALITIS VIRUS

by
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To all my teachers who encouraged and believed in me.

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PATHOGENESIS AND TRANSMISSION OF VENEZUELAN EQUINE ENCEPHALITIS VIRUS

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Venezuelan equine encephalitis virus (VEEV) is an emerging arboviral pathogen that affects the Americas. Outbreaks can involve hundreds- of- thousands of equines and humans, spread over large geographic regions, and can last several years. The principal vector in most major coastal outbreaks is the mosquito *Aedes taeniorhynchus.* This species is more susceptible to most epidemic than to enzootic strains, and the adaptation of VEEV to this vector may be an important determinant of epidemic transmission. However, studies on the infection, dissemination, and transmission of VEEV regarding this important vector are lacking.

The major determinant of *Ae. taeniorhynchus* infection with VEEV is the E2 envelope glycoprotein, which interacts with cellular receptors. I therefore hypothesized that differential interactions of VEEV with receptors on midgut epithelial cells determine the ability of a representative epidemic versus a representative enzootic strain to infect this mosquito. In support of this hypothesis, I found that significantly more epidemic VEEV bound to and infected mosquito midguts compared to the enzootic strain. The dissemination from the midgut of an epidemic VEEV strain was compared to that of an enzootic strain. Following initial infection, the epidemic strain was pantropic in tissues of the mosquito, including the salivary glands, whereas the enzootic strain did not infect the midgut efficiently and replicated only in muscles and nervous tissue upon dissemination.

Following the infection of the mosquito salivary glands with an epidemic strain, the amount of VEEV transmitted was estimated. I hypothesized that the method of mosquito infection and saliva collection significantly affects estimates of the amount of virus transmitted and that differing infection routes affect the viremia and mortality of mice. Both the mosquito species and infection route used affected the amount of virus

detected in the saliva. The amount of VEEV transmitted *in vivo* by mosquitoes during blood feeding was significantly less than *in vitro* transmission estimates and mosquito transmission had little or no effect on murine viremia or mortality compared to needle inoculations. These results have important implications for evaluating the vector competence of *Ae. taeniorhynchus* and other VEEV vectors, for designing pathogenesis experiments, and for modeling transmission in nature.

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LIST OF ABBREVIATIONS

Ae.	*Aedes*
AST	average survival time
BHK	baby hamster kidney
C6/36	*Aedes albopictus* larval cells
cDNA	complementary DNA
CNS	central nervous system
CPE	cytopathic effect
d	day
DAPI	4',6-Diamidino-2-phenylindole
DNA	deoxyribonucleic acid
EEEV	Eastern equine encephalitis virus
EIP	extrinsic incubation period
FBS	fetal bovine serum
GFP	green fluorescent protein
h	hour
IFN	interferon
IHC	immunohistochemistry
$ICLD_{50}$	intracranial lethal dose 50%
kB	kilobase, 1000 nucleotides
kDa	kiloDalton
LD_{50}	lethal dose 50%
MEM	minimal essential medium
min	minutes
MOI	multiplicity of infection
mRNA	messenger RNA
nsP	nonstructural protein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFU	plaque forming unit
PI	post infection
PRNT	plaque reduction neutralization test
RNA	ribonucleic acid
SC	subcutaneously
SINV	Sindbis virus
$SMICLD_{50}$	suckling mouse intracranial lethal dose 50%
VEEV	Venezuelan equine encephalitis virus
Vero	African green monkey kidney cells
WEEV	Western equine encephalitis virus

CHAPTER 1: INTRODUCTION

ALPHAVIRUSES

The *Alphavirus* genus is in the family *Togaviridae* and includes 29 virus species, which are mostly transmitted by arthropods and are thus termed "arboviruses." The alphavirus lifecycle occurs mainly between mosquitoes and small mammals or birds. Dead-end hosts important to epidemic alphavirus transmission cycles include larger mammals such as humans and horses. The alphaviruses have a very wide geographic distribution and are divided into the Old and New World viruses. The Old World viruses generally cause a human disease characterized by rash and arthritis. The prototype Old World alphavirus, Sindbis virus (SINV), has a wide distribution including Europe, Asia, Australia, and many parts of Africa. Other Old World alphaviruses, such as Ross River virus (RRV), which is primarily found in Australia, have a more restricted distribution (64).

Some of the alphaviruses primarily found in the New World cause encephalitis and are widely distributed throughout the Americas. Western equine encephalitis (WEE), Eastern equine encephalitis (EEE) and Venezuelan equine encephalitis (VEE) all occur in both North and South America (64). VEE virus (VEEV) is the most important human and equine pathogen of the New World alphaviruses. Many outbreaks occurring primarily in Latin America during the past century have involved tens-to hundreds- of- thousands of equine and human cases (230).

Genome and Virion Structure

VEEV is a single-stranded RNA virus with a plus or messenger sense RNA genome of approximately 11,400 nucleotides. The 5' end of the genome encodes four nonstructural proteins (nsP1-4), which aid in the replication of the genome and viral protein processing. The 3' end of the genome encodes three structural proteins: the

capsid and E1 and E2 envelope glycoproteins. The structural proteins are under the control of a 26S sub-genomic promoter (Figure 1) (64).

VEEV is a spherical virus that has a 70 nm diameter with icosahedral T = 4 symmetry. The RNA genome is surrounded by 240 copies of the capsid protein and this nucleocapsid is surrounded by a lipid bilayer derived from the host cell plasma membrane. The plasma membrane-derived envelope contains 240 copies of each of the E1 and E2 structural glycoproteins, which are assembled in 80 trimers of E1-E2 heterodimers that form spikes on the surface of the virion. The E1 protein lies at the base of the E2-derived spikes and is adjacent to lipid envelope from the host cell (Figure 2) (143).

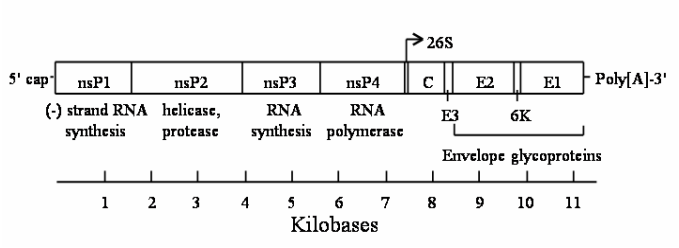

Figure 1. VEEV Genome Organization.

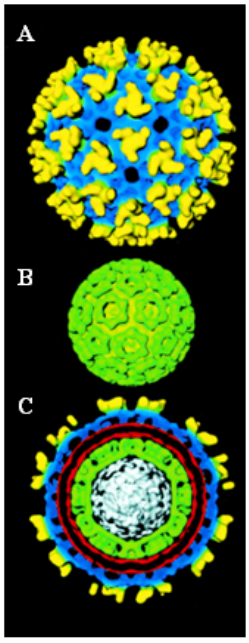

Figure 2. VEEV Cryo-EM Reconstruction. A) External view of the virion particle. B) Nucleocapsid showing the capsid proteins in T=4 icosahedral symmetry. C) Virion cross section; genomic RNA is white, capsid proteins are green, viral membrane is red, envelope proteins comprising spikes are blue/yellow. Adapted from Paredes et al. (143)

Replication Process

Attachment, Entry, and Uncoating

Alphaviruses replicate in many different host species and cell types and replication begins with the virion attaching to the

host cell receptor (Figure 3). For alphaviruses, evidence suggests that the E2 envelope glycoprotein binds to host cell receptors for virus entry. Previous studies determined that viral infectivity is neutralized with anti-E2 glycoprotein antibodies (189) and E2 gene mutations affect cell binding and replication in mice (108, 206). The E2 glycoprotein is a transmembrane protein and contains the important epitopes for neutralizing antibody. For VEEV the E2 gene contains two (most IC, ID and IE strains) or three (most IAB strains) N-linked glycosylation sites (151). The lipid content and glycosylation patterns are determined by the host cell (92, 158). The E2 glycoproteins of viruses defining the VEEV

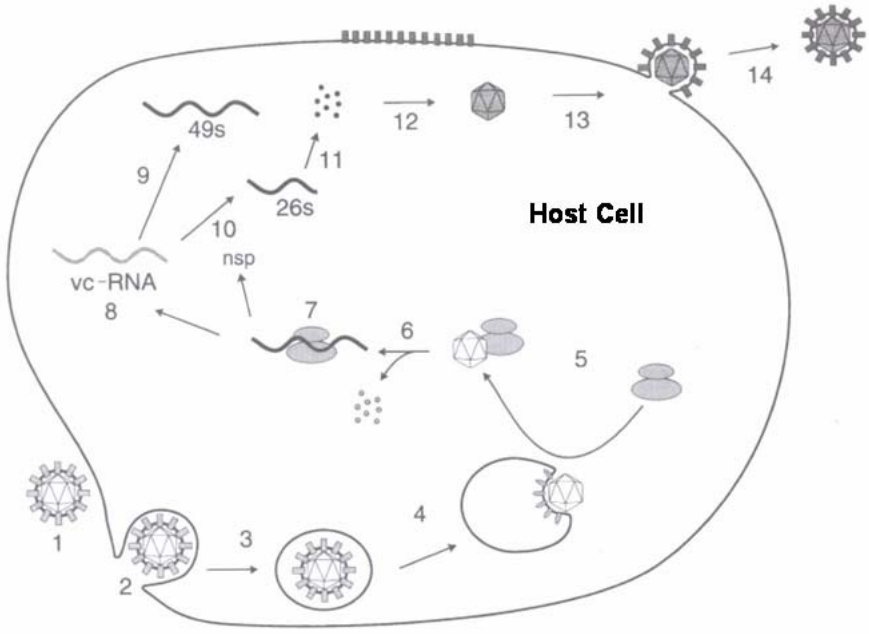

Figure 3. Replication of Alphaviruses. 1) virus binds to host cell via envelope glycoproteins; 2) internalization via receptor-mediated endocytosis; 3) viral and endosomal membrane fusion; 4) nucleocapsid released into host cell cytoplasm; 5) nucleocapsid binds to host cell ribosomes; 6) nucleocapsid uncoated, freeing viral RNA for 7) translation to begin; 8) nonstructural proteins are translated and cleaved forming the replicative complex needed for synthesis of complementary negative-sense RNA, which is the template for synthesis of 9) genomic positive-sense RNA and 10) subgenomic 26S RNA; 11) translation of structural proteins from subgenomic RNA to produce capsid and envelope proteins, which are modified in the secretory pathway prior to insertion in the host cell membrane; 12) packaging of genomic RNA with capsid proteins into nucleocapsid cores; 13) nucleocapsids associate with envelope glycoproteins; 14) viral budding. Adapted from Tsai et al. (204).

antigenic complex demonstrate considerable variability in the number of potential sites for Asn-linked glycosylation (95). The molecular weights of different VEE virus E2 proteins do not correlate uniformly with the number of potential glycosylation sites present in the amino acid sequence of E2 (46, 96); therefore, the number of potential glycosylation sites used or the extent of glycosylation at particular sites must vary among VEE viruses.

SINV uses the high affinity laminin receptor to enter some mammalian cells *in vitro* (221), but additional protein receptors have been identified for SINV in mouse neural (213) and chicken cells (222). Recently, lectin molecules have been implicated as attachment receptors in human monocytic cells for SINV *in vitro*, and virus derived from mosquito cells exhibits increased binding compared to mammalian cell derived virus, most likely due to complex carbohydrate content (101). The mechanism of entry of VEEV *in vivo* is not described, but *in vitro* VEEV enters mosquito cells via the laminin receptor (115). Adaptation of VEEV to a heparin sulfate receptor is known to occur following cell culture passage (11). Some alphaviruses, including VEEV, have a very specific infectivity pattern for their mosquito vectors (184, 185, 236) suggesting the involvement of a less conserved protein receptor or the use of co-receptors or accessory factors during midgut epithelial cell binding. Alphavirus attachment to receptors on the host cell leads to conformational changes, which most likely disrupt protein-protein associations in the envelope for the initiation of envelope disassembly (2, 6, 7). Alphaviruses enter the cell cytoplasm via receptor-mediated endocytosis (94). Following entry into the cell, alphaviruses fuse with the membrane of endosomes at a low pH via a hydrophobic amino acid sequence that is located in the E1 protein. Following entry, the viral nucleocapsid is released into the host cell cytoplasm. Host cell ribosomes bind the nucleocapsid, which triggers uncoating and initiates translation (186).

Transcription, Translation and Genome Replication

The replication of the alphaviral genome occurs on the cytoplasmic surface of endosomes where the genomic RNA acts as messenger RNA (mRNA) for viral non-structural protein translation and as a template for the generation of the viral genome

complementary minus strand. The minus strand serves as the template for the synthesis of both new genomic and subgenomic RNA. The nonstructural proteins are translated from the genomic RNA as two polyproteins due to a termination codon at the end of nsP3, which forms P123 and P1234, encoding nsP1-3 and nsP1-4 respectively. The nonstructural polyprotein is co- and post-translationally cleaved into four distinct polypeptides designated nsP1, nsP2, nsP3, and nsP4. These cleavages are due to a protease in the C-terminal domain of nsP2. The nonstructural proteins form a replicative complex, which is necessary for replication of the viral RNA. Nonstructural protein 1 acts as a methyl transferase and is involved in the synthesis of minus strand RNA. Non-structural protein 2 is a helicase and protease and is involved in the regulation of minus strand RNA synthesis and for the initiation of subgenomic RNA synthesis. Nonstructural protein 3 is involved in RNA synthesis, but its function is not well understood. Nonstructural protein 4 functions as the viral polymerase (186).

The structural proteins are translated from the subgenomic RNA soon after its formation. Translation is initiated at the 5' end and continues uninterrupted to a termination site around 150-300 nucleotides from the 3' poly A terminus. The first protein encoded is the capsid protein, which proteolytically cleaves itself. This cleavage exposes a signal sequence that facilitates the translocation and insertion of the PE2 polyprotein into the endoplasmic reticulum where the envelope proteins are modified by the attachment of oligosaccharides and proteolytic cleavages. These envelope proteins are further modified during transport through the Golgi apparatus. The insertion of the 6 kDa protein and translocation and cleavage of E1 is due to signals on PE2. Dimers of PE2-E1 form in the ER and during transport through the Golgi, cleavage of PE2 leads to the formation of E2 and E3. The E3 protein contains a signal peptide responsible for directing the placement of the E2 into the endoplasmic reticulum. The E1 and E2 proteins are transported in secretory vesicles to the host cell plasma membrane (186).

Virion Assembly and Release

One molecule of genomic RNA interacts with 240 copies of the capsid protein in the cell cytoplasm to generate the nucleocapsid. The cytoplasmic domain of the E2

glycoprotein interacts with nucleocapsids to promote virus assembly at the plasma membrane. Following binding of the glycoproteins to the nucleocapsid and cell membrane, the plasma membrane bends to envelope the particle. New virions bud from the cell and are now able to infect new susceptible host cells (186).

Cell Culture Replication Characteristics

Once most vertebrate cells are infected by alphaviruses, extensive cytopathic effects (CPE) are evident *in vitro*. In contrast, invertebrate cells usually show no CPE when infected *in vitro*, but a persistent infection is established. The maturation of alphaviruses in invertebrate cells has been observed to occur within cytoplasmic membrane-bound "virus factories" that are extruded from the cell to release progeny virus (19). The "virus factories" have not been observed *in vivo* where maturation is known to occur via plasma membrane budding (225).

VENEZUELAN EQUINE ENCEPHALITIS COMPLEX VIRUSES

History of VEE Complex Viruses

VEE epidemics and epizootics were first identified in 1935 in Colombia, but retrospective studies of epidemiological data reveal that outbreaks date back to the 1920's (70, 140). VEEV was first isolated from the brains of fatal equine cases in 1938 from Yaracuy State, Venezuela. From 1938 to 1956 only epizootic VEEV strain isolates were made in the Northern part of South America, which were later classified as antigenic subtype IAB. The IAB along with the IC subtype is designated as epidemic or epizootic since they have only been isolated during outbreaks involving equines and humans. In the late 1950's to 1970's antigenically related virus isolates were made in Central America (90, 183), South America (193), Mexico (182) and Florida (25). These isolates were made from sylvatic and swamp habitats where the presence of equine disease was not noted. These isolates include subtypes/varieties ID-F, II-VI. Further studies revealed that these enzootic subtypes occasionally cause disease, which is sometimes fatal in humans (89, 217, 224). Antigenic studies (247) demonstrated that the epizootic and enzootic

viruses make up a serocomplex of related alphaviruses. This VEE complex now contains 7 different virus species and 14 subtypes and varieties (Table 1) (229).

Table 1. VEEV Antigenic Complex Viruses. Adapted from Weaver et al. (230)

Subtype	Species	Variety	Transmission Pattern	Equine Virulence	Location(s)	Vector(s)
I	VEE Virus	AB	Epizootic	Yes	Central, South, North America	Mammalophilic Mosquitoes
	VEE Virus	C	Epizootic	Yes	South America	Mammalophilic Mosquitoes
	VEE Virus	D	Enzootic	No	Central, South America	*Culex (Melanoconion) aikenii s.sl (ocossa, panocassa); vomerifer, pedroi, adamesi*
	VEE Virus	E	Enzootic	Variable	Central America, Mexico	*Cx. (Mel.) taeniopus*
	Mosso Das Pedras Virus	F	Enzootic	Unknown	Brazil	Unknown
II	Everglades Virus		Enzootic	No	Southern Florida	*Cx. (Mel.) cedecei*
III	Mucambo Virus	A	Enzootic	No	South America	*Cx. (Mel.) portesi*
	Tonate Virus	B	Enzootic	Unknown	South America	Unknown, *Oeciacus vicarius**
	Bijou Bridge Virus	B	Enzootic	Unknown	Western North America	Cliff Swallow Bug
	Mucambo Virus	C	Enzootic	Unknown	Western Peru	Unknown
	Mucambo Virus	D	Enzootic	Unknown	Western Peru	Unknown
IV	Pixuna Virus		Enzootic	Unknown	Brazil	Unknown
V	Cabassou Virus		Enzootic	Unknown	French Guiana	Unknown
VI	Rio Negro Virus		Enzootic	Unknown	Northern Argentina	*Cx. (Mel.) delpontei**

*Possible vector incrimination based only on virus isolation

VEEV Outbreaks

VEE has been an important human and equine disease throughout the Americas for over 70 years (Figure 4) (112). Equines are important in Latin America for agriculture and transportation; therefore, VEE outbreaks cause significant indirect (social and economic) and direct effects on human health (89, 112, 217, 224).

The first recognized VEE outbreak occurred in Colombia during 1935 and spread into Venezuela one year later and finally to the island of Trinidad in 1943 (230). Major VEE outbreaks then occurred intermittently until 1973, with interepizootic periods of around 10 years without any activity. In the 1940's a major outbreak occurred along the Pacific coast of Peru (112, 217). During the 1960's, very large outbreaks occurred in central Colombia, causing over 200,000 human cases and more than 100,000 equine deaths (66). From 1969-1972, an outbreak spread through Central America, Mexico, and into southern Texas involving tens of thousands of equines and people. The outbreak was finally stopped by using extensive equine vaccination and vector reduction efforts (197).

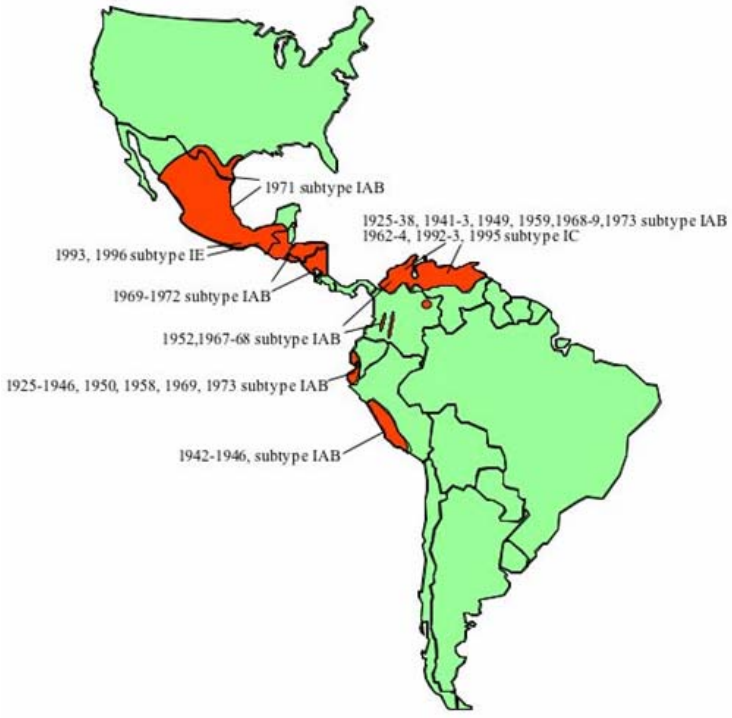

Figure 4. Locations of VEEV Outbreaks. Adapted from Weaver et al. (230)

After 1973, no VEE outbreaks were documented until 1992 in Trujillo State of western Venezuela where a small number of equine and human cases was documented (164). In 1993 and 1996, small equine outbreaks occurred in southern Mexico in Chiapas

and Oaxaca States respectively (137). In 1995, one of the largest outbreaks on record began in Venezuela and spread to Colombia and involved an estimated 75,000 to 100,000 people (235).

Vaccine Development and Use

Soon after the first isolation of VEEV in 1938 equine vaccines were developed from virus obtained from infected animal tissues, which were inactivated with formalin. These vaccines were made in Venezuela, Peru, and Trinidad from subtype IAB strains. However, inactivated virus vaccines generally produce a short-lived immunity and evidence suggests that many outbreaks during this era (1938-1973) were initiated by the use of incompletely inactivated vaccine preparations (98, 199, 234). The epizootic IC strains were never used for vaccine production, so the subtype IC outbreaks are not a result of incompletely inactivated vaccines. Also, these inactivated vaccines have not been produced from wild-type VEEV strains since the early 1970's (230).

A live attenuated vaccine strain, TC-83, was developed in 1961 by passaging the virulent subtype IAB strain, Trinidad donkey, 83 times in guinea pig heart cells (10). This vaccine was shown to be safe and effective during experimental equine trials (215). An inactivated vaccine, which is a multivalent formula also containing WEEV and EEEV in addition to the inactivated TC-83 vaccine, is currently used to vaccinate equines. The U.S. Army Special Immunizations Program offers human vaccination with TC-83 for laboratory personnel with occupational exposure risks, but the availability for civilians has become more restricted in recent years. Additionally, human vaccination with TC-83 is accompanied by high rates of adverse reactions and a low seroconversion rate. Non-responders can receive a formalin-inactivated version, C-84, but multiple boosters are required. A new VEEV vaccine strain, 3526, was found to be more effective than TC-83 in mice (116) and has begun human trials.

Transmission Cycles of VEEV

The major mechanism of VEEV transmission is via infected arthropods within the epizootic or enzootic cycles (see below). However, VEEV can be transmitted via aerosolization of virus particles. Over 150 human laboratory infections presumably

occurred via the generation of aerosols (1). Additionally, intercage transmission through contact or aerosol does occur among laboratory rodents such as rats (83). Since VEEV is highly infectious by aerosol, it is considered a potential biological warfare or terrorism agent (73).

Epizootic Transmission Cycle

Epizootic subtype IAB and IC strains produce a high titered viremia in naturally and experimentally infected equines, which lasts for around 2-4 days with up to 8 $\log_{10}$ infectious units/mL of serum (39, 77, 89, 99, 100, 118, 120, 166, 177, 216). Large numbers of mosquitoes are attracted to and feed on horses resulting in transmission to other equines, humans, and a variety of domestic animals (Figure 5). Many different mosquito species can become infected due to the development of a high titered equine viremia. Field studies suggest that more than one mosquito species can participate in the transmission cycle (177, 197). Proven epizootic vectors following traditional criteria (8) include *Psorophora confinnis* (177), *Ps. columbiae* (197), *Ae. sollicitans* (197, 211), and *Ae. taeniorhynchus* (102, 197, 211). *Aedes taeniorhynchus* mosquitoes are a saltmarsh species implicated in most major coastal VEE outbreaks ranging from northern

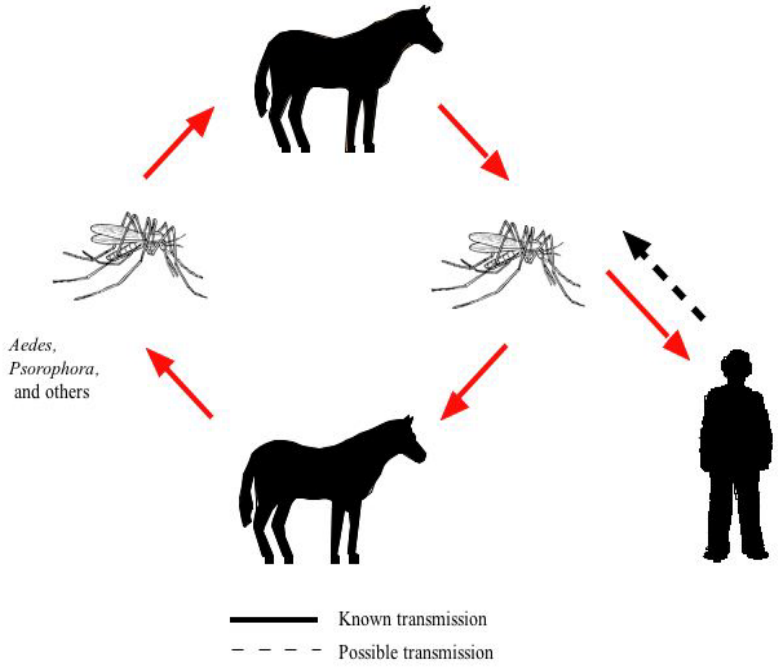

Figure 5. Epizootic VEEV Transmission Cycle.

South America to Texas, including the major 1995 epidemic in Venezuela (165, 235). Additional coastal VEEV vectors include *Culex (Deinocerites)* spp. mosquitoes (60).

Humans can develop viremia levels similar to those of equines (14, 177, 196, 235). During a Venezuelan epidemic, experimental transmission was demonstrated when naturally infected humans were fed upon by *Ae. aegypti* mosquitoes, which were then able to transmit to mice following an extrinsic incubation period (196). Major epidemics without equine amplification have never been reported; therefore, humans are not likely to be important contributors to the epidemic cycle. Additionally, no direct human-human transmission has been reported during epidemics (226).

Enzootic Transmission Cycle

Enzootic VEE complex viruses (subtypes ID-IF, II-VI) circulate in wet tropical forest or shaded swamp habitats and amplify among rodents in the genera *Sigmodon*, *Oryzomys*, *Zygodontomy*, *Heteromys*, *Peromyscus*, and *Proechimys* (Figure 6) (89, 217). Enzootic mosquito vectors are members of the subgenus *Culex (Melanoconion)* within the Spissipes Section and are susceptible to experimental infection, even with small oral virus doses (48, 49, 181, 209, 210, 237). In contrast to the epizootic cycle, enzootic strains generally produce a low serum viremia in equines. However, people can become ill or even die from enzootic infection when they enter the enzootic transmission foci and outbreaks involving tens to hundreds of people have been described (176).

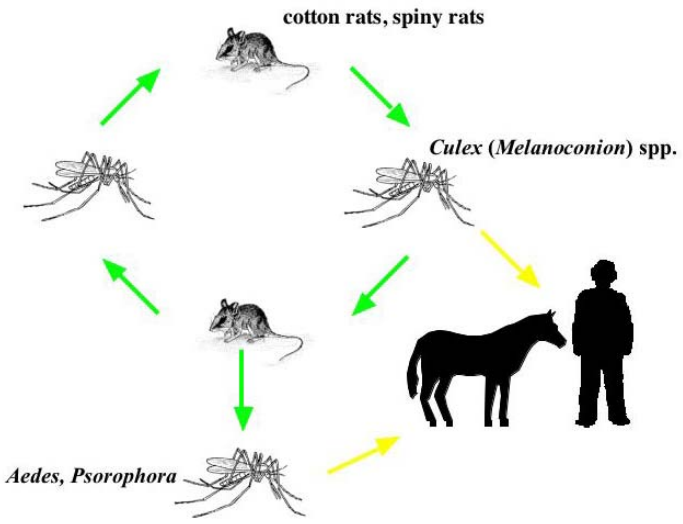

Figure 6. Enzootic VEEV Transmission Cycle.

Determinants of VEE Emergence

Due to the sporadic nature of VEE outbreaks, the source of the subtype IAB and IC strains has been an important topic of research. Many hypotheses have been proposed to explain the origins of the epizootic strains (89), but evidence supports only two. The first hypothesis is that epizootic viruses re-emerge due to the administration of incompletely inactivated vaccines. In support of this hypothesis, genetic studies of the IAB strains isolated during early outbreaks (98, 234) and the isolation of live virus from human vaccines who received "inactivated" vaccines made from IAB strains (199) suggest that some of the early outbreaks may have been caused by improperly inactivated vaccine preparations. However, this hypothesis does not explain the occurrence of recent VEE outbreaks involving subtypes IC and IE, which are not used to make vaccines.

The second hypothesis is that epizootic strains periodically emerge via mutations of enzootic strains. In support of this hypothesis, genetic studies using RNA fingerprinting (163) and sequencing of enzootic and epizootic VEEV strains (97) suggest that IAB and IC subtypes evolved from ID subtypes. When the entire VEE complex was subjected to phylogenetic analyses, evidence was found that the IAB and IC strains evolved at least three times from one of 6 major enzootic lineages, the subtype ID strains, which occur in Colombia, western Venezuela and northern Peru (151, 174, 227). Strong evidence suggesting that epizootic strains emerged from enzootic progenitors comes from IC isolates during the 1992-93 Venezuelan epizootic, which have only 7 amino acid differences from subtype ID strains isolated in the same region in 1997 (164, 218). A single mutation in the E2 envelope glycoprotein was identified to be responsible for generating high-titer equine viremia as well as the IC epidemic serotype (5). Additionally, evidence suggests that the enzootic subtype IE avirulent strains are the progenitors of the equine virulent subtype IE strains isolated during the Mexican epizootics of 1993 and 1996 (15, 137, 138).

In summary, the above evidence strongly suggests that epizootic VEEV strains emerge from enzootic progenitors via a small number of mutations that increase the ability of these viruses to cause high titered equine viremia. Additionally, adaptation to

mosquito vectors could play a role in the emergence of VEE, since vectors implicated during outbreaks are different from those that transmit enzootic strains.

Equine Amplification

Epizootic mosquito vectors generally are not susceptible to infection with low oral doses of epizootic VEEV strains; therefore, the magnitude of equine viremia is an important determinant of vector infection and thus transmission and spread during epizootics. Past studies infecting equines with strains isolated during several outbreaks found a strong correlation between the magnitude of equine viremia and the size of the epizootic (226). The mutations responsible for the emergence of the epizootic phenotype were tested using infectious cDNA clones to generate chimeric viruses. The first chimeric viruses were derived from enzootic subtype IE and epizootic subtype IAB strains due to their differing virulence in guinea pigs. The guinea pig small animal model closely resembles equines in that these rodents exhibit differential responses to some enzootic and epizootic strains (179, 180), but in contrast to equines, guinea pigs succumb to infection with some enzootic subtypes. The chimeric viruses included the nonstructural proteins of an epizootic IAB strain and the structural proteins of an enzootic IE strain (IAB/IE) and the reciprocal construct (IE/IAB). An intermediate virulence and viremia phenotype was generated in the guinea pigs by the chimeric viruses compared to the parental strains; however, the IE/IAB chimera produced a slightly higher viremia and lower survival than the IAB/IE chimera, which suggests that the structural proteins may be more important guinea pig virulence determinants than the nonstructural proteins (150). Additional studies with subtype ID and IC strains and chimeric viruses with swapped E2 genes suggest that envelope and non-envelope genes both affect the virulence phenotype in guinea pigs, although early replication in the lymphoid tissue seemed to be primarily envelope gene dependent (61). In another study, the partial envelope genes from epizootic IAB and IC strains was introduced into the enzootic subtype ID backbone, and when horses were experimentally infected, the equine virulent phenotype was produced (62). Recently, a phylogenetically predicted virus mutation was analyzed by reverse genetics, which identified a single mutation in the E2 envelope

glycoprotein to be responsible for generating high-titer equine viremia as well as the IC epidemic serotype (5). In summary, the above studies support the hypothesis that epizootic VEEV arises from enzootic progenitors and that mutations in the E2 envelope glycoprotein are critical to the acquisition of equine virulence and induction of high serum viremia.

Vector Susceptibility

Epizootic and enzootic VEEV strains are transmitted by different mosquito species; therefore, efficient infection of epizootic vectors may mediate VEE emergence. Kramer et al. found that *Ae. taeniorhynchus*, an established epizootic vector, was more susceptible to infection with epizootic subtype IAB strains compared to enzootic subtype IE strains (102). A more recent study with the epizootic subtype IC strain (3908), which was isolated during the 1995 outbreak, found that *Ae. taeniorhynchus* mosquitoes are more susceptible to this epizootic strain than a closely related enzootic ID strain (17). Additional studies of the susceptibility of *Ae. taeniorhynchus* with strains from the 1993 and 1996 Mexican outbreak indicates that this species is more susceptible to infection with the epizootic strains compared to the enzootic strains (16). These results support the hypothesis that adaptation to epizootic vector species can mediate VEE emergence.

Reciprocal chimeric viruses from epizootic and enzootic VEEV strains were constructed in an effort to identify the genetic determinants of mosquito infection. *Aedes taeniorhynchus* mosquitoes were more susceptible to infection with the chimeras containing the structural genes, more specifically the PE2 envelope glycoprotein E2 precursor gene, from epizootic (IAB or IC) than enzootic (ID) strains (17). A similar reverse genetics study with epizootic and enzootic strains associated with the Mexican outbreaks found that a single mutation in the envelope glycoprotein is responsible for the increased susceptibility of *Ae. taeniorhynchus* mosquitoes to the epizootic strains (16). These studies demonstrate that the E2 envelope glycoprotein, the site of epitopes that define the enzootic and epizootic subtypes, also encodes mosquito infection determinants, which may contribute significantly to VEE emergence. However, this adaptation may be

species-specific because the susceptibility of another proven epizootic vector, *Ps. confinnis*, does not differ between enzootic and epizootic strains (226).

Pathogenesis and Dissemination of VEEV in Vertebrates

VEEV can cause a wide range of disease from inapparent to acute encephalitis in equines and humans. Enzootic strains (subtypes I-E, II, III, and IV) are generally not virulent for equines and produce a low titered viremia and little or no signs of illness (89, 216). In humans most enzootic strains can be pathogenic (47) and even fatal (90, 249). Epizootic strains (IAB and IC) can be fatal for equines and humans and produce a high viremia. Mortality rates in equines during epizootics are estimated at 19%-83%. Human fatalities occur less frequently and neurological disease appears in about 4%-14% of cases (89, 217).

In equines, the signs and symptoms of disease appear around 2 to 5 days after infection with epizootic VEEV. Signs include fever, tachycardia, depression and anorexia (89, 216, 219). A large percentage of animals develop encephalitis 5 to 10 days after infection with signs of circling, ataxia, and hyperexcitability, with death occurring around one week after infection. The level of serum viremia correlates with the development of encephalitis and death, although enzootic strains that are not virulent in equines can cause death when injected intracerebrally, suggesting that virulence is related to the ability of VEEV to replicate extracerebrally and spread to the brain (230).

In humans, VEE occurs in all age groups with no sex bias, although children are most likely to develop fatal encephalitis and suffer from permanent neurological sequelae. In pregnant women, VEEV can infect the fetus and cause birth defects or spontaneous abortions or stillbirths. The incubation period in humans is 2 to 5 days following infection (89). The majority of infections are apparent with abrupt signs and symptoms including malaise, fever, chills, and severe retro-orbital or occipital headache. Myalgia occurs in the lumbar region of the back and the thighs. Signs of VEEV infection include leucopenia, tachycardia, and fever, and are often accompanied by nausea, vomiting, and diarrhea. Central nervous system involvement occurs less frequently with signs and symptoms including convulsions, confusion, and photophobia. Acute disease

often subsides around day 4 to 6 following onset, but the infected individual often feels weak for several weeks. The illness can occasionally be biphasic with a recurrence 4 to 8 days after onset. Lethal human VEEV infection occurs in less than 1% of the cases and is accompanied by diffuse congestion and edema with hemorrhage in the brain, gastrointestinal tract, and lungs (38). Some individuals develop meningoencephalitis associated with intense necrotizing vasculitis and cerebritis. Similar to equines and laboratory rodents, a major depletion of lymphocytes occurs in the lymph nodes, spleen, and gastrointestinal tract (230).

Natural reservoir hosts infected with VEEV do become viremic, but do not show signs and symptoms of disease following experimental infection (12, 247, 248). Experimental VEEV infection of non-human primates causes a nonspecific febrile disease similar to what is observed in human cases. Fatal disease is observed in domestic rabbits, sheep, goats, and dogs during epizootics (89). Laboratory rodents such as hamsters and mice are extremely susceptible to VEEV infection with all subtypes and some subtypes cause fatal disease (217). Epizootic IAB and IC and some ID VEEV strains are known to be lethal for guinea pigs, but other enzootic subtypes tested do not cause death (180). The replication and dissemination of VEEV following needle inoculation in mice results in lymphotropic and neurotropic phases (Figure 7) (54). This mimics what is seen in both humans and horses, making mice a good model. However, mice are not an ideal model, due to their high susceptibility to fatal disease, with mortality often reaching 100%. Several studies suggest that the initial site of VEEV replication is the draining lymph node. Grieder et al. demonstrated that after subcutaneous inoculation of VEEV in the foot pad of the mouse, the closest draining lymph node contained more virus than any other tissue (63). The use of replicon particles containing the green fluorescent protein (GFP) identified Langerhan cells as the initial cells infected by VEEV (117). In both equines and rodents, VEEV infection leads to severe myeloid depletion in bone marrow and lymphocyte destruction in both the lymph nodes and spleen (34, 63, 180). In the murine model, VEEV appears to infect the brain via the olfactory bulb, seeded by viremia (34). A wide range of histopathology is

observed in the brains of infected laboratory rodents, such as mild neutrophilic infiltration to neuronal degeneration, necrotizing vasculitis, and Purkinje cell destruction (see Figure 7, step 5).

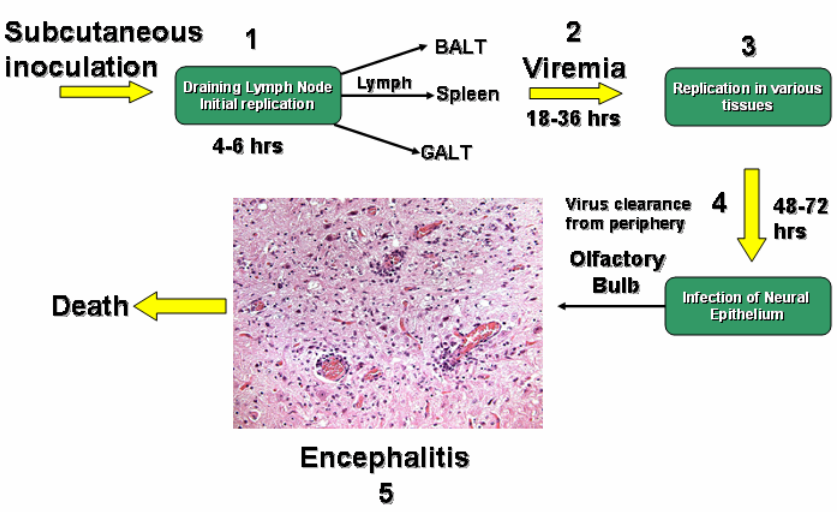

Figure 7. VEEV Pathogenesis in Laboratory Rodents. Following subcutaneous inoculation, virus replicates 1) in draining lymph node 4-6 hrs post infection.; 2) virus spreads through lymphoid tissue with viremia occurring 18-36 hrs post infection; 3) virus replicates in various tissues and then 4) infects the neural epithelium via the olfactory bulb 48-72 h post infection; 5) signs of encephalitis are apparent which ultimately leads to death. BALT=blood associated lymphoid tissue; GALT=gut associated lymphoid tissue.

Laboratory Diagnosis

Diagnosis of VEE can be made by virus isolation from acute phase serum or spinal fluid, or by VEEV-specific IgM antibody detection in cerebral spinal fluid in cases of encephalitis or in the serum of non-encephalitic cases. Virus is most easily isolated from blood on the first three days of illness and declines up to day 8 of illness. Virus can also be recovered from the pharynx in 7 to 40% of patients within the same period. The polymerase chain reaction (PCR) appears to be sensitive for virus detection in acute phase serum samples (204). An IgM capture enzyme-linked immunosorbent assay

(ELISA) and a monoclonal antibody based antigen capture ELISA have been developed for the detection of alphavirus antibodies (21, 119, 173). However, serological diagnosis of VEE can be complicated in horses that have previously been vaccinated for any of the equine encephalitis alphaviruses due to cross-reacting antibodies to the vaccine (162) and it is important to be able to distinguish the subtype and variety of antibodies to VEEV by serologic examination of acute and convalescent sera. The plaque reduction neutralization test (PRNT) is a better method to distinguish between enzootic and epizootic-specific antibodies. However, the similarity of the neutralization domains of closely related strains makes definitive diagnosis difficult. Recently, an epitope blocking ELISA was developed, which is able to distinguish between infections with enzootic ID/E/F and epizootic IAB/C VEEV strains (220). For retrospective studies, four-fold or greater increases in VEEV specific antibody titers detected by any serological assays indicate a recent positive VEEV infection.

Treatment

Considering the high case fatality rate in equines, the prognosis in many cases is likely to be poor; therefore, euthanatization of severely affected equines is the most humane option. When supportive therapy is opted for, treatment should be focused on controlling inflammatory changes in the central nervous system, relieving physical discomfort and minimizing the risk of self-inflicted injuries (162).

In humans, the treatment for VEEV infection is largely supportive and involves analgesics and bed rest. Children with neurologic signs and symptoms are often administered anticonvulsants. Pneumonia is the main extraneural syndrome and is sometimes a result of secondary infection. Due to the severe lymphoid cell depletion, bacterial infections may occur through the gastrointestinal tract; therefore, early antibacterial therapy may improve survival (204).

Prevention and Control

The best way to prevent future VEE outbreaks is to sustain equine vaccinations where epizootic strain progenitors circulate in nature and where outbreaks have been documented in the past. Unfortunately, the governments in these countries often only

provide free equine vaccination during and shortly after periods of outbreaks; therefore, susceptible equine populations are refreshed 5 to 10 years later. If a VEE outbreak is recognized early, vaccination of equines slows the spread of disease. Public and veterinary health officials should ensure that regions in Latin America with a history of VEE receive the live attenuated TC-83 vaccine and not the inactivated vaccines, which are inferior for the protection of equines. Limiting the movement of equines in affected regions is usually not effective because infected animals are asymptomatic for 1 to 3 days; therefore, owners inadvertently move these asymptomatic infected equines to unaffected areas, thinking they are helping to protect them.

Controlling mosquito populations by aerial applications of adulticides may have an impact on reducing the transmission of VEEV. Protecting human populations relies mainly on the personal protection against mosquito bites by limiting exposure and applying mosquito repellants that contain the active ingredient diethylmethylbenzamide (DEET). Applying permethrin to clothing also enhances personal protection. People who live or work near equine herds during outbreaks and people who are in contact with tropical forest or swamp habitats should take seriously the protective measures mentioned above (230).

MOSQUITO ANATOMY AND PHYSIOLOGY

In order to understand the infection and dissemination of VEEV in mosquitoes, it is necessary to have a basic knowledge of mosquito anatomy and physiology. Below is an introduction to the basic internal structure of mosquitoes (Figure 8).

Hemocoel and Tracheal System

The hemocoel is the mosquito's body cavity, which contains the organs and muscles and is an open circulatory system that contains hemolymph fluid. Hemocytes, cells of the mosquito's immune system, are contained within the hemolymph. Tracheal cells provide oxygen to tissues within the mosquito body cavity. These cells consist of external openings (spiracles) from which tubular channels (tracheae) branch, become progressively smaller, and culminate in tracheoblasts, which line tracheoles. The

tracheoblasts deliver oxygen from the tracheae, which are in close association with such organs as the midgut, visceral muscles lining the midgut, and the salivary glands. Trachea have a cuticular lining in the form of helical folds (170).

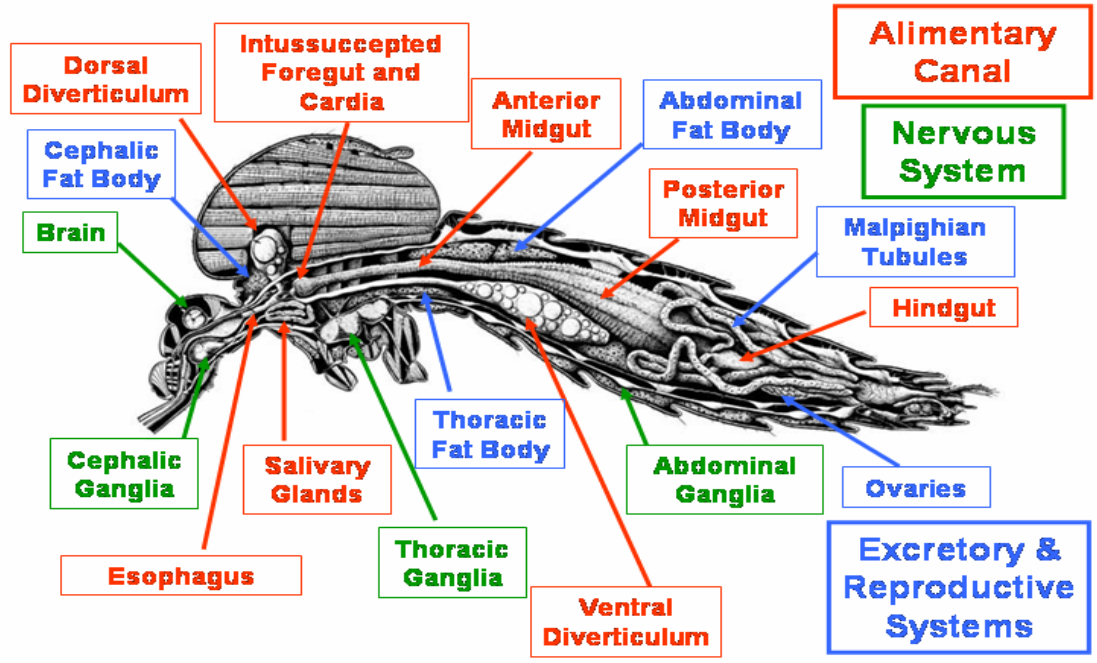

Figure 8. Internal Anatomy of the Mosquito. Organs of the alimentary canal are in red, nervous system in green, and excretory and reproductive systems in blue. Modified from Jobling and Lewis (88), with permission from the Wellcome Library, London.

Alimentary Canal

Esophagus, Diverticula, Intussuscepted Foregut, and Cardia

When a mosquito imbibes a bloodmeal through the proboscis using the cibarial pump, it passes through the labral canal and esophagus on the way to the midgut. The esophagus is a short, soft-walled muscular tube with an inner layer of epithelium lined on the luminal side with cuticle. Located at the posterior end of the esophagus and just anterior to its junction with the cardia are the openings of the diverticula. The diverticula are sac-like structures and have walls consisting of a thin transparent membrane, which is

highly elastic. The walls have an inner layer of epithelium lined with cuticle on the luminal side, and an outer elasto-muscular layer. The walls are impervious to the passage of water. The two dorsal diverticula are smaller in size compared to the ventral diverticulum. When mosquitoes imbibe sugar solutions obtained from plant sources, such as nectar, they are directed into the diverticula and are thought to provide energy to the flight muscles. At the junction of the esophagus and the midgut are the intussuscepted foregut and the cardia (Figure 9). Like the esophagus and diverticula, the intussuscepted foregut is lined with cuticle on the luminal side (27).

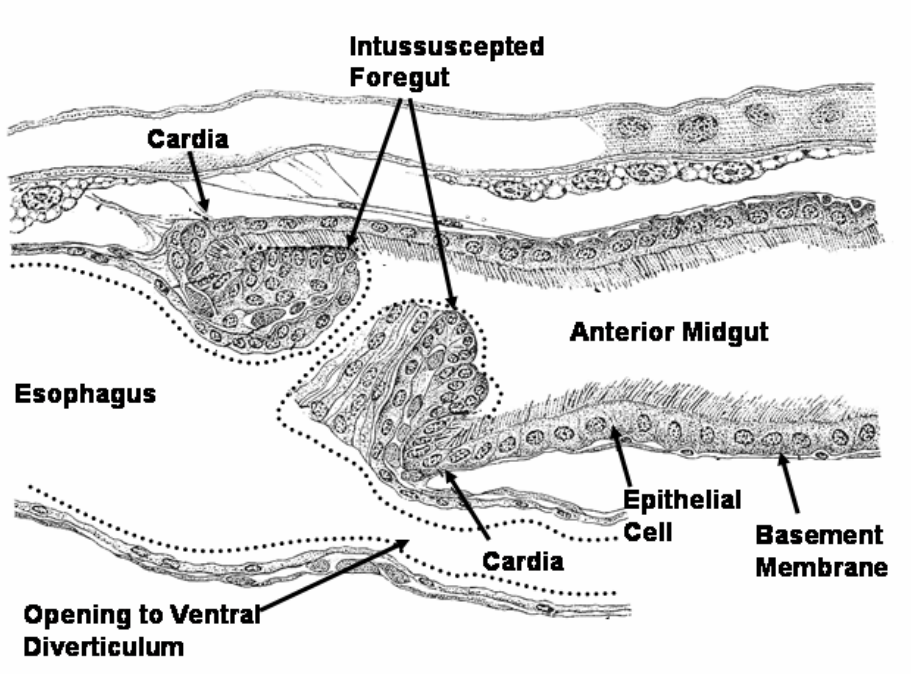

Figure 9. Illustration of the Foregut-midgut Junction. Dotted line indicates location of cuticle. Modified from Joblilng and Lewis (88), with permission from the Wellcome Library, London.

Anterior Midgut, Posterior Midgut, and Hindgut

The anterior midgut is a narrow tubular structure made up of a single layer of epithelial cells. The bloodmeal is directed to the posterior midgut, which can expand in size to accommodate the blood. When empty, it is often configured in many longitudinal folds. The posterior midgut is also made up of a single layer of epithelial cells (Figure 10).

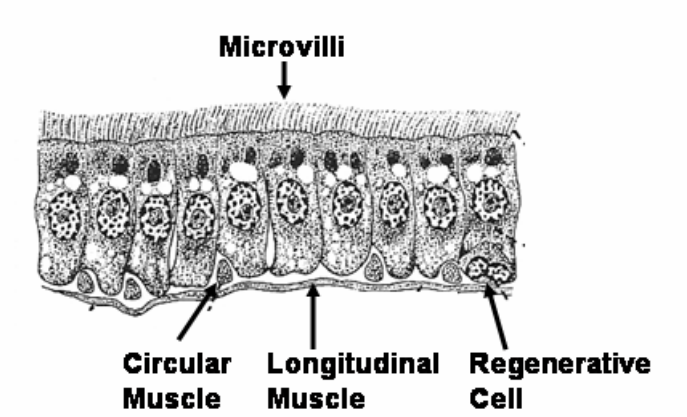

Figure 10. Illustration of the Midgut Epithelium. Modified from Joblilng and Lewis (88), with permission from the Wellcome Library, London.

Midgut cells include the more abundant digestive-absorptive cells and the regenerative and endocrine cells. Scanning electron microscopy studies of the luminal midgut surface of *Ae. aegypti* mosquitoes revealed a group of cells termed "bare cells" that lack microvilli and occur singly or in clusters thoughout the posterior midgut (250). A noncellular basal lamina coats the midgut epithelia and is present during all stages of adult mosquito life (76). The thickness of the basal lamina may be modified by nutritional deprivation of mosquito larvae (65). Following a bloodmeal, the midgut epithelial cells become flattened and the basal lamina layers become stretched, thus reducing the thickness. Within days after the bloodmeal, when most of the blood is digested, the basal lamina becomes irregularly thickened. The basal lamina layers of the anterior midgut are similar to those in the posterior midgut (156). Both the anterior and posterior midgut are surrounded by longitudinal and circular muscles that form a net covering the organ (27). Tracheae, which transport oxygen to various tissues, are associated intimately with the visceral muscle fibers, but do not penetrate the basal lamina according to two studies (75, 76), although a recent study suggests tracheal cells may penetrate the basal lamina in the anterior midgut (170). The hindgut plays an

important role in ion reabsorption and, like the foregut tissue, is lined with cuticle on the luminal side that is impervious to viruses.

Salivary Glands

The salivary glands are paired laterally and each consists of three lobes or acini, two lateral and one medial, which are linked at their anterior ends by the junction of their ducts that allow saliva to flow to the fascicle during feeding (Figure 11). The medial lobe secretions aid in sugar feeding while the lateral lobe secretions aid in blood feeding. The glands are made of a single layer of epithelial cells, which are surrounded by a basal lamina. This layer of acinar cells stores secretory products in apical vesicles and surrounds a central duct, which has a thick cuticular lining. The salivary glands coil irregularly and lie alongside the esophagus, cardia, intussuscepted foregut and the anterior midgut, with fat body in close association.

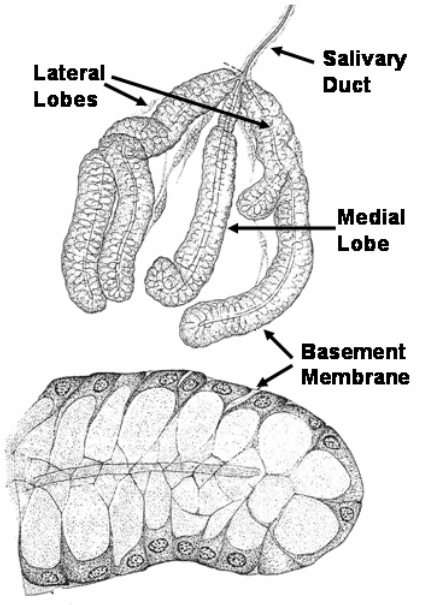

Figure 11. Illustration of the Salivary Glands. Modified from Joblilng and Lewis (88), with permission from the Wellcome Library, London.

Nervous System

The cephalic, thoracic, and abdominal ganglia are all connected throughout the mosquito body and are a part of the ventral nerve cord. Ganglionic connective tissue surrounds the neuropile, which is surrounded by cell bodies. Mosquitoes also have special sense organs such as the ommatidia of the compound eyes and the Johnston's organ, which is considered an organ of hearing. Each eye consists of hundreds of ommatidia, which appear as aligned facets on the surface. Each ommatidium is made up of three parts that include the corneal, iris, and retinal portions. The Johnston's organ is located in the pedicel of the antenna and is made up of a massive collection of sensilla, which respond to sound.

Excretory and Reproductive Systems

Within the mosquito, excretion may be external or internal. The Malpighian tubules are the mosquito's kidney equivalent and are responsible for external excretion, which passes waste products out of the body with the faeces. They filter the hemolymph and secrete the filtrate into the hindgut. A single layer of epithelial cells makes up the tubules. The organs of internal excretion are the fat body cells, in which waste products are accumulated, and is found throughout the entire mosquito body. The fat body is a few cells thick and is surrounded by a permeable basal lamina.

The female mosquito reproductive organs lie in the posterior portion of the mosquito and consist of a pair of ovaries whose oviducts are joined to a common duct, which opens into the genital chamber. The oviducts penetrate the ovary and form the calyx, which contains many follicles that give rise to eggs following a bloodmeal. Each follicle is made up of an outer layer of epithelial cells that have a chorionic membrane that surrounds the oocyte and nurse cells. A muscular ovarian sheath is responsible for holding the follicles together.

INFECTION AND DISSEMINATION OF ARBOVIRUSES IN MOSQUITOES

A mosquito vector's initial exposure to arboviruses occurs most often from a viremic bloodmeal from a vertebrate host, although transovarial or veneral exposure (discussed below) can occur. When the mosquito acquires a bloodmeal, the blood is directed to the posterior midgut where a chitinous peritrophic matrix forms (discussed below) and isolates the blood from the midgut epithelium. Alphaviruses are thought to infect the midgut cells within minutes or hours, which is prior to secretion of the peritrophic matrix (82, 225, 238). Posterior midgut epithelial cells are thought to be the primary site of replication with a concentration at the posterior end near the hindgut for SINV in *Ae. aegypti* mosquitoes (139, 146). VEE-viral replicon particles (VRPs) expressing green fluorescent protein (GFP) infect only midgut epithelial cells when introduced orally (170). A small amount of blood can sometimes be detected in the ventral diverticulum, but most likely cannot be infectious due to the lumen being lined

with cuticle, which is impervious to viruses (232, 241). The foregut and hindgut are also lined by cuticle, which most likely blocks virus infection.

In order for productive infection of a mosquito, enough virus must be ingested to infect the midgut (23). In the posterior midgut the virus is concentrated against the epithelium due to the clotting of the blood cells, which expresses the serum to the periphery (232, 241). When mosquitoes are exposed to an artificial bloodmeal (which does not clot) this concentration does not occur (232) and may explain the reduction in susceptibility of mosquitoes exposed to arboviruses by artificial means. Western equine encephalitis virus binds specifically to isolated microvillar membranes of *Culex tarsalis* mosquitoes within three hours of a bloodmeal and with a higher affinity for susceptible than refractory mosquitoes (80). Initial replication in the midgut can be detected by infectious assay (191) or electron microscopy (223, 239) as early as a few hours to a few days following oral infection.

After infection and amplification of the virus in the midgut epithelium, arboviruses must escape the midgut into the hemocoel and then replicate in the salivary glands in order to be transmitted orally (225). The period of time between the ingestion of the viremic bloodmeal and the ability of the mosquito to transmit the virus is called the extrinsic incubation period. For example, EEEV infects and disseminates very rapidly and can be transmitted by its enzootic mosquito vector *Culiseta melanura* as early as 3 or 4 days post infection (191). The extrinsic incubation period for VEEV is around 7 to 10 days. Alphavirus titers in mosquito vectors usually peak within a few days to a week after infection and then decline over time by about ten-fold (71). RNA interference is the likely mechanism of the decline of alphavirus titers (93, 175). Shortly after the ingestion of the viremic bloodmeal, an “eclipse phase” occurs where titers of virus decline before detectable replication begins (24). The eclipse phase can last anywhere from 1--4 days depending on the bloodmeal titer, the mosquito species, and the temperature of incubation. The decrease in infectious titer is thought to be caused by inactivation of the virus in the hostile environment of the midgut where proteolytic enzymes are secreted and virions are disassembled when entering epithelial cells. In between the midgut

epithelium and the hemocoel is the basal lamina. Electron microscopy reveals that alphaviruses primarily bud from the basolateral membrane of infected midgut epithelial cells. Virions often accumulate in large numbers between the epithelial cells and the basal lamina (223, 239). The basal lamina is a non-cellular layer composed mainly of a fibrous collagen-like structure that coats the epithelium and has pore sizes significantly smaller than arboviruses (70, 154). The salivary glands also contain a basal lamina and the mechanism by which arboviruses pass through the basal lamina of both the midgut and salivary glands is unknown. Romoser et al. suggest that trachea and visceral muscle may act as conduits for VEEV dissemination through the basal lamina of the midgut into the hemocoel (170). Alternatively, cytopathic effects on the epithelial cells of the mosquito midgut by EEEV (240) and WEEV (81) have been suggested to aid in the dissemination of virus into the hemocoel; however, this mechanism has not been reported for VEEV. Japanese encephalitis virus (JEV) (40) and Rift Valley fever virus (RVFV) (167, 168) have been found to replicate in the anterior (thoracic) region of the midgut. The close proximity of the anterior midgut to the salivary glands suggests that viral replication in this region could affect transmission.

Following the dissemination of arboviruses into the hemocoel, secondary tissues and organs are infected. Most arboviruses appear to disseminate to infect the salivary glands via the hemolymph (70) although two studies suggest that dissemination occurs via the neural pathways (106, 128). RVFV sporadically disseminates to the intussuscepted foregut of infected mosquitoes, and subsequently to the fat body, salivary glands, epidermis, neural and endocrine tissues (168). Many arboviruses are detected in the salivary glands at the same time as other tissues in the hemocoel (e.g. fat body); therefore, it is not known whether amplification in these tissues is a requirement for biological transmission (70).

The salivary gland basal lamina must be traversed by arboviruses before the acinar secretory cells can be infected. Nucleocapsids from alphaviruses form in the cytoplasm of acinar cells and mature by budding (239, 240). EEEV budding is random in *Ae. triseriatus* salivary glands, where virions mature on the apical and basal plasma

membrane and into cytoplasmic vesicles (242). In contrast, EEEV buds only from the apical membranes into the salivary matrix in *Cs. melanura* mosquitoes (239). Most salivary gland acinar cells have apical cavities filled with virions late after infection with the majority of arboviruses (225). Arboviruses exhibit different tropisms for the three salivary gland lobes. Most arboviruses replicate first and sometimes entirely within the lateral lobes (70).

Barriers to Transmission within the Mosquito Vector

Many "barriers" exist within the mosquito vector that could prevent transmission of arboviruses. Some are physical barriers while others may represent target tissue/organs with limited susceptibility. These barriers include the midgut infection barrier, where virus is not able to productively infect midgut epithelial cells; the midgut escape barrier, where virus can infect midgut epithelial cells, but cannot disseminate into the hemocoel; the salivary gland infection barrier, where virus is not able to productively infect salivary gland acinar cells; the salivary gland escape barrier, where virus is not shed into apical cavities of infected acinar cells (Figure 12) (70, 71). Mosquito vectors insusceptible to oral infection

are frequently susceptible by intrathoracic infection, indicating that the midgut infection barrier is the most critical barrier to overcome for transmission to occur (71, 122, 125).

Several hypotheses have been proposed to explain the refractoriness of a virus to infect the midgut of a particular mosquito species or the refractoriness of a mosquito for infection with a certain virus. These include: 1) the diversion of the virus into the ventral diverticulum, 2) filtration of viruses by the peritrophic membrane, 3) inactivation of virions by digestive enzymes, 4) cellular charge/charge distribution differences in the midgut epithelia, 5) presence of specific receptor sites on the midgut epithelia of susceptible mosquito species are modified or absent on the midgut epithelia of refractory mosquitoes (71).

Diversion of a viremic bloodmeal into the diverticulum instead of the midgut has been suggested to cause a reduced mosquito susceptibility. It is thought that in a natural

environment sugary solutions (i.e. nectar from plants) are diverted to the diverticulum (56). Diversion of blood into the diverticulum appears to occur most often when sucrose

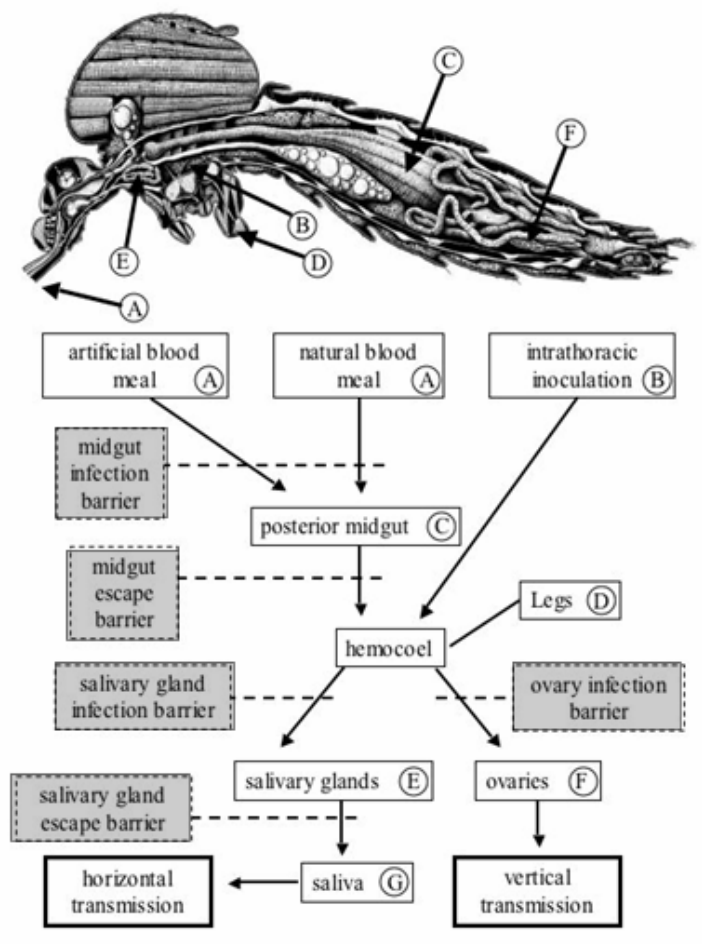

Figure 12. Critical Sites of Arbovirus Infection and Dissemination for Transmission to Occur. Gray shaded boxes are the barriers to dissemination. A) Oral infection occurs by artificial (laboratory setting) or natural bloodmeal. B) Intrathoracic infection bypasses the midgut infection or escape barrier. C) The posterior midgut can be infected following oral bloodmeals. When virus disseminates into the hemocoel, the D) legs E) salivary glands and F) ovaries can become infected leading to either G) virus in the saliva resulting in horizontal transmission or vertical transmission from the infected ovaries. Adapted from Weaver et al. (228).

is mixed with the blood (35, 79, 203). However, diversion of the blood into the diverticulum is most likely a laboratory artifact and does not occur under natural conditions.

The peritrophic matrix forms in the mosquito midgut after blood feeding and is thought to occlude virus from being able to infect the midgut. The pore size of the peritrophic matrix is smaller (20-30nm) than all arboviruses (82, 161). The time required for the formation of the peritrophic matrix is mosquito species specific and ranges from 20 minutes to 32 hours following a bloodmeal. However, most viruses are able to infect the midgut prior to peritrophic matrix formation (71).

Digestive enzymes such as trypsin and chymotrypsin are secreted following a bloodmeal and may inactivate virions. Different mosquito species secrete different digestive enzymes at various times following the bloodmeal and different viruses are affected differently by the digestive enzymes. In most mosquitoes, initial midgut infection occurs prior to digestive enzyme secretion (71). Some viruses bind more efficiently to the midgut in the presence of digestive enzymes potentially found in the midgut lumen. For example, infection of the mosquito midgut for La Crosse virus requires the enzymic cleavage of the G1 and G2 glycoproteins (113, 114). Infection of *Ae. aegypti* mosquito midguts with dengue serotype 2 virus is facilitated by midgut trypsin activity through a nutritional effect and also most likely by direct proteolytic processing of the viral surface (129).

Cell surface charge may affect midgut epithelial cell infection with some arboviruses. For example, the addition of the polycation DEAE dextran to a bloodmeal enhanced the susceptibility of *Ae. aegypti* for SINV, SFV, and West Nile viruses (WNV) (145) and for *Cx. pipiens* for WEEV (71). However, no enhancement of infection occurrs when DEAE dextran is added to bloodmeals for *Ae. aegypti* and 17D yellow fever viruses or Anopheles stephensi and SINV (145).

Houk et al. provide direct evidence that specific receptors on the midgut epithelium may be responsible for differences in midgut infection. They found that Western equine encephalitis virus (WEEV) binds specifically to isolated brushborder

membrane fragments with a higher affinity for membranes recovered from susceptible than refractory mosquitoes (80). Further evidence of the importance of specific receptors on the midgut epithelium is provided by Mourya et al., who isolated two proteins from brushborder membrane fragments of *Ae. aegypti* mosquitoes, which are linked to differential infection of chikungunya virus (130). The 38 and 60 kDa proteins were found to be in lower concentrations in refractory mosquitoes compared to susceptible mosquitoes.

TRANSMISSION OF ARBOVIRUSES

Arboviruses are transmitted either mechanically or biologically (71). Mechanical transmission does not require the replication of the virus within the invertebrate host. The virus is transmitted by contaminated mouthparts of the arthropod vector (59, 91, 121, 147). Soon after an arthropod ingests a viremic bloodmeal, the virus on the mouthparts is inactivated, so only a short period of time exists for mechanical transmission to occur. Mechanical transmission has occasionally been shown to be important in outbreaks, but is not the main contributor to the arboviral cycle (243).

Biological transmission of arboviruses occurs either vertically or horizontally. Vertical transmission occurs when the female mosquito passes the virus to her progeny in the eggs (transovarial) or on the eggs (transovum). Seminal fluid from infected males can sometimes infect progeny as well. Horizontal transmission occurs when an infected vector transmits the pathogen to the vertebrate host during feeding and an uninfected vector ingests the virus during vertebrate viremia. This is the most common route of arbovirus transmission. Arboviruses can also be venerally transmitted from male to female mosquitoes (243) (Figure 13).

When the female mosquito comes into contact with the vertebrate host, she begins to probe by inserting her feeding stylets, or fascicle, intradermally into the host skin. The fascicle is flexible and curves anteriorly within the skin. Saliva is ejected during probing, which aids in the location of blood vessels and has pharmacological effects including anti-hemostatic, vasodialatory, or anti-inflammatory/immunosuppressive activity to aid in

maintaining blood flow. If the mosquito is infected, pathogens within the saliva are transmitted to the host during salivation. The mosquito feeds from both venules and arterioles within the dermal layer, but superficial venules are cannulated more often

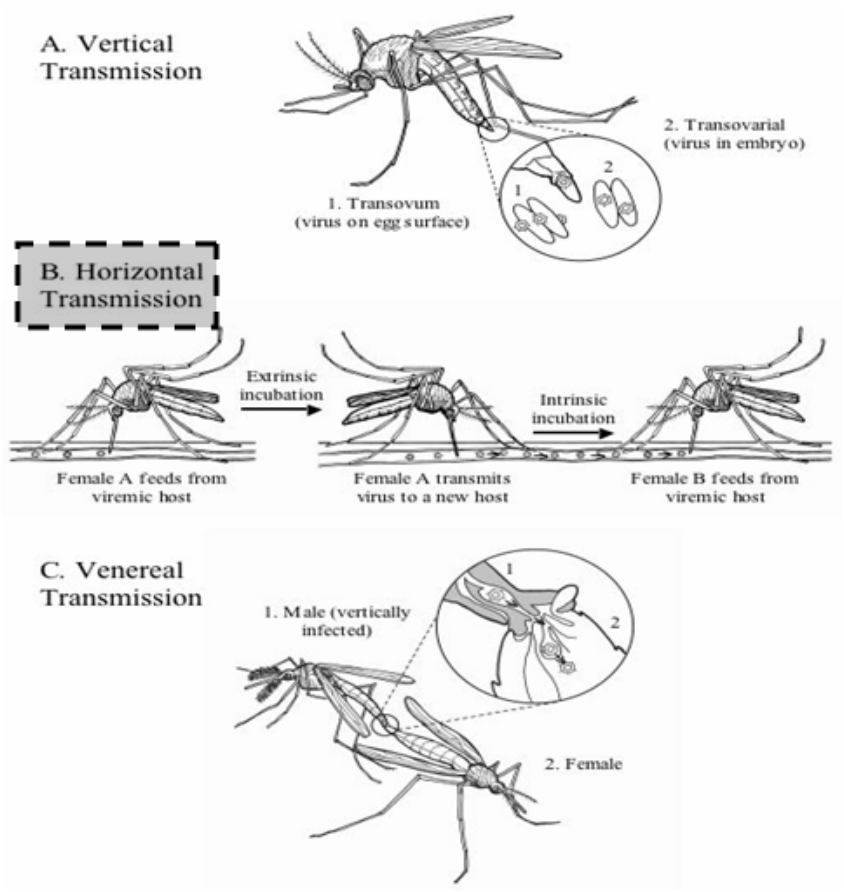

Figure 13. Types of Arbovirus Transmission by Mosquitoes. A) Vertical transmission occurs when the female mosquito passes virus to either its male or female progeny. B) Horizontal transmission occurs when an infected mosquito feeds on a naïve vertebrate host. C) Veneral transmission is a type of horizontal transmission that occurs when a male (vertically infected) copulates with a female. Adapted from Weaver et al. (228).

because they are larger and thus easier targets to the thrusting fascicle. The mosquito will feed directly from a venule or arteriole or from a pool of blood created by laceration during the action of probing (159). Turell et al. found that mosquitoes primarily feed and thus deposit pathogens extravascularly (208, 212). The amount of virus transmitted is

most likely different depending on the virus, mosquito species, and method of estimating the transmission dose (4, 22, 30, 33, 68, 84, 124, 171, 172, 214, 239).

EFFECT OF MOSQUITO SALIVA ON THE PATHOGENESIS OF ARBOVIRUSES

Saliva from sand flies and ticks has been shown to enhance infection with some pathogens (135, 160, 192, 200-202) and mosquito saliva potentiates infection by a few arboviruses including LaCrosse virus (LAC) (142), Cache Valley virus [(CVV) (43) (both in the family *Bunyaviridae*)], and vesicular stomatitis New Jersey (VSNJ) virus (109) (family *Rhabdoviridae*). When saliva was co-inoculated with virus, enhanced viremia levels and increases in virus specific antibody were observed compared to responses in animals inoculated with virus alone. These studies suggest that mosquito saliva may modulate the early innate immune response in the host. Previous studies have suggested that the potentiation of arbovirus infection by saliva is modulated by the vertebrate host's interferon (IFN)α/β response (69, 110). IFN levels play a key role in the immunopathogenesis of VEE. No distinct tissue tropism is observed in mice deficient in the IFN response, with VEEV replicating in nearly all tissues (26).

The above studies suggest that saliva may enhance the infection of arboviruses and that the early pathogenesis following the bite of an infected mosquito may be different compared to a needle infection. However, other studies suggest that vector saliva does not cause an enhancement of infection. Sbrana et al. (178) showed that adult hamsters infected with WNV by mosquitoes or needle inoculation do not differ in the level or duration of viremia, clinical manifestations, pathology, or antibody response. Reisen et al. (157) concluded that birds infected with WEEV or SLEV by mosquito bite or needle show no difference in viremia responses. An *in vitro* study with dengue virus found that mosquito saliva inhibits infection of dendritic cells (3).

PROJECT SUMMARY AND SIGNIFICANCE

Summary

To understand arboviral pathogenesis it is essential to determine the sites of viral replication and the pathways of viral spread throughout the body of both vectors and vertebrate hosts. This knowledge will aid in the development of effective antivirals and vaccines by providing insight for new viral and host targets. Important questions remain regarding the transmission and pathogenesis of VEEV in both the mosquito vector and the vertebrate host; therefore, the goals of this dissertation are to:

1. Determine mechanisms of dissemination and refractoriness of VEEV in *Ae. taeniorhynchus* mosquitoes following an artificial bloodmeal by comparing two virus strains with markedly differing infectiousness. *I hypothesize that enzootic VEEV strain 68U201 fails to be transmitted by this mosquito vector because the virus cannot productively infect midgut epithelial cells.*

Ae. taeniorhynchus mosquitoes are more susceptible to most epizootic than to enzootic VEEV strains. Adaptation of VEEV to utilize this vector may be an important determinant of epidemic transmission. The dissemination of VEEV in the epizootic mosquito vector is poorly studied. Comprehending mechanisms of infection and dissemination in competent vectors is needed in order to define barriers to successful infection in incompetent vectors. Therefore, the replication of VEEV will be compared from the initial site of replication in the midgut to the final replication site in the salivary glands for an epizootic and enzootic strain of VEEV that show different infectivities in *Ae. taeniorhynchus* mosquitoes. I chose to focus on epidemic subtype IC strain 3908 and enzootic subtype IE strain 68U201 due to their relatively low passage histories before undergoing cDNA clone production (17, 150), the existing studies on their characterization for *Ae. taeniorhynchus* infection (16, 17, 102, 141, 226), and the availability of infectious cDNA clones within the laboratory. The divergence of the subtype IE strain from the subtype IC strain is around 11% of their amino acid sequences.

2. Determine the amount of VEEV transmitted from the bite of an infected mosquito. ***I hypothesize that the method of infection and saliva collection significantly affects estimates of the amount of virus transmitted***.

Many basic questions concerning the transmission of a virus by a mosquito have not yet been addressed, such as the amount of virus transmitted. Answering these questions are important for pathogenesis studies, which are not just important for VEE, but for other arboviruses as well. Additionally, experimental protocols for pathogenesis and transmission studies simulating a natural infection have never been optimized.

3. Determine the tissue tropism of VEEV following infection by needle compared to the bite of an infected mosquito. ***I hypothesize that initial sites of viral replication differ by a natural infection route compared to sub-cutaneous inoculation with a needle.***

The primary sites of replication have not yet been determined for VEEV infection following exposure to the bite of an infected mosquito. Pathogenesis studies of VEE have primarily been conducted by needle inoculations. In addition, mosquito saliva has been shown to enhance the infection by a few arboviruses, but this phenomenon has not yet been studied specifically for VEEV. The addition of mosquito saliva may alter VEE pathogenesis.

Significance

For the past 70 years, VEE has remained an important human and equine disease in the Americas, but unfortunately the natural transmission and pathogenesis of the virus has not been adequately addressed. This study addresses the initial infection and dissemination of VEEV in the mosquito vector followed by the transmission of the virus to a vertebrate host.

Questions remain unanswered about the initial infection and dissemination of VEEV in the important epidemic mosquito vector, *Ae. taeniorhynchus*. This study enhances the understanding of the interactions of VEEV and this epidemic mosquito vector, which adds to our comprehension of the mechanisms of VEE emergence. Understanding mechanisms of arbovirus dissemination is important to ensure vaccine

candidates will be unable to disseminate in mosquitoes. Additionally, many different methods are used in the laboratory setting to infect mosquitoes. These methods may affect the amount of virus transmitted by infected mosquitoes, which is important to know for designing transmission and pathogenesis studies of arboviruses. In order to conduct pathogenesis studies simulating natural infection it is necessary to determine the amount of virus a mosquito deposits when imbibing a bloodmeal. The primary sites of replication in a vertebrate host have not yet been determined following VEEV infection by the bite of an infected mosquito. Since mosquitoes probe and deposit virus intradermally, the initial natural site of replication will likely be different from that following the artificial method of infecting animals, which is typically by the subcutaneous route. This study is the first to compare the natural route of VEEV infection to artificial needle-delivery methods.

CHAPTER 2: CHARACTERIZATION OF THE MIDGUT INFECTION BARRIER FOR VENEZUELAN EQUINE ENCEPHALITIS VIRUS IN THE EPIDEMIC MOSQUITO VECTOR *AE. TAENIORHYNCHUS*

ABSTRACT

Aedes taeniorhynchus mosquitoes, important epidemic vectors of Venezuelan equine encephalitis virus (VEEV), are highly susceptible to infection with most epidemic, subtype IAB and IC VEEV strains, but are refractory to infection with enzootic, subtype IE strains. The VEEV E2 envelope glycoprotein, which is a component of spikes on the virion surface and probably interacts with cellular receptors, is the major determinant of *Ae. taeniorhynchus* infection. We therefore hypothesized that differential interactions of VEEV with receptors on midgut epithelial cells determine the ability of epidemic versus enzootic VEEV strains to infect this mosquito. To test this hypothesis, we used purified VEEV labeled with [^{3}H] uridine, and replicon particles expressing green fluorescent protein (GFP). Mosquitoes received a high titer artificial bloodmeal containing either the radiolabelled epidemic IC or enzootic IE strain, or the corresponding replicon particles. Significantly more epidemic IC strain virus bound to and infected mosquito midguts compared to the enzootic IE VEEV. Replicon particles were also injected intrathoracically to test the hypothesis that midgut infection is the only barrier affecting the transmissibility of epidemic versus enzootic VEEV strains. The GFP was detected in mosquitoes intrathoracically infected with both epidemic and enzootic strains, indicating that midgut infection is the primary barrier to enzootic VEEV infection. These results suggest that interactions of the virus with receptors on midgut epithelial cells allow epidemic VEEV strains to infect efficiently *Ae. taeniorhynchus*.

INTRODUCTION

Arthopod-borne viruses (arboviruses) are often transmitted by mosquito vectors to vertebrate hosts though horizontal transmission cycles, where the mosquito is first exposed to the virus by ingesting a viremic bloodmeal from the infected vertebrate host.

The bloodmeal is directed to the posterior midgut of the mosquito where the virus must be able to infect and replicate within the midgut epithelial cells. From the midgut, the virus has to cross the basal lamina to escape into the hemocoel, which is the mosquito's body cavity and contains important secondary amplification tissues such as the fat body, nervous system, and the salivary glands. Following infection of the salivary glands and secretion of virus into the saliva, the mosquito can transmit the virus to a naïve vertebrate host when ingesting a subsequent bloodmeal.

Mosquito Infection Barriers

Certain mosquito species are refractory to oral infection with some, but not other arboviruses. This variable susceptibility and ability to transmit depends on several "barriers" or abortive stages of infection. In the posterior midgut, the virus encounters its first potential barrier--the ability to productively infect midgut epithelial cells. Several hypotheses have been proposed to explain the species-specific refractoriness of midgut epithelial cells to infection, including: 1) diversion of the virus into the ventral diverticulum, a chitin-lined sac used for nutrient storage; 2) filtration of virus by the peritrophic matrix, a chitinous sac secreted by the midgut epithelium during blood digestion; 3) inactivation of virions by midgut digestive enzymes; 4) virus/midgut cell charge interactions that preclude binding, and; 5) the absence of appropriate receptors on the apical surfaces of epithelial cells. Hypotheses 1-4 have not received experimental support (71), whereas hypothesis 5 is supported by the studies discussed below.

Houk et al. (80) provided evidence that specific receptors on the midgut epithelium may be responsible for differences in oral infection by arboviruses. Western equine encephalitis virus (WEEV) binds with higher affinity to isolated midgut brush border membrane fragments of susceptible than refractory mosquito strains (80). Further evidence of the importance of specific receptors on the midgut epithelium was provided by Mourya et al., who identified 2 proteins from brush border membrane fragments of *Ae. aegypti* mosquito midguts that were linked to differential susceptibility to chikungunya virus infection (130). The 38 and 60 kDa proteins were found to be in lower concentrations in refractory mosquitoes compared to susceptible mosquitoes.

Genetic Determinants of Mosquito Infection

Viral determinants of mosquito midgut infection have been studied for alphaviruses, flaviviruses, bunyaviruses and orbiviruses (113, 114, 126, 129, 132, 146, 244, 245). For alphaviruses, all determinants of midgut infection studied to date lie within the E2 envelope glycoprotein, which forms spikes on the virion surface (149) and probably interacts with cellular receptors. A major determinant of midgut infection of *Ae. aegypti* mosquitoes with Sindbis virus (SINV) is the E2 envelope glycoprotein (132, 146). A monoclonal antibody resistant mutant of the Venezuelan equine encephalitis virus (VEEV; *Togaviridae*: *Alphavirus*) vaccine strain with a single amino acid substitution in the E2 glycoprotein gene exhibits reduced infection and dissemination from the midgut of *Ae. aegypti* mosquitoes compared to the parent vaccine, also implicating E2 as an infection determinant (244). Brault et al. determined that mutations in the E2 envelope glycoprotein could enhance the ability of epidemic strains to infect the epidemic mosquito vector, *Ae. taeniorhynchus* (16, 17).

Although these results suggest that mosquito infectivity is determined by specific midgut/alphavirus interactions, such associations have not been studied in detail nor in comparative experiments using different VEEV subtypes. Venezuelan equine encephalitis virus also warrants further study because it is an important emerging arbovirus that causes periodic epidemics, including a 1995 outbreak in Venezuela and Colombia that affected about 100,000 people (165, 235). *Aedes taeniorhynchus,* a saltmarsh species implicated in most major coastal VEE outbreaks ranging from northern South America to Texas, are probably the most important epidemic vector (230). Because *Ae. taeniorhynchus* are more susceptible to most epidemic than enzootic VEEV strains, they serve as an important model vector for understanding the role of adaptation in VEE emergence as well as for understanding alphavirus-mosquito interactions.

We hypothesized that the interactions of VEEV with receptors on midgut epithelial cells determine the ability of epidemic versus enzootic strains to infect *Ae. taeniorhynchus*. To test this hypothesis, we used purified VEEV labeled with [^{3}H] uridine to assess virus binding to mosquito midguts, and replicon particles expressing green

fluorescent protein (GFP) to determine sites of initial infection. Additionally, GFP replicon particles were injected intrathoracically to test the hypothesis that midgut infection is the only barrier to transmissibility of epidemic versus enzootic VEEV strains. The results of this study enhance understanding of the virus/vector interactions necessary for epidemic VEEV transmission.

MATERIALS AND METHODS

Virus

Venezuelan equine encephalitis virus was rescued from infectious cDNA clones derived from either epidemic strain 3908 (subtype IC), a 1995 human isolate from Zulia State, Venezuela during a major outbreak (235), or enzootic strain 68U201 (subtype IE) isolated from a sylvatic Guatemalan focus in 1968 (183). Strain 3908 strain was passaged once in C6/36 mosquito cells before undergoing RNA extraction and infectious cDNA clone production (17), and strain 68U201 was passaged once in suckling mice and twice in BHK-21 cells before undergoing infectious cDNA clone production (150). Virus recovered from BHK-21 cells electroporated with transcribed RNA was used for all experiments without further passage. The use of virus derived from an infectious clone minimizes the development of confounding attenuating mutations that occur when VEEV is passaged in cell culture (11).

Mosquitoes

First generation *Ae. taeniorhynchus* mosquitoes were reared from eggs laid by wild-caught females from Galveston, TX (latitude 29°13.128′ N; longitude 94°56.063′ W). Mosquitoes were reared in an insectary at 27°C with 80% relative humidity using a light:dark cycle of 12:12 h. Adult female mosquitoes were presented with artificial infectious bloodmeals or intrathoracically inoculated 6 to 8 days after emergence, and incubated at 27°C with 10% sucrose provided *ad libitum*.

Radiolabelled Virus

The VEEV strains 3908 and 68U201 were radiolabelled with [^{3}H] uridine in BHK-21 cells. Cell monolayers were infected at a multiplicity of approximately 10

PFU/cell and [^{3}H] uridine (MP Biomedicals, Irvine, CA) was added to the culture medium at a concentration of 12.5 μCi/ml. At 24 hours post infection (PI), the culture medium was harvested, clarified by centrifugation at 3,000 x g for 10 minutes, and virus was precipitated at 4°C overnight in polyethylene glycol 8000 and sodium chloride to final concentrations of 7% and 2.3% (W/V) respectively. Following centrifugation at 6,000 x g for 30 minutes, the pellet was resuspended in 1X TEN [0.05M Tris-HCl (pH 7.2), 0.1M NaCl, 0.001M EDTA] buffer and purified on continuous 20-70% (W/V) sucrose gradients in TEN buffer at 270,000 x g for one h. Virus bands were harvested and pelleted at 270,000 x g for 3 hours using a 30% sucrose/TEN buffer cushion. Virus pellets were resuspended in MEM containing 10% FBS and stored at -80°C prior to mosquito infections.

Mosquito Infections with Radiolabelled Virus

Aedes taeniorhynchus mosquitoes received an artificial bloodmeal containing 20% (V/V) FBS, 10% (V/V) Eagles minimal essential medium (MEM), and 70% (V/V) packed sheep red blood cells and the radiolabelled virus in MEM. Mosquitoes ingested an average of 8.7 $\log_{10}$ PFU/mosquito and 50250 CPM of strain 3908 and 8.4 $\log_{10}$ PFU/mosquito and 27150 CPM of strain 68U201. The two virus strains differed in their incorporation of the radionuclide, but we used the same number of virus particles to infect the mosquitoes with each strain to ensure a fair comparison of virus-midgut cell interaction. At 30 minutes, 90 minutes, and 3 h after feeding, mosquitoes were anesthetized by chilling them and their midguts were dissected and cut in half longitudinally. Later time points were not observed due to peritrophic matrix formation. Residual blood was removed by washing thee times in *Aedes* physiological saline (74). Radiolabelled virus was dissociated from the midgut in 1% Triton X-100 (Sigma, St. Louis, MO) and 0.5% Igepal (Sigma, St. Louis, MO) in *Aedes* saline, and midgut and wash samples were counted for 10 minutes using a Tri-Carb 2800TR liquid scintillation analyzer (PerkinElmer, Wellesley, MA) to determine the fraction of the radiolabelled virus that bound to midguts.

An additional cohort of mosquito midguts was analyzed by autoradiography to ensure that binding measured by scintillation counts reflected radiolabelled virus bound to the luminal side of midgut cells, and not artificially, during washing, to the basal side. Midguts were fixed in 10% formol saline and embedded in LR white resin (SPI supplies, West Chester, PA). One μm sections were cut using a glass knife and dried on microscope slides. Slides were coated with liquid autoradiography emulsion (Eastman Kodak, Rochester, NY) and exposed at 4°C for one month. The slides were then developed using D-19 developer (Eastman Kodak), fixed, and analyzed by brightfield and phase contrast microscopy.

Development of Replicon Particles

Alphavirus genome replication only requires the nonstructural proteins and cis-acting sequences, allowing for the creation of defective replicating genomes (replicons) by deletion of the structural protein genes (18, 50, 152). To visualize primary cells in which VEEV initially replicates, replicon particles were created by replacing the structural proteins within the cDNA clones of strains 3908 and 68U201 with a reporter gene encoding green fluorescent protein (GFP). The structural proteins were expressed from a separate cDNA (helper) clone (Figure 14). Transcribed RNAs (4μg) from both replicon and helper clones were co-electroporated into BHK-21 cells for packaging into virus-like particles. These replicon particles, which are structurally nearly identical to wild-type virus, can infect cells via the same mechanism,

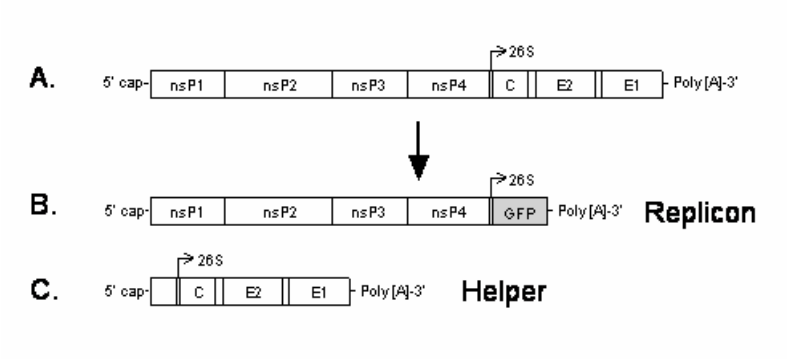

Figure 14. Schematic Depiction of the (A) Alphavirus Genome (B) the VEEV Replicon Encoding GFP, and (C) the Helper RNA Required for Packaging the Replicon RNA.

but no infectious virus can be released to initiate infection of secondary cells because the helper RNA, which lacks an encapsidation signal, is not packaged.

Mosquito Infections with Replicon Particles

Aedes taeniorhynchus mosquitoes were infected with artificial bloodmeals or intrathoracically with VEEV 3908 or 68U201 replicon particles. Mosquitoes were allowed to feed for 1 h from an artificial bloodmeal (described above) containing replicon particles in MEM. Bloodmeal titers were 8 $\log_{10}$ fluorescent forming units (FFU)/ml for both VEEV strains. An additional cohort of mosquitoes received a bloodmeal of 9 $\log_{10}$ FFU/ml of VEEV strain 68U201 replicons to increase chances for midgut infection. Another cohort of mosquitoes was infected intrathoracically (IT) with approximately 1µl containing 4 $\log_{10}$ FFU of VEEV 3908 or 68U201 replicon particles. Twenty-four h PI, midguts and, for IT inoculated mosquitoes, salivary glands, were dissected, washed once in *Aedes* saline, and fixed in 4% paraformaldehyde (PFA). Tissues were mounted and observed using an Olympus FluoView-1000 scanning confocal microscope (Olympus, Melville, NY) to examine sites of GFP expression. An additional cohort of mosquitoes was fixed with 4% PFA by intrathoracic injection, frozen in OCT compound (Sakura Finetek, Torrance, CA), and 6 µM sections were made with a cryostat, mounted with ProLong Gold antifade reagent with DAPI (Molecular Probes, Eugene, OR), and observed by confocal microscopy.

Statistical Analyses

The fraction of radiolabelled VEEV strains 3908 or 68U201 that bound to midguts was analyzed by unpaired t-tests using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA).

RESULTS

VEEV Binding in Mosquito Midguts

Ae. taeniorhynchus mosquitoes were fed high titered bloodmeals containing radiolabelled epidemic or enzootic VEEV to determine whether the subtypes exhibit differential midgut binding. Significantly more epidemic VEEV strain 3908 bound to

midguts compared to enzootic strain 68U201 at all time points post feed (30min $p=0.0015$, 90min $p<0.0001$, 3h $p=0.0006$; Figure 15). This significant difference in binding between the epidemic and enzootic strains was repeated in an additional independent experiment (data not shown). The percentage of virus that bound to the midgut increased for both virus strains over time, with the lowest percentage being detected 30 min post feed (3908 mean 0.046%; 68U201 mean 0.016%) followed by 90 minutes (3908 mean 0.094%; 68U201 mean 0.018%) and 3 hours (3908 mean 0.147%; 68U201 mean 0.033%). All scintillation counts for both virus strains was significantly above background counts at all time points post-infection. Autoradiography confirmed qualitatively the increased binding of VEEV strain 3908 compared to 68U201 (Figure 15) and indicated that virus particles, resulting in exposed silver grains, were concentrated along the luminal brush border of the midgut for strain 3908 (Figure 16A-C); enzootic strain 68U201 binding was not detected using this method (Figure 16D). The absence of virus binding to the basal (hemocoel) side of the midgut (Figure 16) confirmed that virus binding was specific to the luminal side where exposure to the bloodmeal occurred.

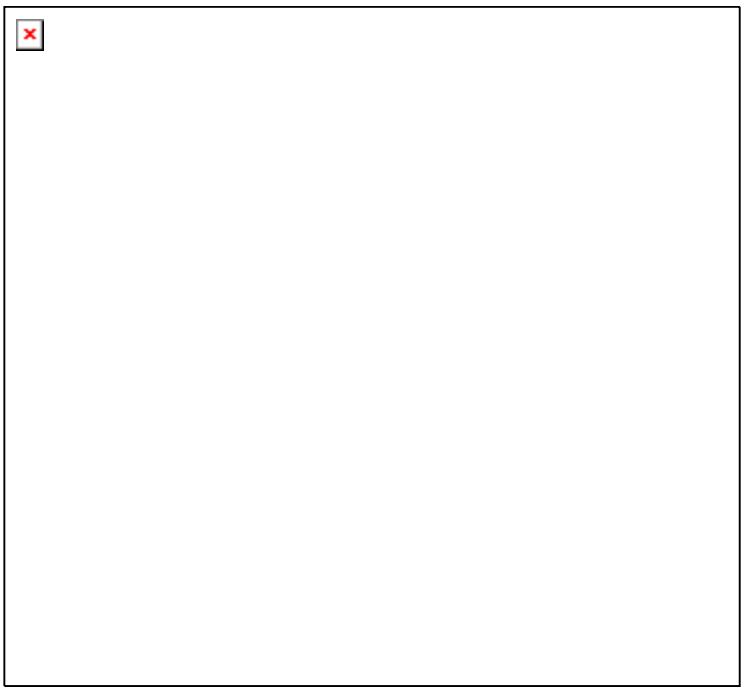

Figure 15. Percentage of [^{3}H] Labeled Virus Bound to the Midgut of *Ae. taeniorhynchus* Mosquitoes Following Oral Infection With VEEV Strains 3908 and 68U201. Each cohort represents VEEV virus strain-time post infection. Horizontal line represents the geometric mean.

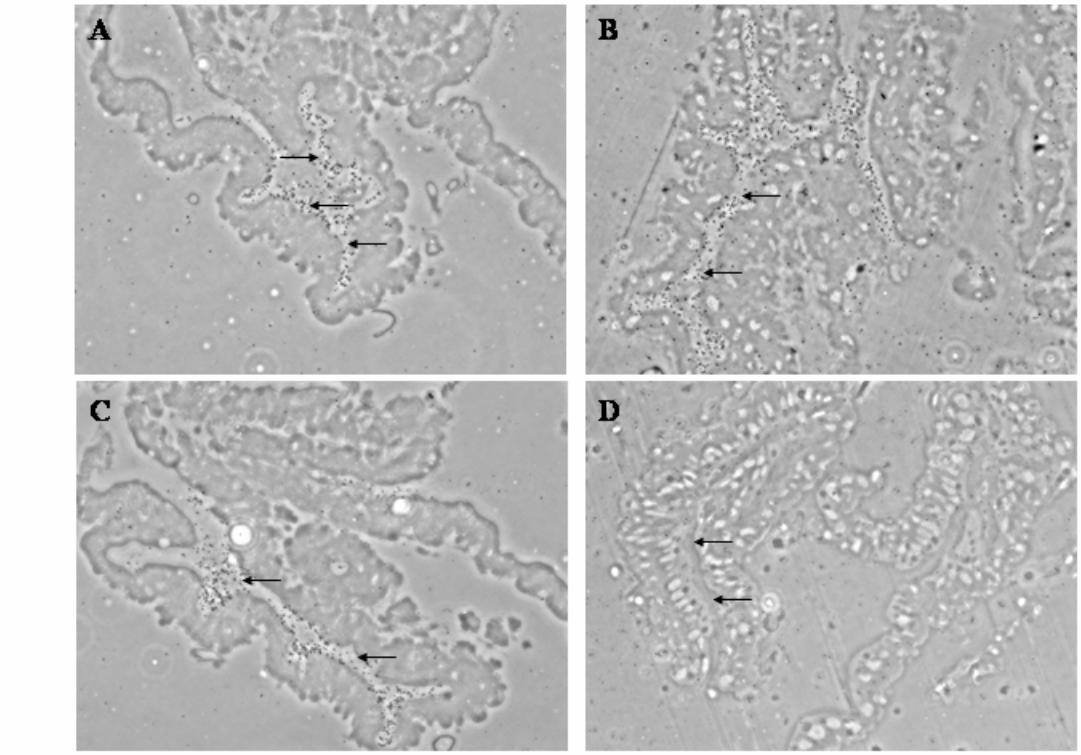

Figure 16. Autoradiography of *Ae. taeniorhynchus* Mosquitoes Infected Orally With [^{3}H] Labeled VEEV Strain 3908 (A-C) and 68U201 (D). Arrows point to the lumen of the midgut.

Primary Sites of VEEV Replication in Orally Infected Mosquitoes

Aedes taeniorhynchus mosquitoes were infected orally with either epidemic or enzootic VEEV replicon particles expressing GFP to determine primary sites of replication. The GFP fluorescence was detected in 80% (8/10) of midguts of mosquitoes fed epidemic strain 3908 replicon particles, compared to significantly less [0% (0/20)] of midguts from mosquitoes fed enzootic strain 68U201 ($p<0.0001$, Fisher's exact test). Even when mosquitoes ingested a strain 68U201 bloodmeal titer that was 10-fold higher

than strain 3908-fed mosquitoes (9 versus 8 $\log_{10}$ FFU/ml), GFP was not detected in any midgut epithelial cells.

The number of infected midgut epithelial cells in mosquitoes that ingested epidemic strain 3908 replicon particles was highly variable, ranging from 1-70 per midgut with a mean of 14 (n=10; Figures. 17, 18). The high variability in the number of infected midgut cells in individual mosquitoes was similar to the variability seen in the percentage of virus bound to the midgut for the same VEEV strain. The epithelial cells expressing GFP were sometimes observed in clusters (Figure 18C), suggesting preferential infection for a certain group of epithelial cells. The only cells observed to be expressing GFP were midgut epithelial cells, except for unidentified small cells in a few samples (Figure 18D).

Orally infected whole mosquitoes were sectioned to determine if primary sites of replication occurred outside of the midgut (i.e. the hemocoel). No GFP expressing cells were found in the hemocoel associated cells and tissues. These results suggest that primary sites of replication in orally infected mosquitoes are restricted to the midgut.

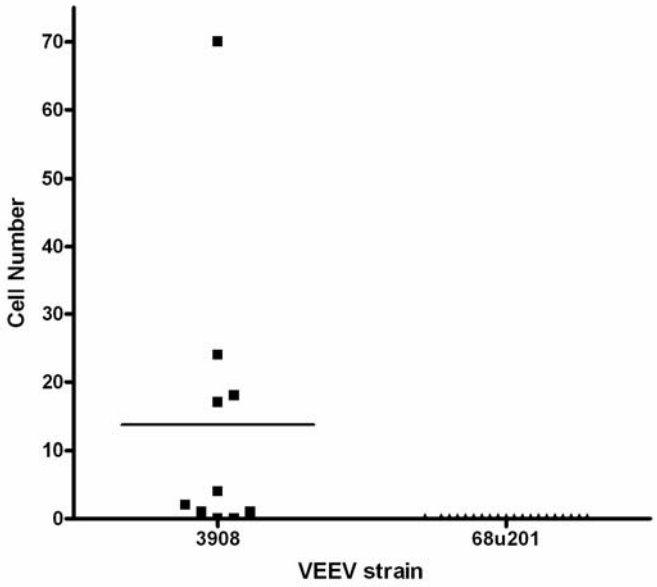

Figure 17. The Number of *Ae. taeniorhynchus* Midgut Epithelial Cells Expressing GFP Following Oral Infection With VEEV Strain 3908 and 68U201 Replicon Particles. Horizontal line represents the geometric mean.

Primary Sites of VEEV Replication in Intrathoracically Infected Mosquitoes

Aedes taeniorhynchus mosquitoes were injected intrathoracically with either epidemic or enzootic VEEV replicon particles expressing GFP to model primary sites of replication following virus

dissemination from the midgut. The GFP fluorescence was detected in 100% (20/20) of mosquitoes injected intrathoracically with epidemic strain 3908 and 80% (16/20) injected with enzootic strain 68U201, which was not statistically significant (p=0.106, Fisher's exact test). For both virus strains, anterior midgut muscles, fat body, tracheal and nerve cells associated with the midgut and malphigian tubules were most often infected (Figure 19). More GFP expressing cells were observed in mosquitoes injected with VEEV 3908 replicons compared to VEEV 68U201 (Table 2). Additional organs infected by VEEV 3908 replicon particles included muscles of the posterior midgut, hindgut and diverticulum, special sense organs (Johnston's organ and ommatidia of the compound eyes), Ganglionic connectives such as in the thoracic ganglia and epithelial cells of the intussuscepted foregut (Table 2, Figure 20).

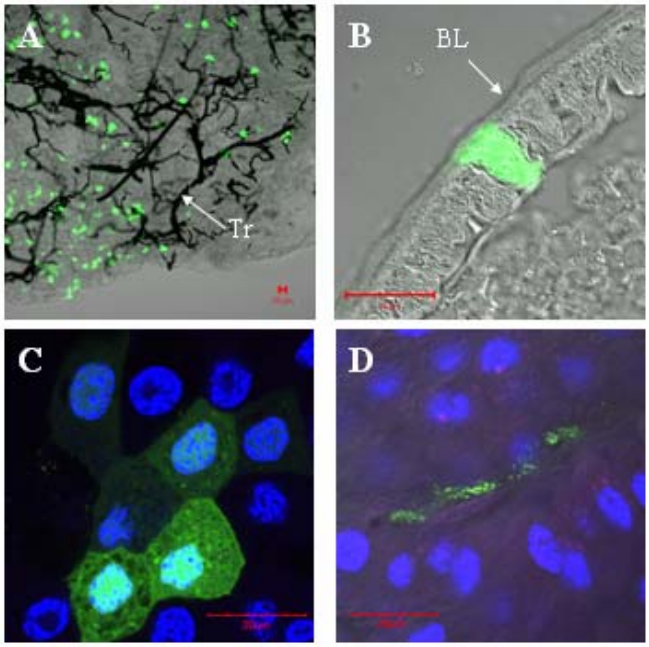

Figure 18. Confocal Micrographs of Midguts From *Ae. taeniorhynchus* Mosquitoes Orally Infected With VEEV 3908 Replicon Particles. Transmitted image is overlayed with GFP fluorescent image (except for C). DAPI staining was added for images C and D. A) Dissected posterior midgut where numerous epithelial cells expressing GFP were detected. Tr = treacheoles, which appear black in the transmitted image. B) Single infected posterior midgut epithelial from a whole mosquito section. BL = basal lamina. C) Cluster of posterior midgut epithelial cells expressing GFP at high magnification. D) Unidentified cells expressing GFP in the posterior midgut. Red bar in lower corner represents 20µm.

DISCUSSION

VEEV Binding in Mosquito Midguts

Previous studies indicate that *Ae. taeniorhynchus* vector infection determinants lie within the VEEV E2 envelope glycoprotein (16, 17), which is a

component of spikes on the virion surface (149) and is involved in cell binding. We hypothesized that differential interactions of epidemic versus enzootic VEEV strains with receptors on midgut epithelial cells determine infection in *Ae. taeniorhynchus*. Using [^{3}H] uridine labeled virus, we found that significantly more epidemic strain 3908 VEEV bound to *Ae. taeniorhynchus* midguts compared to enzootic strain 68U201, and that binding increased for both strains up to 3 h post feed (Figure 15). Binding at later time points was not a focus of this study because alphaviruses are believed to enter midgut cells within minutes or a few hours post feed, prior to formation of the chitinous peritrophic matrix, which blocks virus access to midgut epithelial cells (71, 82, 225, 238).

Table 2. Percentage of *Ae. taeniorhynchus* Mosquito Tissues Infected by Intrathoracic Injection of VEEV 3908 or 68U201 Replicon Particles.

Tissue	VEEV 3908	VEEV 68U201
Anterior Midgut Muscles	100% (20/20)	80% (16/20)
Posterior Midgut Muscles	100% (20/20)	0% (0/20)
Hindgut Muscles	100% (20/20)	0% (0/20)
Ventral Diverticulum Muscles	100% (20/20)	0% (0/20)
Intussuscepted Foregut	25% (5/20)	0% (0/20)
Tracheal Cells Associated with the Midgut	45% (9/20)	15% (3/20)
Tracheal Cells Associated with the Malpighian Tubules	100% (20/20)	45% (9/20)
Tracheal Cells Associated with the Ovaries	40% (8/20)	30% (6/20)
Nerves Associated with the Midgut	40% (8/20)	15% (3/20)
Ganglionic Connectives*	100% (10/10)	0% (0/10)
Johnston's Organ*	100% (10/10)	0% (0/10)
Ommatidia*	100% (10/10)	0% (0/10)
Fat Body*	100% (10/10)	80% (8/10)
Salivary Glands	0% (0/20)	0% (0/20)

*Tissues only observed in whole mosquito sections.

Autoradiography confirmed the differential binding results, depicting a concentration of silver grains reflecting VEEV strain 3908 virus binding along the luminal surface of the midgut (Figure 16 A-C), and no detectable concentration of silver grains for VEEV strain 68U201 (Figure 16D). Additionally, the autoradiography results confirmed that virus binding occurred specifically to the luminal side of the midgut, the site of oral mosquito infection, and not on the basal side that might reflect artificial, nonspecific binding during dissection or washing. However, we cannot conclude that the binding on the luminal side specifically involves receptors on midgut epithelial cells; therefore, the binding observed in this study only represents virus associated with the lumen on the midgut. Competitive binding studies are needed to determine if this binding is specific.

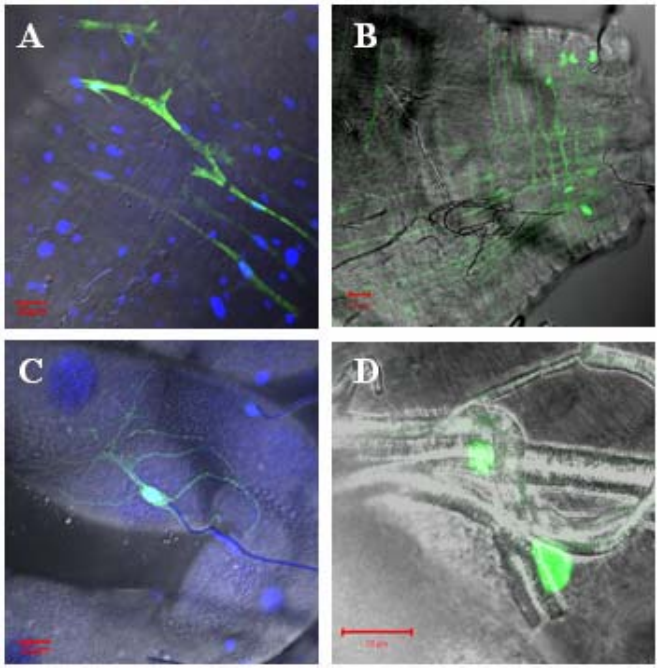

Figure 19. Confocal Micrographs of Dissected Tissues From *Ae. taeniorhynchus* Mosquitoes Intrathoracically Infected With VEEV 3908 (A and B) and 68U201 (C and D) Replicon Particles. Transmitted image is overlayed with GFP fluorescent image. DAPI staining was added for images A and C. A) Circular muscles of the anterior midgut. B) Circular and longitudinal muscles of the posterior midgut. C) Tracheoles associated with the Malpighian tubules. D) Cells associated with Tracheoles of the midgut. Red bar in lower corner represents 20μm.

Primary Sites of VEEV Replication in Orally Infected Mosquitoes

The primary sites of VEEV replication in orally infected *Ae. taeniorhynchus* mosquitoes were determined using replicon particles expressing GFP. Coincident with increased binding to midguts, more GFP expressing cells were detected in mosquitoes fed epidemic strain 3908 (80%) compared to enzootic strain 68U201 (0%). Even when

mosquitoes were fed a 10-fold higher bloodmeal titer of strain 68U201, no GFP expressing cells were detected. Previous studies determined that *Ae. taeniorhynchus* mosquitoes are more susceptible to most epidemic compared to enzootic VEEV strains (102), and that vector infection determinants lie within the E2 protein (2, 3). The results of our study suggest that VEEV epidemic subtype IC strains infect *Ae. taeniorhynchus* mosquitoes more efficiently than enzootic subtype IE due to more efficient initial interactions (possibly with receptors) on the apical surface of midgut epithelial cells.

For epidemic VEEV strain 3908, both the number of midgut epithelial cells infected by replicon particles and the percentage of radiolabelled virus bound were highly variable between individual mosquitoes. This variability could reflect the use of F_1 outbred mosquitoes, which are probably genetically less homogenous compared to colonized mosquitoes used in previous studies (45, 139, 170, 190). The average number of midgut cells infected with strain 3908 replicons was 14 (Figure 17). Other studies of arbovirus infection report similar, low numbers of infected mosquito midgut cells, even with high titer bloodmeals. Scholle et al. (190) observed that $\leq$ 15 midgut cells were infected when *Culex pipiens quinquefasciatus* mosquitoes ingested West Nile (WNV) virus-like particles (VLPs). Additionally, only a small number of infected cells was initially observed in the midgut of mosquitoes infected orally with SINV expressing GFP (45, 139).

The midgut epithelium of mosquitoes is composed primarily of columnar epithelial cells whose function is secretory and absorptive, and which aid in bloodmeal digestion (75). Another midgut epithelium cell type is the endocrine cell, which tends to be smaller than secretory cells (20). In our study, GFP expressing midgut cells occurred both singly and in clusters (Figure 18). The repeated occurrence of infected clusters suggests that selected cells are preferentially targeted by VEEV. Scanning electron microscopy studies of the luminal midgut surface of *Ae. aegypti* mosquitoes revealed a group of cells termed “bare cells” that lack microvilli and occur singly or in clusters thoughout the posterior midgut. The authors speculate that these bare cells correspond to a subset of cells preferentially invaded by malaria parasites (250). Although further

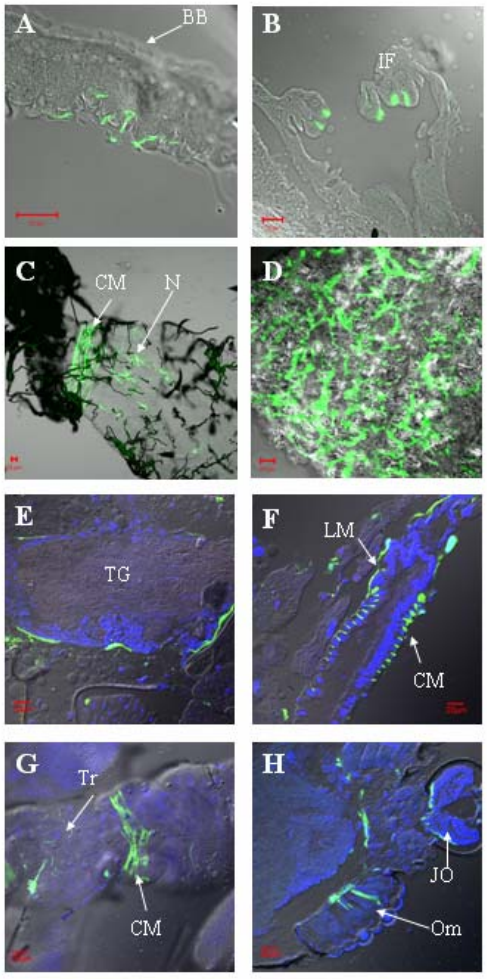

Figure 20. Confocal Micrographs of Tissues From *Ae. taeniorhynchus* Mosquitoes Intrathoracically Infected With VEEV 3908 Replicon Particles. Transmitted image is overlayed with GFP fluorescent image. DAPI staining was added for images E-H. Images taken from whole mosquito sections: A, B, E-H. Images taken from dissected tissue: C, D. A) Tracheoles associated with the posterior midgut. BB = brush border. B) Epithelial cells of the intussuscepted foregut (IF). C) Circular muscles (CM) and nerves (N) of the posterior midgut. D) Ventral diverticulum muscles. E) Ganglionic connective tissue of the thoracic ganglia (TG). F) Longitudinal muscles (LM) and circular muscles (CM) of the posterior midgut. G) Circular muscles (CM) and tracheoles (Tr) of the hindgut. H) Johnston's organ (JO) and ommatidia (Om). Red bar in lower corner represents 20µm.

investigation is necessary, we speculate that VEEV-infected cells may share similar characteristics with "bare cells" as a preferential target for VEEV infection. Scholle et al. reported finding antigen positive clusters of cells in the midgut of *Cx. p. quinquefasciatus* mosquitoes infected with WNV VLPs, possibly due to mitotic division of infected cells (190). However, we did not observe mitotic figures and, since midgut cells in adult mosquitoes are not rapidly dividing unless damaged (36), we believe the clusters of cells may represent preferentially infected cells.

The only *Ae.*

taeniorhynchus cells that expressed GFP after bloodmeals containing replicon particles were midgut epithelial cells. However, in a few samples, unidentified smaller midgut cells were found to be expressing GFP. The morphology of these cells suggests the possibility that they are endocrine cells, but further investigation is needed for definitive identification.

The rapid appearance of arboviruses in the hemocoel of mosquito vectors prior to replication and dissemination within the midgut suggest the role of a "leaky" midgut following a bloodmeal (13, 71, 128, 223, 239, 241). Orally infected whole mosquitoes were sectioned to determine if primary sites of replication could occur outside of the midgut, within the hemocoel. No GFP expressing cells were detected in the hemocoel associated cells and tissues suggesting no role of a "leaky" midgut in this study. However, midgut disruption is most likely a rare event and a larger sample size is needed to determine more conclusively the role in dissemination of a "leaky" midgut.

Primary Sites of VEEV Replication in Intrathoracically Infected Mosquitoes

GFP replicon particles were injected intrathoracically to test the hypothesis that midgut infection is the only barrier that determines the transmissibility of epidemic versus enzootic VEEV strains. GFP fluorescence was detected in 100% of mosquitoes injected intrathoracically with epidemic strain 3908 and in 80% of mosquitoes infected with enzootic strain 68U201. For both virus strains, anterior midgut muscles, fat body and tracheal and nerve cells associated with the midgut and malpighian tubules were most often infected (Figure 19). Our results agree with those of Romoser et al. (170), except that we did not find infected tracheal cells associated with the salivary glands. This difference may be due to the different VEEV and *Ae. taeniorhynchus* mosquito strains used. However, in agreement with our results, Romoser et al. (170) also reported consistent infection of tracheal cells in the alimentary tract, suggesting they may serve as conduits for virus dissemination from the midgut. Scholle et al. also reported consistent infection of tracheal cells associated with the midgut of *Cx. p. quinquefasciatus* mosquitoes infected with WNV VLPs (190). Our results add to the growing evidence of

susceptibility of tracheal cells to oral infection by arboviruses, suggesting that dissemination from the midgut may occur via these cells.

More GFP expressing cells were detected in mosquitoes intrathoracically injected with the strain 3908 replicon compared to the 68U201 replicon. However, when fully infectious VEEV is injected intrathoracically, strain 68U201 replicates to only slightly lower titers compared to strain 3908 (DRS, unpublished). Additional organs infected by VEEV strain 3908 replicon particles included muscles of the posterior midgut, hindgut and diverticulum, nerves, Johnston's organ, ommatidia, thoracic ganglia and epithelial cells of the intussuscepted foregut (Figure 20). We did not detect infection of salivary glands. The number of cells expressing GFP was dose dependent for both virus strains (data not shown).

In summary, epidemic VEEV strain 3908 bound to and infected significantly more midgut epithelial cells in *Ae. taeniorhynchus* mosquitoes compared to enzootic strain 68U201. When the midgut was bypassed and replicon particles were injected directly into the hemocoel, most of the same tissues were susceptible to infection with both epidemic and enzootic strains. Therefore, we conclude that interactions of VEEV with midgut epithelial cells (possibly with receptors) probably determine their ability to infect this important vector. Future studies with additional epidemic (subtype IAB) and enzootic (subtype ID) VEEV strains are needed to determine if comparable virus-vector interactions mediate the emergence of all epidemic VEEV strains via changes in epidemic vector infectivity. Additionally, we plan to assess the role of nonstructural vs. structural protein genes in initial midgut cell infection and replication.

CHAPTER 3: INFECTION AND DISSEMINATION OF VENEZUELAN EQUINE ENCEPHALITIS VIRUS IN THE EPIZOOTIC MOSQUITO VECTOR, *AEDES TAENIORHYNCHUS*

ABSTRACT

A detailed study addressing the initial infection, dissemination, and transmission of Venezuelan equine encephalitis virus (VEEV) in the epidemic mosquito vector, *Aedes taeniorhynchus*, is lacking. We therefore compared infection of an epidemic VEEV subtype IC strain to that of an enzootic subtype IE strain using virus titrations, immunohistochemistry, and a virus construct expressing green fluorescent protein. This mosquito was more susceptible to the epidemic strain, which initially infected the posterior midgut and occasionally the anterior midgut and cardia. Once dissemination beyond the midgut occured, virus was present in nearly all tissues and organs. Transmission to mice of the epidemic strain was first detected on day 4 post-infection. In contrast, the less infectious enzootic strain did not efficiently infect midgut epithelial cells, but replicated in muscles and nervous tissue upon dissemination. This study provides a better understanding of the patterns of replication and dissemination of epizootic and enzootic VEEV in this important vector.

INTRODUCTION

Understanding the infection of mosquitoes by arboviruses is necessary to comprehend the epidemiology of diseases caused by these agents. This topic has therefore received considerable attention in the past (24, 70, 71, 123, 131), but only minor emphasis recently. Many barriers exist to the infection, dissemination, and transmission of arboviruses by their mosquito vectors and an understanding of these mechanisms is important for the design of safer vaccines and novel strategies to interrupt transmission.

Arbovirus Infection Patterns in the Mosquito Vector

Most studies agree that the posterior midgut epithelial cells are the primary site of replication after ingestion of a viremic bloodmeal (139, 146, 170). A threshold of infection, the minimum dose required to infect the midgut, has been demonstrated for many viruses in mosquitoes (23). After infection and amplification of the virus in the midgut epithelium, the virus must escape the midgut into the hemocoel, where secondary tissues and organs are infected including the salivary glands. Most arboviruses appear to disseminate via the hemolymph (70) although two studies suggest that dissemination occurs via neural pathways (106, 128). Many arboviruses are detected in the salivary glands at the same time as other tissues in the hemocoel; therefore, it is not known whether amplification in these tissues is a requirement for biological transmission (70).

An important emerging arbovirus lacking attention in recent years regarding virus/vector interactions is Venezuelan equine encephalitis virus (VEEV; *Togaviridae*: *Alphavirus*). The principal vector in most major coastal outbreaks, including the 1995 epidemic in Venezuela involving about 100,000 people, is the mosquito *Aedes taeniorhynchus* (165, 235). This species is more susceptible to most epidemic than enzootic strains, and the adaptation of VEEV to this vector may be an important determinant of epidemic transmission. However, no detailed studies of the infection, replication, and dissemination of VEEV in this vector have been reported. To more fully understand the differential susceptibility of *Ae. taeniorhynchus*, we compared the infection and dissemination patterns of an epidemic, subtype IC VEEV strain to that of an enzootic, subtype IE strain using virus titration, immunohistochemistry, and a virus construct expressing green fluorescent protein (GFP). We also determined the earliest time point when this mosquito can transmit virus to a vertebrate host.

METHODS

Virus

VEEV strains were rescued from infectious cDNA clones derived from epidemic subtype IC strain 3908, enzootic subtype IE strain 68U201, or strain 3908 expressing

GFP. Strain 3908 is a 1995 human isolate from Zulia State, Venezuela during a major epidemic (235) and was passaged once in C6/36 mosquito cells before undergoing infectious cDNA clone production (17). Enzootic strain 68U201 was isolated from a sentinel hamster in La Avellana, Guatemala, in 1968 and was passaged once in suckling mice and twice in BHK cells before undergoing infectious cDNA clone production (150). The GFP gene and an additional sub-genomic promoter were inserted between the structural and non-structural protein gene regions within the cDNA clone of VEEV strain 3908 (3908/GFP; Figure 21). Virus recovered from BHK cells electroporated with transcribed RNA was used for all experiments without further passage. The use of virus derived from an infectious clone minimized attenuating mutations that occur when VEEV is passaged in cell culture (11).

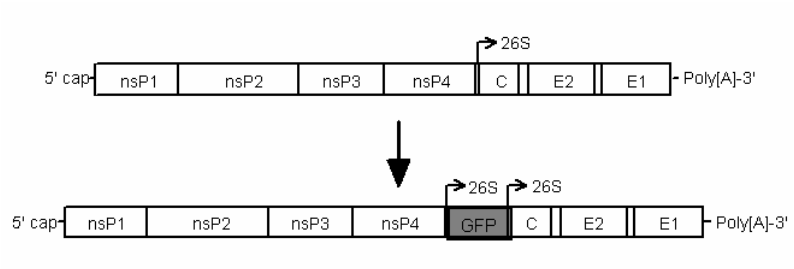

Figure 21. Schematic Depiction of the Alphavirus Genome and the Location of the Inserted GFP.

Mosquitoes

Aedes taeniorhynchus F1 mosquitoes were derived from adults collected in Galveston, TX (latitude 29°13.128′ N; longitude 94°56.063′ W). Mosquitoes were reared in an insectary at 27°C, 80% relative humidity using a light/dark cycle of 12:12 hr. Adult female mosquitoes were infected 6 to 8 days after emergence and incubated at 27°C with 10% sucrose provided *ad libitum*.

Mosquito Infection for Titration and Immunohistochemistry

Mosquitoes were fed an artificial bloodmeal containing 20% FBS, 10% Eagles minimal essential medium (MEM), and 70% (V/V) packed sheep red blood cells. Bloodmeal titers were 5 (low titer) and 7 (high titer) $\log_{10}$ PFU/mL for VEEV strain 3908 and 7 $\log_{10}$ PFU/mL for strain 68U201. An additional cohort of mosquitoes was infected intrathoracically with approximately 1µl containing 4 $\log_{10}$ PFU of each VEEV strain.

Five mosquitoes per cohort were collected on days 1-11, 16, and 21 for trituration in 300 µL of 20% MEM using a Mixer Mill 300 (Retsch, Inc., Newton, PA), and titrated on Vero cell monolayers. Additionally, 3 mosquitoes/day were collected for fixation and paraffin embedding. The legs/wings, which contain hemolymph, were removed for infectious dissemination assays and the mosquito bodies were injected intrathoracically with 10% formol saline. Mosquitoes were stored at 4°C in 1 mL of 10% formol saline for 24 hours and then transferred to 1 mL of 70% ethanol until further processing. Legs/wings were triturated in 300 µL of 20% MEM and 75 µL of the supernatant were added to Vero cells and observed for cytopathic effects (CPE) for 5 days.

Immunohistochemistry

Mosquitoes were embedded in paraffin (78) and 6µm serial sagittal sections were dried on slides treated with Vectabond following manufacturer's protocol (Vector Laboratories, Burlingame, CA). Slides were incubated overnight at 56°C prior to deparaffinization in xylene and graded alcohol series for hydration. Slides were re-fixed in 10% neutral buffered formalin and antigen was retrieved using proteinase K (Sigma-Aldrich, St. Louis, MO) prepared as a 50 mg/mL stock, diluted 1:500 in PBS. Endogenous peroxidase was inactivated in 3% hydrogen peroxide in methanol and slides were blocked in 3% FBS in PBS (blocking buffer). Primary antibody was VEEV mouse ascitic fluid (ATCC, Manassa, VA) diluted 1:300 in blocking buffer and goat anti-mouse HRP-conjugated secondary antibody (KPL, Gaithersburg, MD) was diluted 1:1000 in blocking buffer. Aminoethylcarbazole (AEC) peroxidase substrate allowed the visualization of antigen as a red precipitate, and the slides were developed according to the manufacturer's protocol (Enzo Life Sciences, Inc., Farmingdale, NY). Slides were counterstained with Mayer's hematoxylin (Poly Scientific, Bay Shore, NY) diluted 1:3 in distilled water and mounted using aqueous mounting medium (Daido Sangyo Co., LTD. Japan), examined and photographed using a Nikon Optiphot-2 microscope (Nikon Corp., Tokyo, Japan). Antigen staining was scored on a scale from 1 to 3 for light staining and very few cells of a specific tissue positive (1+), medium staining and half the cells positive (2+), and dark staining and more than half the cells positive (3+) (see Appendix).

Mosquito Infection for Fluorescence Detection

Mosquitoes were infected with 7 $\log_{10}$ PFU/mL of VEEV strain 3908/GFP in an artificial bloodmeal (as described above) or by intrathoracic inoculation with 4 $\log_{10}$ PFU in a volume of 1μL. Five mosquitoes per cohort were collected for titration on days 1-11, 16, and 21. In addition, 3 mosquitoes/day were collected for midgut and salivary gland dissection and 3 mosquitoes were collected for frozen sectioning. Prior to dissection/sectioning, the legs/wings were removed for dissemination determination as described above. The dissected tissues were fixed in 4% paraformaldehyde (PFA) and mounted with ProLong Gold antifade reagent with DAPI (Molecular Probes, Eugene, OR). The mosquitoes used for frozen sections were injected intrathoracically with 4% PFA, stored at 4°C in 1 mL of 4% PFA for 24 hours, and then transferred to 1 mL of PBS. The mosquitoes were then frozen in OCT compound (Sakura Finetek, Torrance, CA), 6 μM sagittal sections made with a cryostat, and every 5th section collected and mounted with DAPI. The dissected tissue and frozen sections were observed using an Olympus FluoView-1000 scanning confocal microscope (Olympus, Melville, NY) and a scoring system was used to determine infected tissues (described above).

Mosquito Transmission

Mosquitoes were infected with 7 $\log_{10}$ PFU/mL of VEEV strain 3908 in an artificial bloodmeal (described above). Cohorts of 10 to 24 fully engorged mosquitoes were sorted randomly into separate cartons and on various days after infection (see Table 3), the mosquitoes were allowed to feed on a naïve mouse to evaluate transmission. The mice were held in individual cages and monitored for signs and symptoms of VEEV infection.

Statistics

Mosquito titers were analyzed by two-way ANOVA (virus x day effect) with day as the repeated measure, followed by Bonferroni post tests for comparison between viruses on specific days. A p-value <0.05 was considered significant using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA) for statistical analyses.

RESULTS

Mosquito Titrations and Transmission

Virus titration of *Ae. taeniorhynchus* mosquitoes infected orally demonstrated significant variability in the mean virus concentration (Figure 22). Many samples were below the assay's limit of detection for mosquitoes infected with VEEV strain 3908 at a

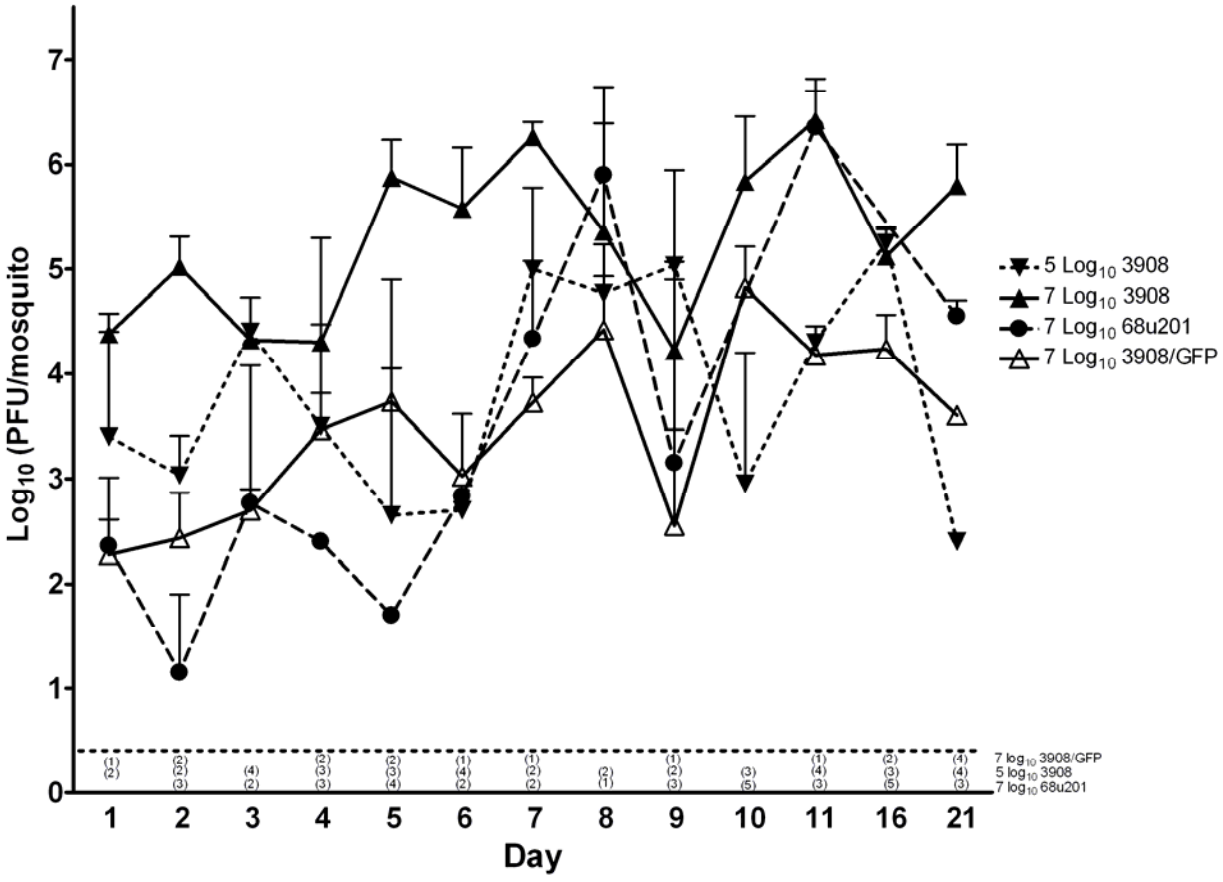

Figure 22. Replication of VEEV in Mosquitoes Following Oral Infection. The titer of infection is followed by the VEEV strain used. N=5 mosquitoes per day. The dotted line represents the limit of detection and the values in parenthesis below this line represent the number of samples below this detection limit. For statistical analysis these values were set in between zero and the limit of detection (0.2 PFU).

low titer, 68U201, and 3908/GFP bloodmeals; however, all mosquitoes infected with the strain 3908 high titer bloodmeal had detectable levels of virus. A two-way ANOVA comparing the virus strain used and day post-infection resulted in highly significant virus strain effects ($p<0.0001$), day effects ($p=0.0036$), and insignificant interactions ($p=0.0767$). Because no significant interactions were detected, Bonferroni post test p-values for comparing viruses on specific days were not meaningful and therefore not shown.

In contrast to orally infected mosquitoes, very little variability in the mean virus concentration was observed after intrathoracic infections (Figure 23). A two-way

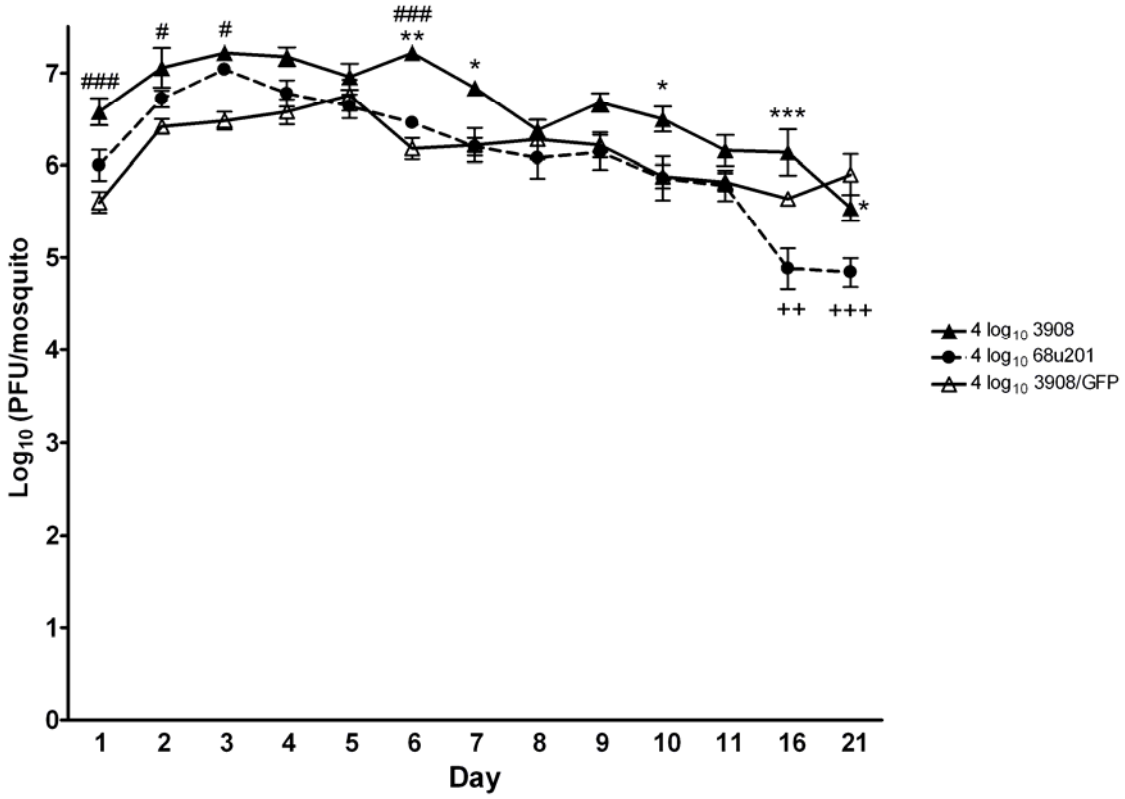

Figure 23. Replication of VEEV in Mosquitoes Following Intrathoracic Infection. The titer of the inoculum is followed by the VEEV strain used. N=5 mosquitoes per day. The symbols represent the p-values from the Bonferroni post tests where one symbol is considered significant, two symbols very significant, and three symbols extremely significant. * 3908 vs. 68U201; # 3908 vs. 3908/GFP; + 68u201 vs. 3908/GFP

ANOVA comparing virus strain and day post-infection resulted in highly significant interactions ($p<0.0001$) making p-values for the virus strain effect ($p<0.0001$) and day effect ($p<0.0001$) difficult to interpret. According to the Bonferroni post test, mosquitoes infected intrathoracically with strain 3908 contained significantly more virus than mosquitoes infected with strain 68U201 and 3908/GFP on days 6, 7, 10, 16, 21 and days 1-3 and 6 post-infection respectively. Mosquitoes infected intrathoracically with 3908/GFP had significantly higher concentrations of virus compared to 68U201-infected mosquitoes on days 16 and 21 post-infection (Figure 23).

Exposure of mice to strain 3908-infected *Ae. taeniorhynchus* indicated that the earliest time that mosquitoes can transmit is day 4 post-infection, although considerable variation was observed (Table 3). In 3 separate experiments, initial transmission was detected on different days. Consistent transmission was not detected until day 8 post-infection.

IHC Analysis of VEEV Dissemination

VEEV antigen was detected by day 1 in mosquitoes receiving the high titer strain 3908 bloodmeal. Within the posterior midgut, epithelial cells in the posterior portion were found to be infected either singly or in clusters. Viral antigen was seen concentrated along the apical brush border and adjacent midgut lumen of epithelial cells (Figure 24). Additional infected tissues on day 1 post-infection included the anterior portion of the anterior midgut, the cardial epithelium, and the ventral and dorsal diverticula (Figure 25). By day 2 post-infection, viral antigen was detected in the intussuscepted foregut epithelial cells. In contrast, infected tissues were not observed in mosquitoes infected with strain 68U201 and the low titer 3908 bloodmeals until days 4 and 5, respectively. Then, only a few weakly infected epithelial cells were observed in the posterior portion of the posterior midgut. Within the posterior midgut, cell-to-cell spread was suggested by the presence of adjacent, infected cells (Figure 24F).

Table 3. Transmission of VEEV Strain 3908 to Mice.

Days Post Infection	# Infected Mosquitoes Exposed	% of Infected Mosquitoes that Engorged	Mouse Status*	MTR** from Engorged Mosquitoes	MTR** from Engorged and Non-engorged Mosquitoes	Experiment #
2	0	NA	NA	NA	NA	1
	0	NA	NA	NA	NA	2
3	5	20% (1/5)	No Viremia	0% (0/1)	0% (0/5)	1
	6	17% (1/6)	Survived	0% (0/1)	0% (0/6)	2
	1	100% (1/1)	Survived	0% (0/1)	0% (0/1)	3
4	10	20% (2/10)	Viremia	50% (1/2)	10% (1/10)	1
	9	78% (7/9)	Survived	0% (0/7)	0% (0/9)	2
	6	17% (1/6)	Survived	0% (0/1)	0% (0/6)	3
5	9	33% (3/9)	No Viremia	0% (0/3)	0% (0/9)	1
	11	46% (5/11)	Survived	0% (0/5)	0% (0/11)	2
	12	17% (2/12)	Survived	0% (0/2)	0% (0/12)	3
6	12	17% (2/12)	Survived	0% (0/2)	0% (0/12)	2
	19	21% (4/19)	Dead	25% (1/4)	5% (1/19)	3
7	22	5% (1/22)	Survived	0% (0/1)	0% (0/22)	3
8	24	25% (6/24)	Dead	17% (1/6)	4% (1/24)	3
9	24	21% (5/24)	Dead	20% (1/5)	4% (1/24)	3
10	24	25% (6/24)	Dead	17% (1/6)	4% (1/24)	3

*Mice from experiment 1 were sacrificed pre-maturely due to the approaching hurricane Rita. The mice were bled before sacrifice for viremia detection.
**MTR = minimum transmission rate

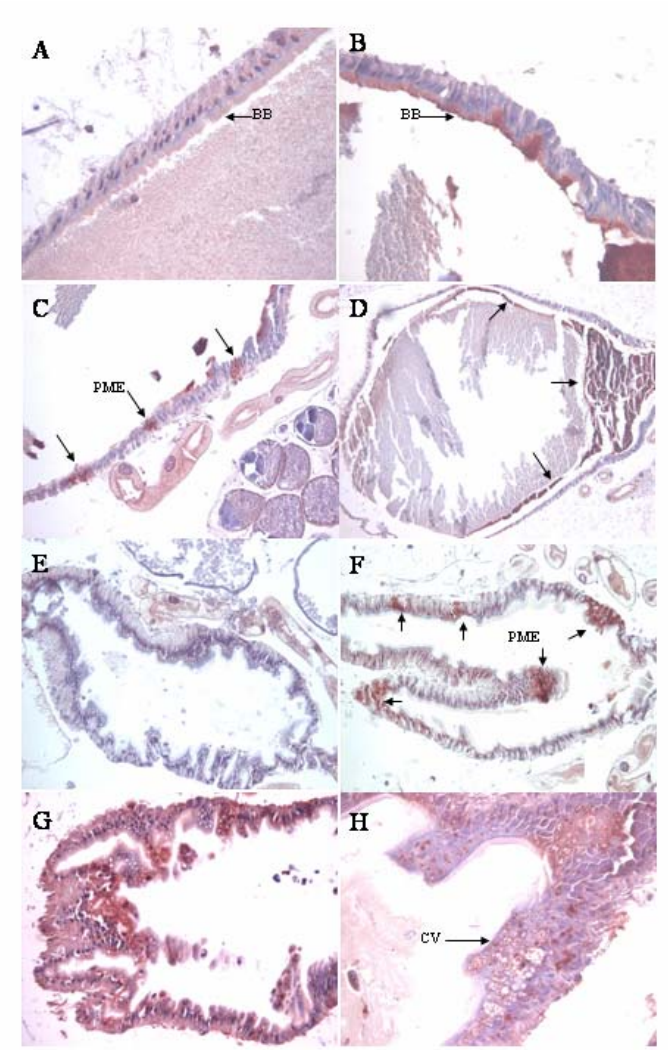

Figure 24. Immunohistochemical Staining of VEEV in the Posterior Midgut During Early (A-D) and Late (E-H) Infection. (A) control mosquito, day 1, 40X. BB, brush border. (B) high titer 3908-infected mosquito, day 1, 40X. (C) high titer 3908-infected mosquito, day 1, 20X. Arrows point to infected posterior midgut epithelial cells (PME). (D) high titer 3908-infected mosquito, day 1, 10X. Arrows point to antigen staining in the bloodmeal surrounding the posterior portion of the posterior midgut epithelium. (E) control mosquito, day 8, 20X. (F) low titer 3908-infected mosquito, day 8, 20X. (G) high titer 3908-infected mosquito, day 11, 40X. (H) high titer 3908-infected mosquito, day 16, 40X. CV, cellular vacuolization.

Dissemination from the midgut in mosquitoes infected with the high titer strain 3908 bloodmeal was detected by day 2 post-infection by assaying the legs and wings for infectious VEEV (Figure 26) and by day 4 by antigen detection in hemocoel-associated tissues including the intussuscepted foregut, cardia, posterior midgut, hindgut, abdominal, thoracic, and cephalic ganglia (Figures 25, 27, 28), and the abdominal and thoracic fat body. The salivary glands were not found to be infected until day 7 (Figure 27B-D) and the anterior portions of the lateral lobes were most often antigen positive. By day 11, viral antigen was abundant throughout the alimentary tract (Figures 24G, 25F, 27D), as well as in the fat body (Figure 29B) by day 16. Other tissues infected on day 16 included the malphigian tubules and ovarian follicles (Figure 29C, D, F). The only sign of pathology, observed on day 16, and only in a single mosquito, was cellular vacuolization in the posterior midgut (Figure 24H), which was not observed in negative controls.

Dissemination from the midgut of mosquitoes infected with a strain 68U201 bloodmeal was detected by day 4 post-infection in the legs/wings (infectious virus; Figure 26). The only tissue outside of the posterior midgut with detectable antigen on day 4 post-infection was the cephalic ganglion (Figure 28G). By day 6 post-infection, the intussuscepted foregut, abdominal, thoracic and cephalic ganglia, fat body, and Johnston's organ all contained VEEV antigen. By days 9 and 10 post-infection muscles of the gut tissue, but not epithelial cells, were also infected.

Dissemination from the midgut of mosquitoes infected with the low titer strain 3908 bloodmeal was detected by day 5 by assaying the legs/wings for infectious virus (Figure 26) and day 7 by the presence of antigen in the intussuscepted foregut, cardia, and thoracic fat body. By day 8, the anterior portion of the anterior midgut was infected. Not until day 21 was more widespread dissemination noted in the abdominal and cephalic ganglia and the abdominal, thoracic, and cephalic fat body.

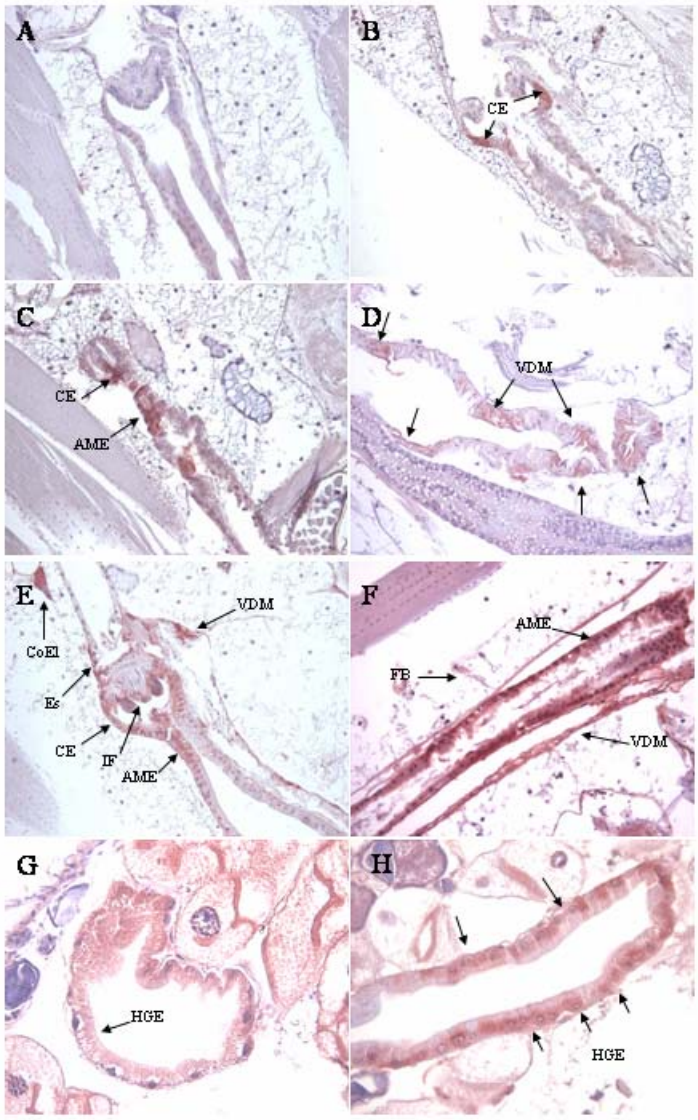

Figure 25. Immunohistochemical Staining of VEEV in the Anterior Midgut (A-F) and Hindgut (G-H). (A) control mosquito, day 1, 10X. (B) high titer 3908-infected mosquito, day 1, 10X. CE, cardia epithelium. (C) high titer 3908-infected mosquito, day 1, 10X. AME, anterior midgut epithelium. (D) high titer 3908-infected mosquito, day 1, 20X. VDM, ventral diverticulum muscle. (E) high titer 3908-infected mosquito, day 7, 20X. CoEl, corpus ellatum; Es, esophagus; IF, intussuscepted foregut. (F) high titer 3908-infected mosquito, day 11, 40X. FB, fat body. (G) Control, day 10, 40X. HGE, hindgut epithelium. (H) 68U201-infected mosquito, day 10, 40X.

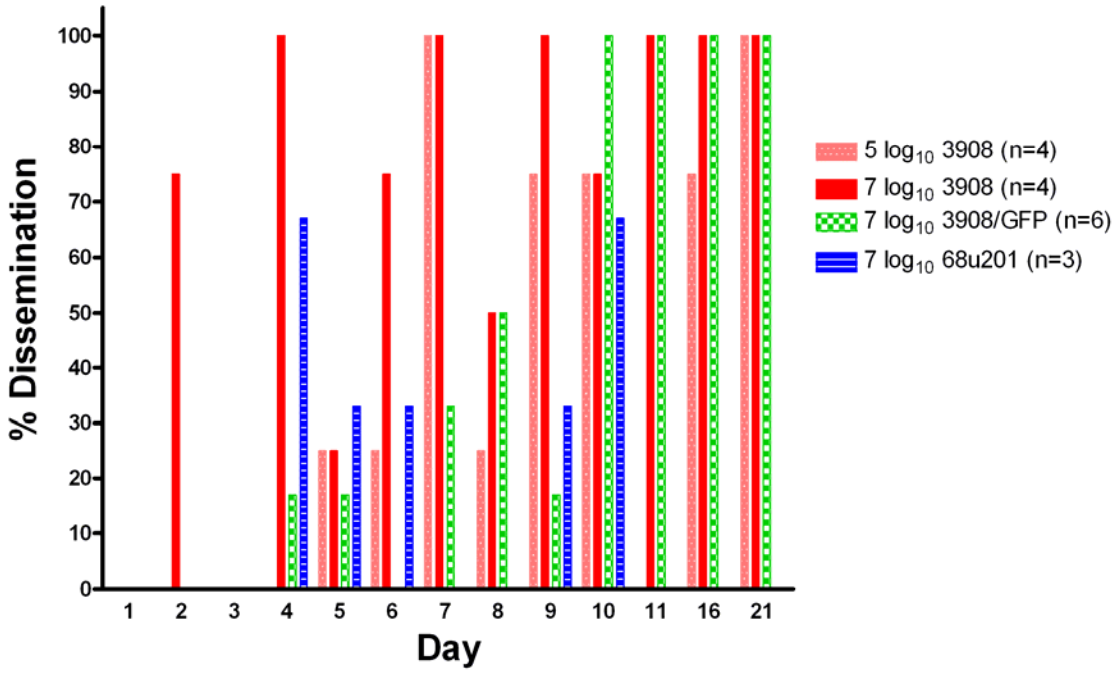

Figure 26. Dissemination of VEEV to the Legs and Wings of Orally Infected Mosquitoes. The titer of infection is followed by the VEEV strain used.

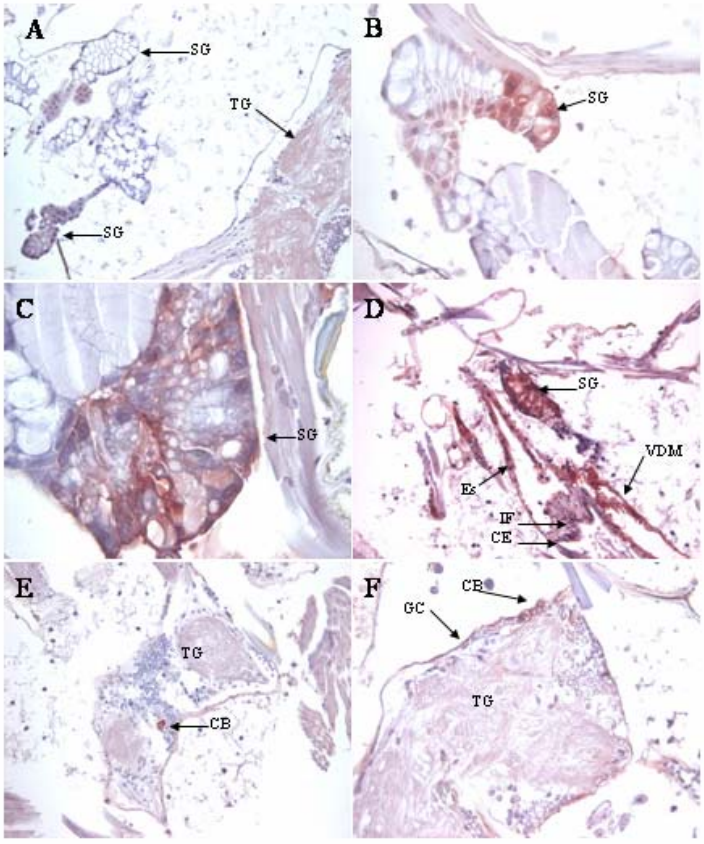

Figure 27. Immunohistochemical Staining of VEEV in the Salivary Glands (A-D) and Thoracic Ganglia (E-F). (A) Control mosquito, day 8, 10X. SG, salivary gland; TG, thoracic ganglia. (B) high titer 3908-infected mosquito, day 7, 20X. (C) high titer 3908-infected mosquito, day 8, 40X. (D) high titer 3908-infected mosquito, day 11, 10X. Es, esophagus; CE, cardia epithelium; IF, intussuscepted foregut; VDM, ventral diverticulum muscle. (E) high titer 3908-infected mosquito, day 4, 20X. CB, cell body. (F) 68U201-infected mosquito, day 6, 40X. GC, ganglionic connective tissue.

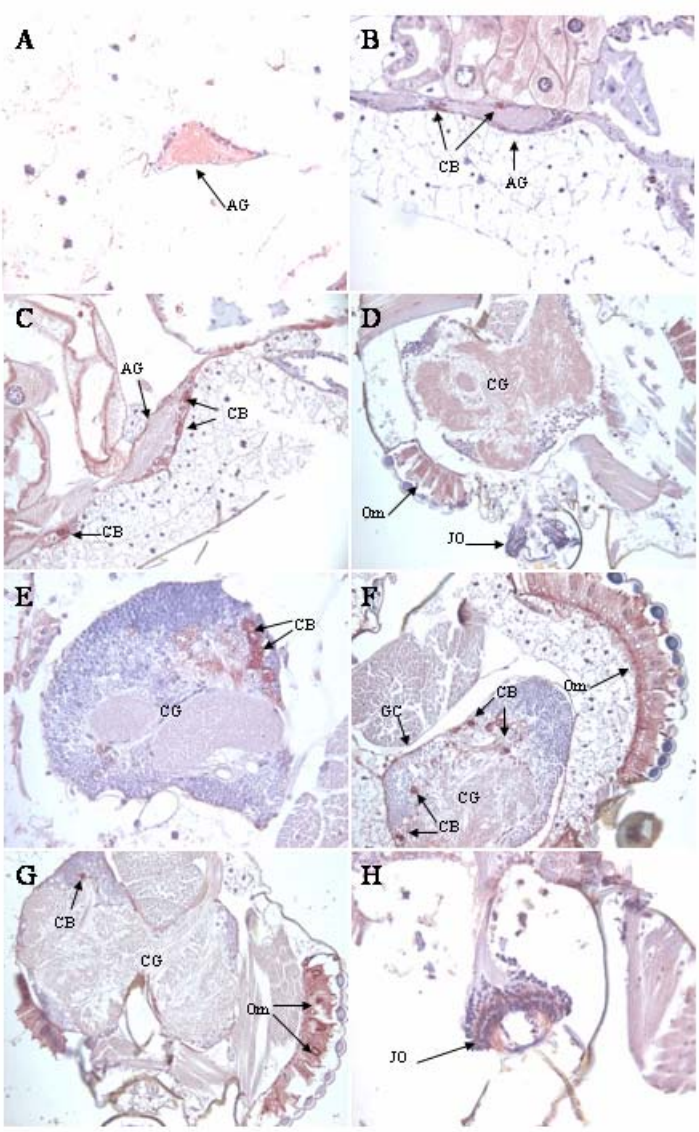

Figure 28. Immunohistochemical Staining of VEEV in the Nervous Tissue (A-F) and Sense Organs (G-H). (A) Control mosquito, day 7, 20X. AG, abdominal ganglia. (B) high titer 3908-infected mosquito, day 4, 20X. CB, cell body. (C) high titer 3908-infected mosquito, day 8, 20X. CB, cell body. (D) Control mosquito, day 8, 20X. CG, cephalic ganglia; Om, ommatidia; JO, Johnston's organ. (E) high titer 3908-infected mosquito, day 7, 40X. (F) high titer 3908-infected mosquito, day 8, 20X. GC, ganglionic connective tissue (G) 68U201-infected mosquito, day 5, 20X. (H) 68U201-infected mosquito, day 6, 40X.

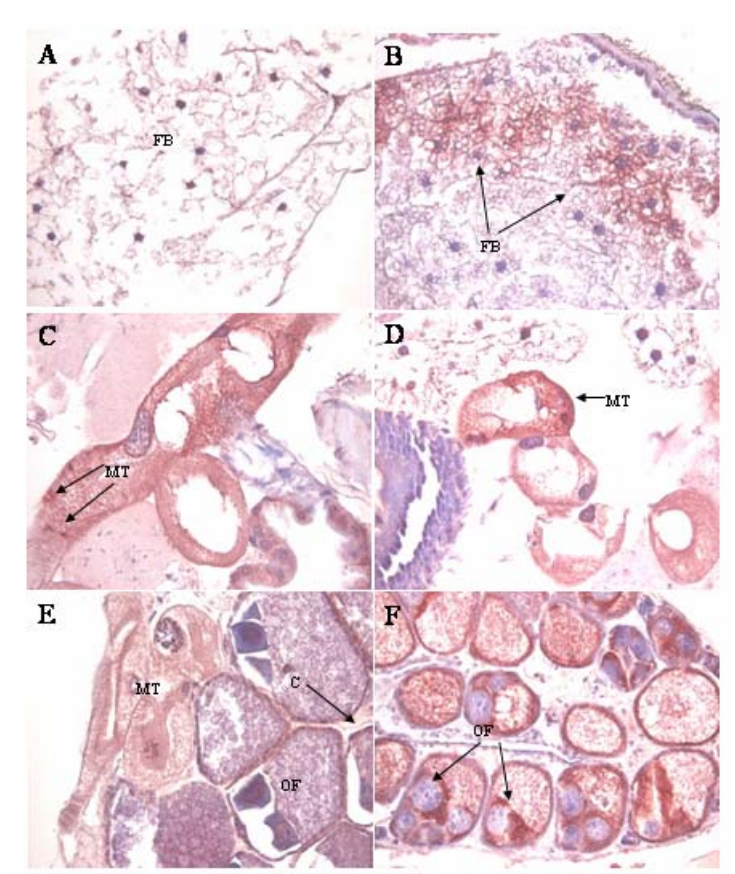

Figure 29. Immunohistochemical Staining of VEEV in the Excretory (A-D) and Reproductive (E-F) Systems. (A) Control mosquito, day 7, 40X. FB, fat body. (B) high titer 3908-infected mosquito, day 16, 20X. (C) high titer 3908-infected mosquito, day 16, 40X. MT, malpighian tubule. (D) high titer 3908-infected mosquito, day 16, 40X. (E) Control mosquito, day 5, 40X. C, calyx; OF, ovarian follicle. (F) high titer 3908-infected mosquito, day 16, 40X.

GFP-labeled VEEV Dissemination

The VEEV 3908/GFP virus exhibited an attenuated phenotype in comparison to its wild-type counterpart with no reporter gene (Figures 22 and 23). Mosquitoes infected orally did not develop a disseminated infection until day 4 post-infection (Figure 26). In contrast, GFP fluorescence was detected weakly by day 1 in mosquitoes infected intrathoracically. By day 4 after intrathoracic infection, GFP fluorescence was detected in

most tissues such as the posterior midgut, fat body, intussuscepted foregut, esophagus, cardia, anterior midgut, rectal area, the anterior portion of the salivary gland lateral lobe, the cephalic ganglia, and the abdominal and thoracic ganglia (Figure 30A-H). By day 8, the epithelial cells of the anterior and posterior midgut (Figure 30I-J) expressed GFP, along with cells in the ovary calyx and malpighian tubules (Figure 30K-L).

DISCUSSION

Understanding the movement of arboviruses from the lumen of the midgut to the hemocoel, which is required for virus transmission by mosquitoes, is important for understanding vector-virus interactions. Our study offers a more thorough understanding of the initial infection, dissemination, and transmission of two VEEV subtypes in the epidemic mosquito vector, *Ae. taeniorhynchus*.

Mosquito Titrations

A high level of variability in the mean virus concentration was observed after mosquitoes were infected orally (Figure 22), in contrast to little variability in mosquitoes infected intrathoracically (Figure 23). This demonstrates the sporadic nature of VEEV oral infection and dissemination from the midgut, most likely due to the midgut infection and/or escape barriers. The virus strain used for oral infections had a highly significant effect ($p<0.0001$) on the titers of virus in the mosquito and the effect of incubation days was also significant ($p=0.0036$), while the interaction between the two effects was not significant ($p=0.0767$). In contrast, the interaction between the virus strain and the day post-infection was highly significant for intrathoracic infections ($p<0.0001$). Although significant differences in virus titers were found on certain days post-infection for mosquitoes infected intrathoracically, the overall trend was similar for both virus strains.

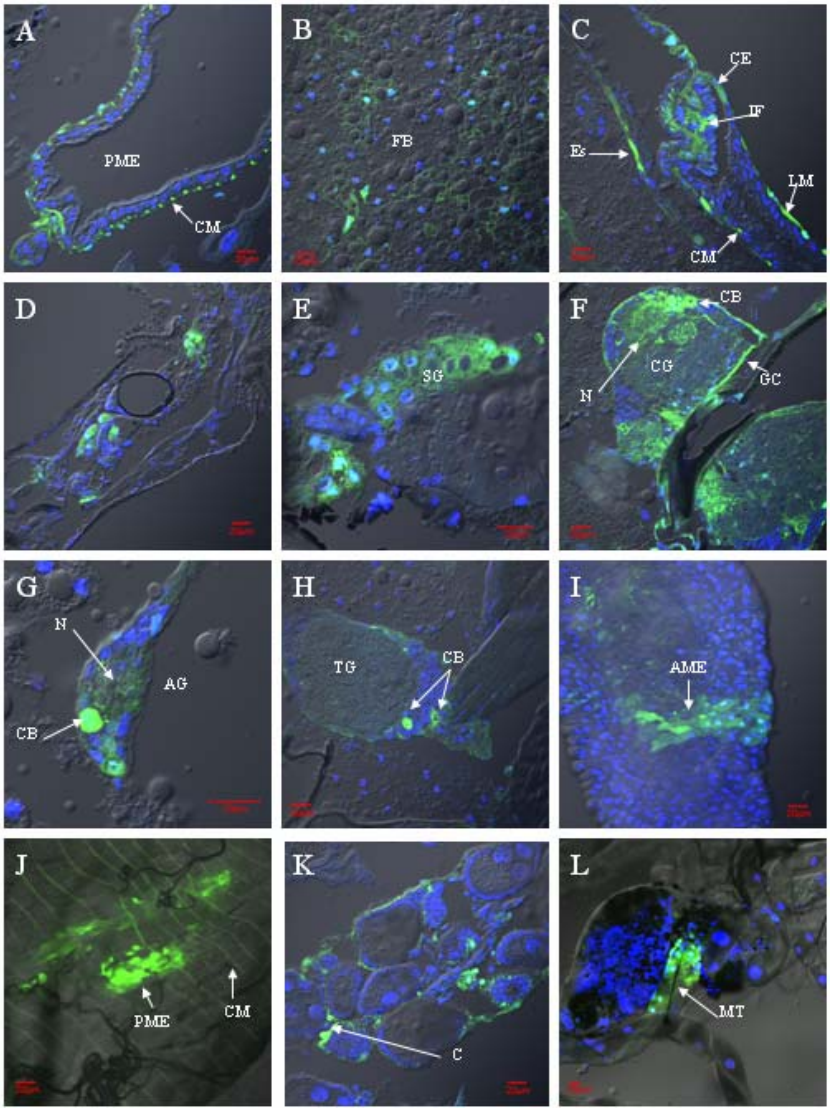

Figure 30**.** Confocal Micrographs of Mosquitoes Infected Intrathoracically with VEEV strain 3908 Expressing GFP. The transmitted image is overlayed with DAPI and GFP images. (A-H) day 4 post-infection. (I-L) day 8 post-infection. (A) PME, posterior midgut epithelium; CM, circular muscle. (B) FB, fat body. (C) Es, esophagus; IF, intussuscepted foregut; CE, cardia epithelium; CM, circular muscle; LM, longitudinal muscle. (D) rectal area (E) SG, salivary gland. (F) CG, cephalic ganglion; CB, cell body; GC, ganglionic connective tissue; N, neuropile. (G) AG, abdominal ganglion. (H) TG, thoracic ganglion. (I) AME, anterior midgut epithelium. (J) PME, posterior midgut epithelium. (K) Ovary; C, calyx (L) MT, malpighian tubule. Red bar in lower corner represents 20µm.

IHC Analysis of VEEV Dissemination

In agreement with other studies of alphaviruses (139, 146, 170), the posterior midgut epithelial cells were the initial site of VEEV replication. Virus appeared by day 1 in mosquitoes infected with the high titer strain 3908 bloodmeal. In addition to the cytoplasm of the midgut epithelial cells, the antigen staining seemed to concentrate along the brush border and in the surrounding lumen, which was not apparent in negative controls (Figure 24). Further studies are needed to confirm that viral antigen accumulates along the brush border and whether it is possibly shed into the lumen. A possible explanation for virus accumulation along the apical side of the midgut may be an effect of bloodmeal digestion. Shortly after the bloodmeal, the midgut epithelial cells begin absorbing nutrients from the bloodmeal, which pass through the cells to the hemocoel from the basolateral membranes. The activity occurring on the basal side of the midgut (i.e. nutrient transport to the hemocoel) shortly after the bloodmeal may cause virus to accumulate along the apical side. However, other studies found that alphaviruses bud primarily from the basolateral membranes of infected midgut epithelial cells (223, 239); therefore, the antigen detection along the brush border may be due to non-specific staining, and requires further investigation. Additional initial sites of replication in *Ae. taeniorhynchus* infected with the high titer 3908 bloodmeals included the epithelial cells of the anterior portion of the anterior midgut and the cardia (Figure 25). Dissemination was detected by day 2 post-infection when the intussuscepted foregut contained viral antigen. It is possible that VEEV spreads in a cell-to-cell manner from the cardia to the adjacent intussuscepted foregut, where virus can hypothetically escape into the hemocoel without traversing a basal lamina (167). Because a disseminated infection was detected in some mosquitoes even before the intussuscepted foregut was infected, virus most likely disseminated to the hemocoel via the more common route from the posterior midgut. A similar pattern of tissue infection was observed for mosquitoes infected with the low titer 3908 bloodmeal, although dissemination occurred much later (day 7) compared to mosquitoes infected with the higher titer.

Several other arboviruses, including two alphaviruses, have been found to replicate in the anterior region of the midgut soon after infection (16, 40, 107, 167, 168, 241). Although studies of Western equine encephalitis virus using hanging drop bloodmeals suggest that anterior midgut infection could be an artifact of artificial bloodmeals (232), we have found that mosquitoes that feed from hanging drops accumulate more blood in their diverticulum compared to those that feed through an artificial membrane (DRS, unpublished). This difference may be due to the penetration of the artificial membrane by the mosquito's proboscis and requires further investigation. We did observe antigen staining in the diverticulum on day 1 post-infection with the high titer strain 3908 bloodmeal, but this may be non-specific staining because virus presumably cannot infect this organ due to its luminal cuticular lining (232, 241). Electron microscopy is needed to clarify this observation.

In contrast to mosquitoes infected with the high titer strain 3908 bloodmeal, mosquitoes infected with strain 68U201 and the low titer strain 3908 bloodmeals did not have detectable antigen in the posterior midgut until days 4 and 5, respectively. The virus was likely undetectable before day 4 due to a low level of replication, below the detection limit for our assays (3 $\log_{10}$ PFU/gram of tissue). Once VEEV escaped into the hemocoel, amplification mainly took place in epithelial cells of the gut tissue, neural tissue, and fat body for mosquitoes infected with strain 3908. In contrast, when virus escaped into the hemocoel of mosquitoes infected with strain 68U201, amplification primarily occured in muscles of the gut (not epithelial cells) and neural tissue. This may reflect a dearth of strain 68U201-specific receptors on the epithelial cells.

For both virus strains, the nervous tissue of mosqitoes was frequently infected. Several studies of mosquitoes infected with dengue viruses report heavy infection of the nervous system (103, 111, 246). Platt et al. (148) demonstrated that *Ae. aegypti* mosquitoes infected with dengue-3 virus require more time to feed on a vertebrate host than uninfected mosquitoes, which could enhance transmission. Because ours and previous studies (105, 223) demonstrate VEEV infection of mosquito nervous tissue, it would be interesting to determine if mosquito behavior is altered.

Salivary gland infection is required for biological transmission to a vertebrate host. Mosquitoes infected with the high titer strain 3908 bloodmeal first had detectable antigen in their salivary glands by day 7 post-infection, beginning with the anterior portion of the lateral lobe. Weaver et al. (223) reported that the salivary glands of *Culex (Melanoconion) taeniopus* mosquitoes first appear to be infected with VEEV by day 4 post-infection, consistent with initial transmission on day 5 post-infection (236). Our results are similar, in that the earliest VEEV transmission detected occurred on day 4 post-infection, although high variability continued until day 8 (Table 3). Virus was never detected in the salivary glands of mosquitoes infected with strain 68U201 and the low titer strain 3908 bloodmeals, possibly due to a salivary gland infection barrier.

Additional organs infected by the high titer strain 3908 bloodmeal included the malpighian tubules and the ovaries. This is in contrast to the study of Weaver et al. (223) who reported no VEEV strain 68U201 in these organs of the enzootic vector, *Cx. taeniopus*. Larsen et al. (105) did report VEEV in the ovaries and malpighian tubules of *Ae. aegypti*, but did not detect virus by electron microscopy in the ovarian follicles, in contrast to our findings.

We detected signs of pathology only late in infection in the posterior midgut of a single mosquito infected with the high titer strain 3908 bloodmeal where cellular vacuolization was apparent (Figure 24H). Two other alphaviruses cause cytopathology in their mosquito vectors; WEEV appears to cause vacuolization and luminal extensions of midgut epithelial cells of *Ochlerotatus dorsalis* mosquitoes and EEEV causes ultrastructural changes in the posterior midgut epithelial cells of *Culiseta melanura* mosquitoes (233, 240).

Analysis of GFP-labeled VEEV Dissemination

Virus derived from the VEEV 3908/GFP construct yielded attenuated virus in comparison to virus from the construct with no additional reporter gene (Figure 22 and 23). Orally infected 3908/GFP mosquitoes did not develop a disseminated infection until much later than mosquitoes infected with strain 3908 with no GFP (figure 26). In contrast, GFP was detected weakly by day 1 post-infection and by day 4 post-infection

throughout most tissues in mosquitoes infected intrathoracically (Figure 30). The same tissues were observed to be infected by GFP detection in tissues compared to IHC detection of viral antigen following a disseminated infection with VEEV 3908. One exception is that GFP was detected in the ovary calyx while IHC viral antigen was detected in the ovarian follicle. One explanation is that the IHC detection could be a result of background staining. However, non-specific staining was not detected in the ovaries of negative controls.

In conclusion, *Ae. taeniorhynchus* mosquitoes orally infected by VEEV exhibit significant variability in the virus titer while intrathoracically infected mosquitoes do not, portraying the significance of the midgut barrier to dissemination. The first day VEEV strain 3908-infected mosquitoes can transmit to a vertebrate host is day 4 post-infection, although this may be variable. Following the replication of VEEV 3908 in the epithelial cells of the posterior midgut and sometimes in the anterior midgut and cardia, the virus disseminates and infects more epithelial cells, muscles and nervous tissue prior to infection of the salivary glands. For VEEV strain 3908, epithelial cells serve as an important initial and secondary site of amplication. In contrast, VEEV strain 68U201 does not efficiently infect epithelial cells and, when dissemination beyond the midgut occurs, the muscles associated with the gut and nervous tissue serves as important sites of replication. This study contributes to a better understanding of the pathogenesis of VEEV in the important epidemic mosquito vector, *Ae. taeniorhynchus*.

Some common patterns of infection and dissemination occur with VEEV in *Ae. taeniorhynchus* mosquitoes that have been observed for other arboviruses and their vectors, such as consistent infection of tracheal cells (170, 190), early replication in the anterior region of the midgut (16, 40, 107, 167, 168, 241), and frequent infection of the nervous tissue (53). It appears that some arboviruses may infect and disseminate within their vectors via common mechanisms, which represents an important topic of future research leading to a better understanding of mechanisms affecting vector competence of arboviruses.

CHAPTER 4: EVALUATION OF METHODS TO ASSESS TRANSMISSION POTENTIAL OF VENEZUELAN EQUINE ENCEPHALITIS VIRUS BY MOSQUITOES AND ESTIMATION OF MOSQUITO SALIVA TITERS[1]

ABSTRACT

Determining the dose of an arbovirus transmitted by a mosquito is important to design transmission and pathogenesis studies simulating natural infection. Several different artificial infection and transmission methods used to assess vector competence and to estimate the dose injected during mosquito feeding have not been fully evaluated to determine whether they accurately reflect natural transmission. Additionally, it is not known whether different mosquito vectors transmit similar amounts of a given virus. Therefore, we compared three traditional artificial transmission methods using Venezuelan equine encephalitis virus (VEEV) and *Aedes albopictus* and *Aedes taeniorhynchus* mosquitoes. Both the mosquito species and the infection route used affected the amount of virus detected in the saliva after a 10 day extrinsic incubation period. Median titers of virus detected in saliva of *Ae. albopictus* and *Ae. taeniorhynchus* mosquitoes ranged from 0.2 to 1.1 $\log_{10}$ (mean 0.7–1.4 $\log_{10}$) and 0.2 to 3.2 $\log_{10}$ (mean 1.0–3.6 $\log_{10}$) plaque-forming units, respectively. The results of this study will aid in the design of transmission and pathogenesis studies involving arboviruses.

INTRODUCTION

Determining the amount of infectious arbovirus transmitted by a mosquito during a bloodmeal is important to design transmission and pathogenesis studies simulating natural infection. Limitations on live animal research have increased the use of several

[1] A substantial portion of this chapter was previously published in the American Journal of Tropical Medicine and Hygiene. This journal allows including information without copyright as long as it is properly cited. The citation for the article is Smith DR, Carrara AS, Aguilar PV, Weaver SC, 2005. Evaluation of methods to assess transmission potential of Venezuelan equine encephalitis virus by mosquitoes and estimation of mosquito saliva titers. Am J Trop Med Hyg 73: 33-39.

different artificial infection and transmission methods to assess laboratory vector competence and to estimate the amount of virus injected during mosquito feeding. However, these methods have not been fully evaluated to determine whether they accurately reflect natural transmission potential and events. The methods used for infecting mosquitoes, the titers of virus used, and the vector species could all affect the amount of virus transmitted by the mosquito, yet these variables have never been evaluated systematically.

Methods of Mosquito Infection and Saliva Collection

Three methods are typically used for experimental infection of adult female mosquitoes: 1) intrathoracic inoculation, 2) oral exposure by using an artificial blood meal, or 3) oral exposure by feeding on a viremic vertebrate host. Different methods can also be used to estimate the amount of virus delivered in a mosquito's saliva during transmission, including both indirect and direct methods. Indirect methods include comparing the time of death of an animal exposed to a mosquito bite to that of animals infected with a known lethal dose, or comparing the time between mosquito feeding and viremia to the time between a known infectious dose delivered by needle, and viremia. Direct methods include detecting virus in hanging drops of blood fed upon by mosquitoes, detection of virus in vertebrate tissue immediately after mosquito feeding, detection of virus in blood-agar fed upon by mosquitoes, and detection of virus after mosquito salivation into a fluid such as immersion oil (84). Saliva collection into a capillary tube containing fetal bovine serum (FBS) (4, 55), may be more sensitive for virus detection due to the stabilizing properties of FBS. However, Chamberlain and others (22) compared several indirect and direct methods and concluded that a similar method to saliva collection by capillary tubes may not be an efficient method for virus detection.

Arboviruses encounter several potential infection and dissemination barriers within the mosquito following a viremic bloodmeal. These include the midgut and salivary gland infection barriers, and midgut and salivary gland escape barriers (71). Intrathoracic inoculations circumvent the midgut infection and escape barriers, but not the salivary gland infection and salivary gland escape barriers. Oral exposures by an

artificial bloodmeal or viremic animal most closely resemble natural exposures compared to intrathoracic inoculations, but can suffer from several important limitations. For example, several studies have demonstrated that mosquitoes are less susceptible to infection by artificial than by natural bloodmeals (127, 144, 207). After the clotting of a naturally acquired bloodmeal in the mosquito midgut, the vertebrate blood cells are concentrated centrally while the serum is expressed to the peripheral portion of the posterior end of the mosquito midgut. Thus, virus present in the serum is concentrated adjacent to the midgut epithelium, enhancing infection (241). Exposures with artificial bloodmeals that do not clot, resulting in no concentration of virus against the midgut epithelium, reduce infection efficiency (232). However, one study of *Aedes aegypti* mosquitoes infected with Semliki Forest virus found no difference in the efficiency of artificial versus viremic animal exposure methods (136). Daily infectivity titrations in mice showed no difference in the titers in infected mosquitoes between the groups except in the first 24 hours, when a larger decrease in virus titer (i.e., eclipse phase) occurred in mosquitoes infected by artificial bloodmeals. Additionally, transmission of the virus from mosquitoes infected either naturally or artificially was not significantly affected (136). However, there is no mention of infection rates, and although the infectivity titers were similar, the blood titer for the naturally exposed mosquitoes was higher than that for mosquitoes exposed by an artificial bloodmeal.

The use of viremic animals for assessing vector competence and transmission potential also has some disadvantages. Animal use in biomedical research is expensive, highly regulated, and requires special facilities. In addition, exposing mosquitoes with a predetermined dose requires knowledge about the animal's viremia level after a given intrinsic incubation period. Variation in viremia responses among individuals can make the accurate prediction of viremia levels difficult. Additionally, good laboratory animal models may not exist because some laboratory animals that serve as models for reservoir or amplification hosts do not develop high enough viremia levels to infect even proven vectors.

Estimate of Mosquito Saliva Titers

Many studies have estimated the arbovirus dose delivered by an infected mosquito during feeding (4, 22, 30, 33, 68, 84, 124, 171, 172, 214, 238). Chamberlain et al. (22) estimated the amount of eastern equine encephalitis virus (EEEV) inoculated by orally (viremic chicken) infected *Ae. aegypti* mosquitoes to be highly variable, ranging from undetectable up to 5 $\log_{10}$ mouse intracerebral 50% lethal doses ($ICLD_{50}$). A later study reported up to 3 $\log_{10}$ plaque forming units (PFU) of EEEV in the saliva of *Culiseta melanura* deposited artificially into capillary tubes filled with immersion oil (238). A study of Semliki forest virus transmission by *Anopheles albimanus* using artificial blood feeding for both infection of mosquitoes and collection of saliva reported that 3.2 to 4 $\log_{10}$ mouse LD_{50} were transmitted (30). Mellink estimated by the use of indirect methods that 2.7 $\log_{10}$ infectious doses of Venezuelan equine encephalitis virus (VEEV) per mg of saliva are transmitted by *Ae. aegypti* mosquitoes infected intrathoracically (124). Using quantitative real time RT-PCR and capillary collection, Vanlandingham et al. determined that *Culex pipiens pipiens* mosquitoes infected by an artificial bloodmeal contain an average of 4.3 $\log_{10}$ PFU of West Nile virus in their saliva with a range of 0.5 to 5.3 $\log_{10}$ (214).

Despite numerous studies cited above, important gaps remain in our knowledge regarding horizontal transmission of arboviruses. Different mosquito species have never been compared directly to determine if they transmit different amounts of a given virus. Different methods for virus exposure to mosquitoes have never been systematically evaluated for their effect on transmission and infectious doses delivered following an appropriate extrinsic incubation period in the infected mosquito. Also, the effect on transmission of the virus dose used to infect mosquitoes has not been evaluated. Additionally, the media used for saliva collection, oil and FBS, have never been compared. To address these issues, I used VEEV, an emerging arboviral pathogen of humans and equines (230) and two mosquito species: *Aedes albopictus*, a species that is susceptible to infection with VEEV and is often used as a model species for arbovirus transmission studies because it feeds readily in the laboratory (9, 44), and *Aedes*

taeniorhynchus, a proven epizootic VEEV vector (165, 198, 235). Three different routes for mosquito infection were compared along with two methods for collecting saliva. I estimated the effect of mosquito species, route of infection, and the method of saliva collection on the amount of VEEV injected by mosquitoes.

MATERIALS AND METHODS

Virus

Venezuelan equine encephalitis virus rescued from an infectious cDNA clone (17) derived from epizootic strain 3908 (subtype IC) was used in this study. Subtype IC viruses have been the etiologic agents of all recent VEE epidemics and strain 3908 is a human isolate made on September 16th, 1995 in Zulia State, Venezuela during a major epidemic (235). This strain was passaged once in C6/36 mosquito cells before undergoing RNA extraction and infectious cDNA clone production. Virus recovered from BHK cells electroporated with transcribed RNA was used for all experiments without further passage. The use of virus derived from an infectious clone avoided attenuating mutations that occur when VEEV is passaged in cell culture (11).

Viremia Determinations

Six to eight-week-old female NIH Swiss mice were injected subcutaneously with 1000 PFU of VEEV. Three mice per time point were bled at 10, 14, 18, and 24 hours from the retroorbital sinus for viremia characterization. Serum titers were determined by plaque assay of serial 10-fold dilutions on Vero cell monolayers (Figure 31).

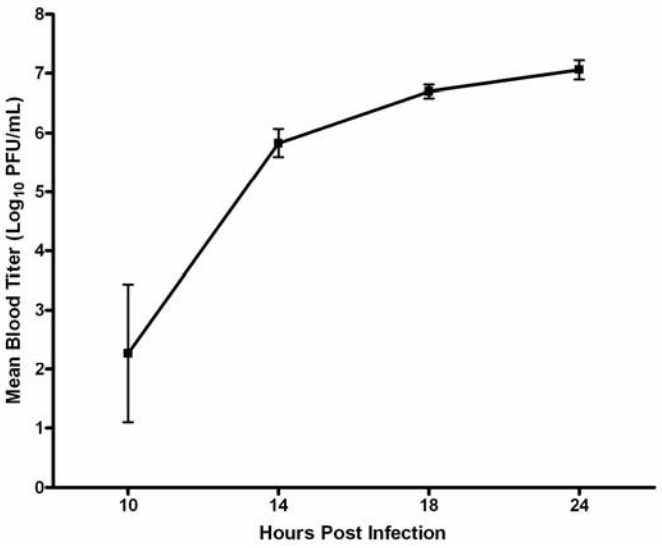

Figure 31. VEEV 3908 Viremia in Mice.

Mosquitoes

Ae. albopictus and *Ae. taeniorhynchus* mosquitoes were used for the reasons described above. Either F1 or F2 *Ae. albopictus,* derived from adult females collected in Galveston, Texas or *Ae. taeniorhynchus* from a colony initiated with mosquitoes from Florida were reared in an insectary at 27°C at 80% relative humidity using a light/dark cycle of 12:12 hr. Adult female mosquitoes were infected 6 to 8 days after emergence and incubated at 27°C for 5 or 10 days following infection with 10% sucrose provided *ad libitum*.

Intrathoracic Inoculation

Mosquitoes were inoculated in the thorax with approximately 1 μL containing 4 $\log_{10}$ PFU of VEEV using glass needles made from heated capillary pipettes.

Artificial Bloodmeals

Mosquitoes were allowed to feed for one hour on an artificial bloodmeal containing 20% FBS, 1% sucrose, and 70% (V/V) packed sheep red blood cells and VEEV in Eagles minimal essential medium (MEM). Bloodmeal titers for each of three feedings were determined by plaque assay to be 6.1 and 8.1 $\log_{10}$ PFU/mL.

Viremic Animal Bloodmeals

Six to eight-week-old female NIH Swiss mice were infected subcutaneously with 1000 PFU of VEEV. Mice were anesthetized using pentobarbital and mosquitoes were allowed to feed on the viremic animal for one hour. The mice were bled from the retro-orbital sinus at the midpoint of their exposure to mosquitoes to estimate the viremia titer. Serum titers for the three mice were 6.1, 6.8, and 7.2 $\log_{10}$ PFU/mL. All experiments were approved by the University of Texas Medical Branch Institutional Animal Care and Use Committee.

Saliva Assays

Saliva samples were obtained by forced salivation into capillary tubes (10 μL capacity, VWR international, West Chester, PA) filled with either immersion oil (type B, Cargille Laboratories Inc., Cedar Grove, NJ) or 50% FBS/50% glycerol. The legs and

wings were removed from individual mosquitoes and the proboscis was inserted into a capillary tube containing the immersion oil or FBS/glycerol. Mosquitoes were allowed to salivate for 30--45 min, and salivation was confirmed in tubes containing hydrophobic immersion oil by the appearance of bubbles at the tip of the proboscis. Saliva could not be observed in tubes containing hydrophilic FBS/glycerol. Oil or FBS/glycerol and saliva were centrifuged into 100 μL of minimum essential medium (MEM) containing 20% FBS and frozen at -80°C until further processing; 30 μL were then added to monolayers of Vero cells and observed for cytopathic effects (CPE) for 5 days. Legs/wings and bodies were triturated separately in 300 μL of 20% MEM using a Mixer Mill 300 (Retsch, Inc., Newton, PA) and 75 μL of supernatant were added to Vero cells and observed for CPE for 5 days. All CPE-positive saliva samples were titrated by plaque assay on Vero cell monolayers. To increase sensitivity of virus detection, some saliva samples from infected mosquitoes with disseminated infections, (infected legs) but with no virus detectable in the saliva by CPE assay were injected intracranially into 1 to 3-day-old NIH Swiss mice, 3 mice/sample, and 20 μL per mouse.

Virus Titration in Mice

Intracerebral inoculation of 1 to 2-day-old mice was used to assay some saliva samples from mosquitoes positive for dissemination (infected legs) that had saliva negative for CPE because it is more sensitive for detection of VEEV than cell culture-based methods (120). Mosquitoes were infected intrathoracically and saliva was collected following a 10 day extrinsic incubation period. Twenty μL of one 2.5-fold and three 10-fold serial dilutions were injected intracranially into 1 to 2-day-old mice. Mice that died were frozen at -80°C and brains were assayed to confirm the presence of virus by CPE. The LD_{50} titers were calculated by the method of Reed and Muench (155).

Statistics

Data that passed a normality test were analyzed by an unpaired t-test, which was used to compare the two different mosquito species infected intrathoracically following a 5 day extrinsic incubation period. Non-normal data were analyzed by the Mann Whitney

test, which was used to evaluate the effect of the saliva collection method, two different mosquito species for the artificial bloodmeal and viremic animal exposure methods, and the varying oral doses for the artificial bloodmeal. The route of mosquito infection and the varying viral doses for viremic animal exposure were analyzed by the Kruskal-Wallis test. All analysis was performed using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA.).

RESULTS

Comparison of Saliva Collection Methods

Fetal bovine serum, an alternative collection medium to oil, may result in recovery of greater quantities of virus in mosquito saliva because of its stabilizing properties (4, 55). To test this hypothesis, saliva from infected *Ae. albopictus* mosquitoes was collected in capillary tubes containing either immersion oil or FBS/glycerol. The largest cohorts for the three different infection routes were used to compare the sensitivity of the two collection media (Figure 32, Table 4). No significant difference ($p>0.05$) occurred in virus detection rates or in saliva titers using immersion oil compared to FBS; therefore, these groups were combined for further analysis.

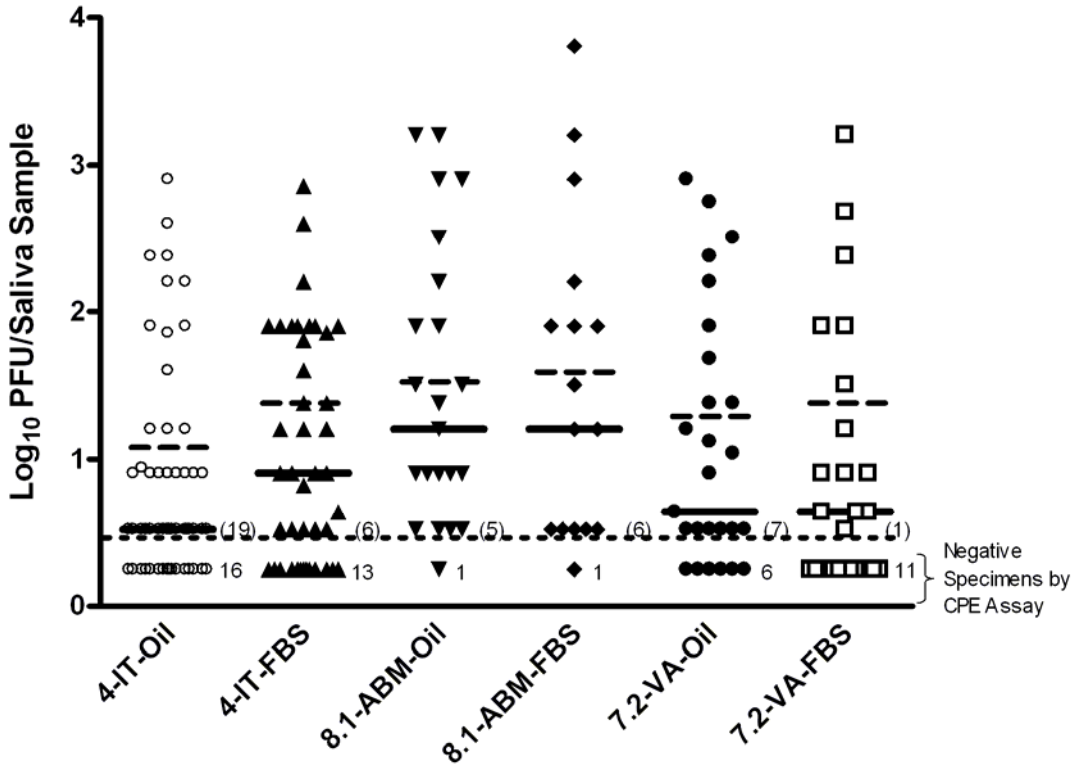

Figure 32. Comparison of Saliva Collection Media Using *Aedes albopictus* Mosquitoes. Titers were determined by cell culture assays (plaque and CPE assay). No significant difference was detected in capillary tubes containing fetal bovine serum compared to immersion oil. Cohorts are labeled as follows: infection titer (log_{10})-method of infection-method of saliva collection. The bar indicates the median saliva titer. The dashed bar indicates the average saliva titer. Numbers in parentheses to the right of the symbols with values of 0.5 indicate the number of samples with titers of 0.5 log_{10} PFU. The dotted line at 0.5 log_{10} PFU indicates the limit of detection for the cell culture assays. The symbols below this line indicate samples negative by the CPE assay, indicating that these samples were below the limit of detection for the assay but the mosquitoes were positive for viral dissemination to the legs and/or wings. Numbers to the right of the symbols with values of 0.25 indicate the number of samples that are in between zero and the limit of detection of the assay, 0.5 log_{10} PFU. IT = intrathoracic; ABM = artificial bloodmeal; VA = viremic animal; FBS = fetal bovine serum. Reproduced with permission (194).

Table 4. Comparison of Immersion Oil and Fetal Bovine Serum as Saliva Collection Media Using *Aedes albopictus* Mosquitoes. Reproduced with permission (194).

Infection method	Titer of infection dose (log_{10} PFU/mL)	Saliva collection medium	% with disseminated infection (fraction)	% negative by CPE assay	% of CPE-negative samples positive by mouse inoculation
Intrathoracic	4.0*	Oil	100% (31/31)	52%	81%
Intrathoracic	4.0*	FBS†	100% (29/29)	45%	92%
Viremic animal	7.2	Oil	100% (27/27)	22.2%	67%
Viremic animal	7.2	FBS†	90% (26/29)	42.3%	73%
Artificial bloodmeal	8.1	Oil	92% (23/25)	4.3%	0%
Artificial bloodmeal	8.1	FBS†	94% (17/18)	6%	100%
Total			96%	31%	79%

CPE, cytopathic effects; FBS, fetal bovine serum
* Total dose delivered intrathoracically
† 50% fetal bovine serum/50% glycerol

Effect of Infection Route on Saliva Titers

Saliva virus content was compared among mosquito cohorts after 3 infection methods: 1) intrathoracic inoculation; 2) artificial bloodmeal, and; 3) viremic animals. Saliva was collected from all orally infected mosquitoes following a 10 day incubation. Significantly lower ($p < 0.001$) saliva titers occurred in *Ae. albopictus* mosquitoes exposed intrathoracically and evaluated following a 10 day incubation period compared to mosquitoes exposed by artificial bloodmeals and viremic animals. Because intrathoracic exposure accelerates mosquito infection, saliva titers may peak earlier. Therefore, one group of intrathoracically exposed mosquitoes was evaluated after 5 days of incubation (Figure 33, Table 5). There was no longer a significant difference when comparing saliva from mosquitoes infected intrathoracically and evaluated following a 5 day incubation period to that from mosquitoes exposed by artificial bloodmeals and viremic animals and incubated for 10 days. Additionally, *Ae. taeniorhynchus* saliva collected following a 5 day incubation period had significantly higher titers ($p < 0.01$) compared to saliva from mosquitoes infected by an artificial bloodmeal or viremic animal and incubated for 10 days (Figure 33, Table 5).

Effect of Infection Dose on Saliva Titers

For *Ae. albopictus*, a range of oral doses for artificial bloodmeal and viremic animal cohorts was used: 6.1, 8.1 and 6.1, 6.8, and 7.2 $\log_{10}$ PFU/mL, respectively. The range of artificial bloodmeal doses for *Ae. albopictus* did significantly affect the saliva titer ($p = 0.0003$). However, this range of oral doses did not significantly affect the amount of virus in the saliva for *Ae. albopictus* mosquitoes exposed to viremic animals (Figure 33, Table 5).

Effect of Mosquito Species on Saliva Titers

For *Ae. albopictus*, the median cohort titers of virus in 208 total saliva samples tested in cell culture ranged from 0.25--1.1 $\log_{10}$ PFU (mean range 0.7--1.4 $\log_{10}$ PFU), with a total range of 0.25--4.2 $\log_{10}$ PFU (Figure 33, Table 5). Overall, *Ae. taeniorhynchus* saliva contained larger amounts of VEEV than that of *Ae. albopictus*, which had median titers of 0.25--3.2 $\log_{10}$ PFU (mean range 1.0--3.6 $\log_{10}$ PFU) and total

range of 0.25--6.2 $\log_{10}$ PFU (Figure 33, Table 5). The difference in saliva titers between *Ae. albopictus* mosquitoes and *Ae. taeniorhynchus* mosquitoes was significant for cohorts exposed intrathoracically ($p < 0.0001$) but not between cohorts exposed to artificial bloodmeals and viremic animals ($p > 0.05$).

Virus Detection by Inoculation of Mice

Intracerebral inoculation into suckling mice is more sensitive for detecting VEEV compared to vertebrate cell culture methods (120), as shown in Table 4. The limit of detection for the CPE assay was 0.5 $\log_{10}$ PFU; therefore, suckling mice were used for some saliva samples if the mosquitoes were positive for virus dissemination in the legs, but their saliva tested negative for CPE. Mouse inoculation was more sensitive than the CPE assay (Table 4). Of 48 saliva samples from mosquitoes with a disseminated infection that were negative by the CPE assay, 38 (79%) were positive by mouse inoculation; therefore, the use of mouse inoculation resulted in a 25% increase in sensitivity. The data gathered initially using mouse inoculation were not quantitative. Therefore, we determined the titers of some saliva samples from *Ae. albopictus* mosquitoes by calculating suckling mice $ICLD_{50}$ values. Simultaneous titrations in Vero cells and mice indicated that the $ICLD_{50}$:PFU ratio was approximately 200:1 (data not shown). Of 16 CPE-negative saliva samples from mosquitoes with disseminated infections, 3 tested positive in suckling mice and the $ICLD_{50}$ values converted to PFU were 0.065, 0.054, and 0.065 $\log_{10}$ PFU. Five samples contained less than one suckling mouse $ICLD_{50}$ (less than 0.04 PFU). The addition of the suckling mouse $ICLD_{50}$ assay decreased the geometric mean titer of saliva by one-tenth of a $\log_{10}$ PFU, and increased sensitivity by 2--31% (Figure 34).

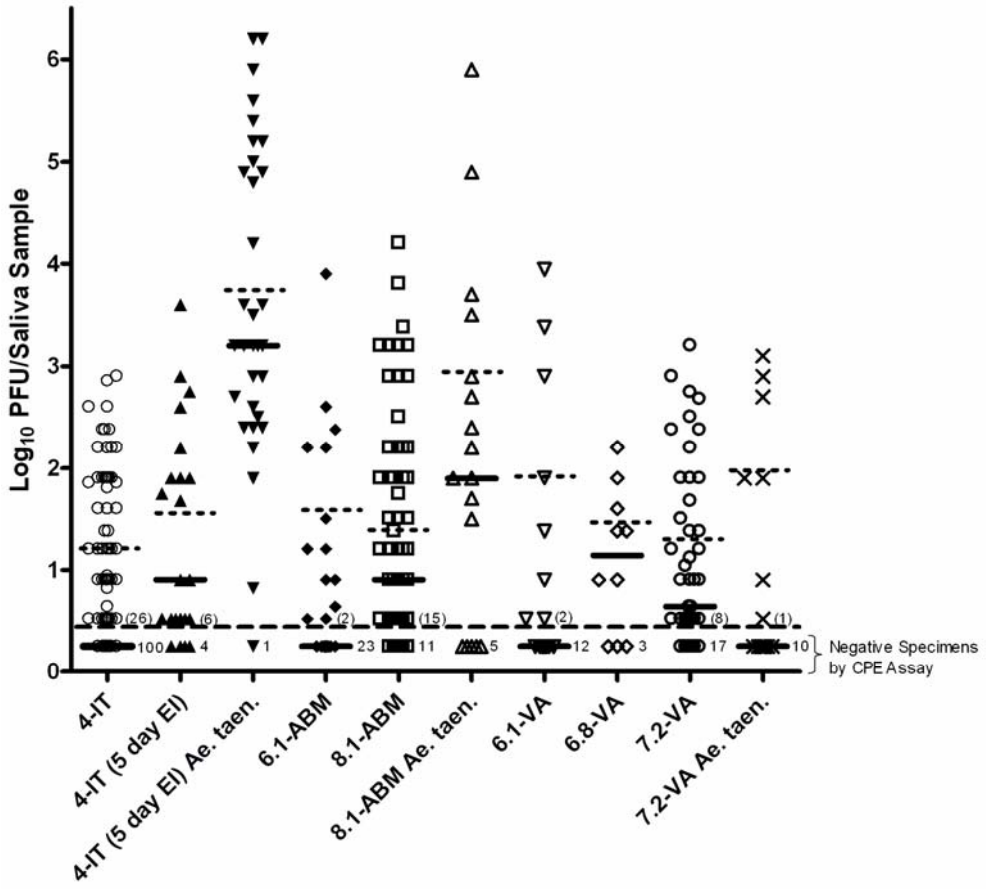

Figure 33. Comparison of Route and Titer of Mosquito Infection. Titers were determined by cell culture assays (plaque and CPE assay). Cohorts are labeled as follows: infection titer (log_{10})-method of infection. Mosquitoes tested were *Aedes albopictus* mosquitoes unless noted as *Aedes taeniorhynchus* (Ae. taen.). The bar indicates the median saliva titer. The dashed bar indicates the average saliva titer. Numbers in parentheses to the right of the symbols with values of 0.5 indicate the number of samples with titers of 0.5 log_{10} PFU. The dotted line at 0.5 log_{10} PFU indicates the limit of detection for the cell culture assays. The symbols below this line indicate samples negative by the CPE assay, indicating that these samples were below the limit of detection for the assay but the mosquitoes were positive for viral dissemination to the legs and/or wings. Numbers to the right of the symbols with values of 0.25 indicate the number of samples that are in between zero and the limit of detection of the assay, 0.5 log_{10} PFU. IT = intrathoracic; ABM = artificial bloodmeal; VA = viremic animal. Reproduced with permission (194).

Table 5. Saliva Assay Results Comparing Virus Titer and Route of Mosquito Infection. Reproduced with permission (194).

Infection method	Mosquito species	Infectious dose (log_{10} PFU/ml)	% with disseminated infection*	% negative by CPE assay	Median saliva titer (log_{10} PFU)	Mean saliva titer (log_{10} PFU)	Range of saliva titers (log_{10} PFU)
Intrathoracic	*Ae. albopictus*	4.0†	100% (172/172)	57%	0.25	1.2	0.5--2.9
Intrathoracic	*Ae. albopictus*	4.0† (5 day incubation)	100% (22/22)	18%	0.9	1.6	0.5--3.6
Intrathoracic	*Ae. taeniorhynchus*	4.0† (5 day incubation)	100% (32/32)	3%	3.2	3.7	0.8--6.2
Artificial bloodmeal	*Ae. albopictus*	6.1	57% (36/63)	63%	0.25	1.6	0.5--3.9
Artificial bloodmeal	*Ae. albopictus*	8.1	92% (61/66)	17%	0.9	1.5	0.5--3.8
Artificial bloodmeal	*Ae. taeniorhynchus*	8.1	61% (17/28)	30%	1.9	2.9	1.5--5.9
Viremic animal	*Ae. albopictus*	6.1	80% (20/25)	60%	0.25	1.9	0.5--3.9
Viremic animal	*Ae. albopictus*	6.8	100% (10/10)	30%	1.1	1.5	0.9--2.2
Viremic animal	*Ae. albopictus*	7.2	94.6% (53/56)	32%	0.6	1.3	0.5--3.2
Viremic animal	*Ae. taeniorhynchus*	7.2	53% (17/32)	59%	0.25	2.0	0.5--3.1

*Numbers tested exceeds totals in Table 4 because only a fraction of samples were tested by mouse inoculation, whereas all were tested using cell culture assays.
† Total dose delivered intrathoracically.

DISCUSSION

Of the methods typically used for infecting mosquitoes with arboviruses, intrathoracic inoculations, artificial bloodmeals and viremic animals, all have advantages and disadvantages as discussed above. However, it is unknown if the infection method affects the dose of an arbovirus delivered in the saliva of a mosquito vector. I therefore systematically evaluated the effect of these infection methods, as well as the virus doses used for infection, on saliva titers using VEEV. Two methods for saliva collection were also compared. Additionally, we compared saliva titers in two different mosquito species to determine if they transmit different amounts of VEEV.

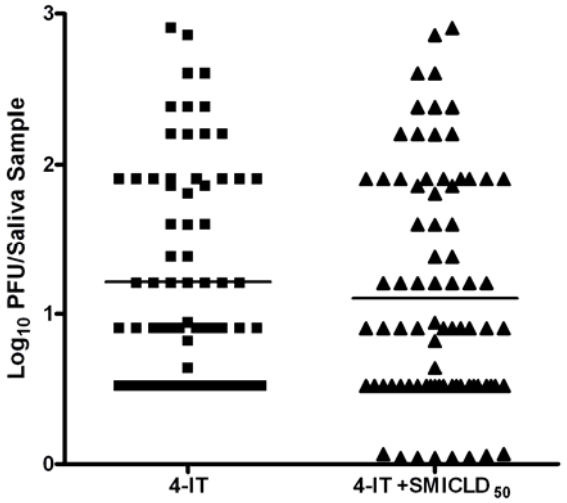

Figure 34. Comparison of Virus Titer With and Without Suckling Mice Titrations [Intracranial Lethal Dose 50% ($ICLD_{50}$)] Using *Aedes albopictus* Mosquitoes. Cohorts are labeled as follows: infection titer (log_{10})-method of infection. The bar indicates the average saliva titer. IT = intrathoracic.

Effect of Saliva Collection Medium

My results indicate no difference in immersion oil versus FBS for the detection and quantification of VEEV in mosquito saliva. Silinized (to reduce possible virus adhesion) capillary tubes were also compared to non-silinized capillary tubes containing FBS spiked with a known amount of virus, and no difference was noted (data not shown). The problems of contamination of the medium by mosquito mouthparts during *in vitro* collection of saliva, as discussed by Vanlandingham et al. (214), were not experienced in my studies.

Effect of Infection Route on Saliva Titers

Significantly less VEEV was detected in *Ae. albopictus* saliva after intrathoracic infection and a 10 day incubation period versus after infection by an artificial bloodmeal and the same incubation. In contrast, when *Ae. albopictus* was infected intrathoracically and incubated 5 days, no significant difference in titers occurred compared to the artificial bloodmeal infection (Figure 33, Table 5). This was most likely due to different kinetics of viral replication in mosquitoes infected intrathoracically versus orally; virus titers peak earlier in intrathoracically infected mosquitoes because the midgut infection barrier is circumvented. My data suggest that when using intrathoracically infected mosquitoes, a shorter extrinsic incubation period should be used to generate peak saliva titers comparable to those following oral infection. Surprisingly, *Ae. taeniorhynchus* infected intrathoracically had a significantly higher mean saliva titer than those exposed via an artificial bloodmeal or viremic animal. Although mosquitoes can be less susceptible to infection using artificial bloodmeals compared to natural infection (127, 144, 207, 232), our study found no difference in the dissemination rates observed by the two oral infection methods although it should be noted that there was almost a 10-fold difference in bloodmeal titers.

Effect of Infection Dose on Saliva Titers

Varying oral doses had a significant effect on *Ae. albopictus* saliva titers for mosquitoes exposed by artificial bloodmeal, but no differences were observed for mosquitoes exposed to varying oral doses by viremic animal (Figure 33, Table 5).

Effect of Mosquito Species on Saliva Titers

The possibility that different mosquito species may transmit different amounts of virus has never been addressed. My results show that saliva from *Ae. taeniorhynchus* mosquitoes contains on average 1.2 $\log_{10}$ PFU more VEEV than that of *Ae. albopictus* infected using the same methods and doses. Previous studies suggest that some epizootic VEEV strains undergo adaptation for *Ae. taeniorhynchus* transmission, and this adaptation may be a significant factor in epidemic emergence (17, 141). The higher saliva titers in this proven vector, compared to *Ae. albopictus* that are susceptible to infection

but have not been implicated in natural transmission, further support this hypothesis for VEEV emergence, although further studies looking at saliva titers and transmission efficiency are needed.

A wide range of VEEV titers was detected in the saliva of individual *Ae. albopictus* (0.25--4.2 $\log_{10}$ PFU) and *Ae. taeniorhynchus* mosquitoes (0.25--6.2 $\log_{10}$ PFU), consistent with previous studies using other arboviruses (22, 30, 214). However, the amount of VEEV in the saliva did not correlate with the amount in the body or legs (data not shown). A previous study also showed no correlation between the amount of dengue-2 virus transmitted by *Ae. albopictus* mosquitoes and the amount of salivary gland tissue infected (68); therefore, the wide range of virus transmitted by mosquitoes is probably determined by factors other than the degree of salivary gland tissue infected once dissemination into the mosquito hemocoel has occurred.

Detection of Low Virus Titers in Saliva Using Mouse Inoculation

As seen in Table 4, a large number of mosquitoes were positive for a disseminated infection (CPE-positive legs), but contained saliva that was negative by the CPE assay (31.4%). Inoculation of suckling mice I.C. demonstrated that small amounts of virus were present in many of these saliva samples, leaving only 6.5% samples negative for virus using both assay methods. The limit of detection for the CPE assay was 0.5 $\log_{10}$ PFU, but suckling mice inoculated intracranially proved to be much more sensitive than cell culture assays (Table 4). Therefore, testing samples collected in capillary tubes by the CPE assay is relatively insensitive for determining the correct transmission rate for mosquitoes.

An important question that remains from this study is if the amount of virus mosquitoes salivates into capillary tubes is the same amount transmitted while feeding on a vertebrate host. In order to salivate the mosquito into a capillary tube the mosquito must first be cold anesthetized and have its legs/wings removed, which may affect the amount of virus transmitted. Additionally, mosquitoes are usually allowed to salivate into the capillary tubes for a much longer time than it takes for a mosquito in nature to acquire a bloodmeal.

In conclusion, this study has shown that the virus titer used for the infection of mosquitoes appears to have little or no effect on the amount of virus found in saliva following extrinsic incubation. The method of oral infection (artificial bloodmeal versus viremic animal) also does not appear to affect the titer of VEEV in saliva. However, intrathoracic inoculation generates lower saliva titers following the same incubation period (10 days). *Ae. albopictus* saliva (median titer of 0.6 $\log_{10}$ PFU, mean titer of 1.0 $\log_{10}$ PFU, range = 0.25-4.2 $\log_{10}$ PFU) contains significantly less VEEV than that of *Ae. taeniorhynchus* (median titer of 1.8 $\log_{10}$ PFU, mean titer of 2.2 $\log_{10}$ PFU, range = 0.25-6.2 $\log_{10}$ PFU), demonstrating that the mosquito species used in transmission studies may affect the quantity of virus transmitted and the resultant pathogenesis. The results of this study should be considered when designing transmission and pathogenesis studies to mimic natural infection by arboviruses.

CHAPTER 5: QUANTIFICATION OF VENEZUELAN EQUINE ENCEPHALITIS VIRUS TRANSMISSION BY MOSQUITOES AND THE EFFECT OF TRANSMISSION MODE ON PATHOGENESIS[2]

ABSTRACT

Quantifying the dose of an arbovirus transmitted by mosquitoes is essential for designing vertebrate pathogenesis studies simulating natural infection. Titration of saliva collected *in vitro* from infected mosquitoes may not accurately estimate titers transmitted during blood feeding, and needle infection may affect vertebrate pathogenesis. I compared the amount of Venezuelan equine encephalitis virus collected from the saliva of *Aedes taeniorhynchus* to the amount inoculated into a mouse during blood feeding. Mosquitoes transmitted significantly less [geometric mean of 11 plaque forming units (PFU)] virus *in vivo* compared to a mean saliva titer of 74 PFU for comparable times of salivation *in vitro*. I also observed slightly lower early and late viremia titers in mice that were needle-inoculated with 8 PFU, representing the low end of the *in vivo* transmission range, compared to infection via a mosquito bite. No significant differences in survival were detected regardless of the dose or infection route.

INTRODUCTION

Designing pathogenesis studies for arboviruses that accurately simulate natural infection requires quantifying the amount of virus transmitted. Artificial collection of mosquito saliva and virus assays can be used to estimate the amount transmitted to

[2] Substantial parts of this Chapter has been accepted for publication in Emerging Infectious Diseases. This journal is published by the Centers for Disease Control and Prevention, a US government agency that places the materials it publishes in the public domain. Information from articles in the journal can be used without permission, with proper citation. The citation for information from this chapter is: Smith DR, Aguilar PV, Coffey LL, Gromowski GD, Wang E, Weaver SC. 2006. Quantification of Venezuelan Equine Encephalitis Virus Transmission by Mosquitoes and the Effect of Transmission Mode on Pathogenesis. Emerging Infectious Diseases, In Press.

vertebrates during blood feeding. However, the amount of virus collected *in vitro* may not accurately reflect mosquito transmission.

In vitro Saliva Collection

Saliva collection in oil-filled capillary tubes was first described by Hurlbut (1) and has become widely used. Chamberlain et al. (22) compared several methods for quantifying arbovirus transmission and concluded that allowing mosquitoes to feed on serum (similar to the capillary method) is less efficient for detecting virus than other methods. Most saliva is expectorated during probing (159), so salivation into capillary tubes may be inaccurate because mosquitoes do not need to salivate to locate a blood vessel.

The amount of several arboviruses transmitted by mosquitoes has been estimated using artificial saliva collection (4, 22, 30, 33, 68, 84, 124, 171, 172, 214, 238). Recently, we estimated that the epidemic Venezuelan equine encephalitis virus (VEEV) vector, *Aedes taeniorhynchus,* salivates from 0.2 to 3.2 $\log_{10}$ PFU into oil-filled capillary tubes (194).

Effect of Vector Saliva on Vertebrate Pathogenesis

Vector saliva enhances infection with many pathogens (135, 160, 192, 200-202), and mosquito saliva is reported to enhance infection by some arboviruses. Deer and chipmunks infected with La Crosse virus by the bite of *Ae. triseriatus* mosquitoes have higher and longer viremias compared to animals infected by needle (142). However, this study used multiple mosquitoes to infect the deer (4-5 mosquitoes) and some chipmunks (1-2 mosquitoes). Additionally, the viremia in deer infected by mosquitoes was compared to needle inoculations of deer from a separate study, which used a virus strain with a different passage history. The viremia in chipmunks infected by mosquito feeding was compared to intramuscular inoculation of virus, which is not the correct anatomical location of mosquito deposition of virus. Mice exposed to uninfected mosquitoes and then injected at the feeding site with Cache Valley virus develop enhanced viremia and seroconversion compared to unbitten mice or to those co-inoculated with virus and mosquito saliva (43). However, this study allowed 20-30 mosquitoes to feed completely

(an additional number of mosquitoes probed without engorging) on the mouse prior to needle inoculation of the virus. Limesand et al. (109) reported that mice have higher seroconversion rates to vesicular stomatitis virus when infected by *Ae. triseriatus* compared to needle inoculation. However, mice exposed to 5 infected mosquitoes, were compared to those receiving a needle inoculation of the estimated dose transmitted by a single mosquito. Schneider et al. (188) demonstrated a difference in cytokine expression in the skin of mice following inoculation of Sindbis virus with mosquito salivary gland extracts, compared to virus alone. Salivary gland extract contains additional proteins and cellular mileu not found in saliva alone, and this study used salivary gland extracts from multiple mosquitoes. More recently, Schneider et al. (187) determined that *Aedes aegypti* (not known to be a natural WNV vector) mosquito feeding potentiates West Nile Virus infection in mice. However, this conclusion is based on the use of multiple mosquitoes (average of 11.5 $\pm$1.5) feeding at the same site prior to needle inoculation of the virus, which was compared to a needle inoculation dose estimate from a single mosquito.

In contrast, other studies report no enhancement of arbovirus infection due to vector saliva or feeding. Sbrana et al. (178) showed that adult hamsters infected with WNV by mosquitoes or needle inoculation do not differ in the level or duration of viremia, clinical manifestations, pathology, or antibody response. However, 10 mosquitoes were allowed to transmit to the hamsters, which was compared to an intraperitoneal (not the anatomical location of mosquito saliva/virus deposition) inoculation of the virus. Reisen et al. (157) concluded that birds infected with western equine encephalitis virus or St. Louis encephalitis virus by mosquito bite or needle have no difference in viremia responses. This study allowed 3 to 4 mosquitoes to transmit to young chicks, which was compared to a subcutaneous needle inoculation with three different virus doses. An *in vitro* study with dengue virus found that mosquito saliva inhibits infection of dendritic cells (3).

Venezuelan equine encephalitis virus (*Togaviridae*: *Alphavirus*) is an important emerging and reemerging pathogen of people and equines in the neotropics of Central and South America. Equines are used extensively in Central and South America for

agriculture and transportation; therefore, VEE outbreaks have negative social and economic impacts, in addition to causing human morbidity and mortality. There are no effective antivirals or a licensed human vaccine for VEEV, so therapy is primarily supportive and prevention relies on avoidance of mosquito exposure. Venezuelan equine encephalitis outbreaks can involve hundreds- of- thousands of equine and human cases, spread over large geographic regions, and can last several years (230).

The effect of vector feeding on vertebrate infections by VEEV has not been studied. We determined the amount of VEEV in mosquito saliva collected *in vitro* (194) but we have not determined whether the amount of virus a mosquito salivates into a capillary tube accurately reflects the amount transmitted during blood feeding. To collect saliva in a capillary tube, the mosquito must first be anesthetized with triethylamine or cold exposure, and immobilized by removal of the legs and wings, traumatic manipulations that may affect salivation. Additionally, mosquitoes are usually allowed to salivate into the capillary tubes for a much longer time (e.g. 30 min) than is required for engorgement on a host. Because determining the infectious dose transmitted by mosquitoes is important for designing vertebrate infection studies, in which needles are typically used for virus delivery, we compared the amount of VEEV transmitted by mosquitoes *in vitro* to that transmitted *in vivo*. We also determined if natural mosquito inoculation results in a difference in viremia or time to death when compared to needle infections. Finally, we used tail amputations to investigate the extra- or intravascular location of VEEV deposition in mice during mosquito feeding.

MATERIALS AND METHODS

Virus

Virus was rescued from an infectious cDNA clone derived from VEEV epidemic strain 3908 (subtype IC), a 1995 human isolate from Zulia State, Venezuela during a major outbreak (235). With the exception of some subtype IE virus strains in Mexico, subtype IC viruses are the etiologic agents of all recent VEE epidemics. The 3908 strain was passaged once in C6/36 mosquito cells before undergoing RNA extraction and

infectious cDNA clone production. Virus recovered from BHK cells electroporated with transcribed RNA was used for all experiments without further passage. The use of virus derived from an infectious clone minimized attenuating mutations that occur when VEEV is passaged in cell culture (11).

Mosquitoes

F1 progeny of *Ae. taeniorhynchus* mosquitoes captured in Florida (29) were used. Mosquitoes were reared at 27°C, 80% relative humidity using a light/dark cycle of 12:12 hr. All adult females were infected intrathoracically with 4 $\log_{10}$ PFU in a 1 μL volume, 6 to 8 days after emergence, and incubated at 27°C for 5 days with 10% sucrose provided *ad libitum*. Intrathoracic infection of mosquitoes with VEEV and incubation for 5 days generates saliva titers comparable to those that occur following oral infection (194).

***In Vivo* Transmission**

Thirty-nine 6- to 8-week-old NIH Swiss mice (Harlan, Indianapolis, IN) were anesthetized with pentobarbital and the distal portion of the tail was exposed to one infected *Ae. taeniorhynchus* mosquito. The mosquito was allowed to probe or engorge, then the tip of the tail was severed and immediately homogenized in 300 μL of Eagles minimal essential medium (MEM) using a Mixer Mill 300 (Retsch, Inc., Newton, PA), with the exception of ten control mice whose tails were left intact. Following centrifugation at 9,000 x g for 5 min, the supernatant was removed for cell culture assays and RNA extraction using a Qiagen kit (Valencia, CA). Vero cells were inoculated with 30 μL of the supernatant and observed 5 days for cytopathic effects (CPE). All CPE-positive samples were titrated by plaque assay on Vero cells. RNA was also extracted from the pellet of the tail homogenate using Trizol (Invitrogen, Carlsbad, CA). The RNA of both the supernatant and tail pellet was tested for VEEV positive strand RNA using real-time RT-PCR with the Qiagen One-step Kit (Qiagen, Valencia, CA) and the Cepheid (Sunnyvale, CA) Smart Cycler . Primers of sequence forward (5'-CATAGTCTAGTCCGCCAAGATGTT-3'), reverse (5'-CGATAGGGCATTGGCTGCAT-3'), and a probe of sequence (5'-[6-

FAM]CCCGTTCCAACCAATGTAT[NFQ-MGB]-3') were used for amplification and detection, respectively. The assay consisted of reverse transcription at 50°C for 20 min, denaturation at 95°C for 10 min, and 45 cycles of 95°C for 15 sec, 63°C for 30 sec, and 72°C for 30 sec. Virus titers were extrapolated from real time RT-PCR results by comparison with a standard curve generated from serial dilutions of a VEEV stock quantified by plaque assay to determine real time RT-PCR to PFU equivalents.

After a mosquito probed/fed on the distal portion of the mouse tail, it was confirmed to be infected by forced salivation into an oil-filled capillary tube, as described below. All 39 mice used were held in individual cages, monitored for signs of infection, and bled retroorbitally 2 weeks later for seroconversion plaque reduction neutralization tests (PRNT).

A control experiment was performed to ensure that no virus inoculated into mouse tails was lost using our methods. A series of known doses (2.9 $\log_{10}$, 2 $\log_{10}$, and 1 $\log_{10}$ PFU in 1μl) were inoculated intradermally (in a volume of 1μl) into the tip of a mouse tail. The tip was then severed and processed as described above. Each dose was tested in duplicate and nearly all of the inoculated virus was recovered (means of 2.7 $\log_{10}$ PFU recovered for 2.9 $\log_{10}$ inoculum; 1.9 $\log_{10}$ PFU recovered for 2.0 $\log_{10}$ inoculum; 0.8 $\log_{10}$ PFU recovered for 1.0 $\log_{10}$ inoculum), indicating that most virus inoculated was detected using our assay methods. Additional VEEV samples of known virus titers were tested to ensure that freezing/thawing samples once did not significantly alter virus content.

Saliva Assays

Thirty-nine saliva samples from intrathoracically infected mosquitoes that fed on a mouse tail were obtained by immobilization (legs and wings removed) and forced salivation into capillary tubes (10 μL, VWR international, West Chester, PA) filled with immersion oil (type B, Cargille Laboratories Inc., Cedar Grove, NJ) for 30-45 min. An additional cohort of mosquitoes was allowed to salivate for timed intervals (repeated in triplicate) to duplicate the times of mosquito feeding observed on a mouse tail. The oil/saliva was centrifuged into 100 μL of MEM and frozen at -80°C; 30 μL was then

added to Vero cells for detection of CPE, followed by plaque assay. Mosquito infection was confirmed by assaying triturated bodies and legs/wings for CPE.

Viremia and Mortality

Ten, 6- to 8-week-old NIH Swiss mice were infected either by a single *Ae. taeniorhynchus* mosquito, or by inoculation of VEEV intradermally in the ear with either 8 PFU or 3.4 $\log_{10}$ PFU. These doses represented the range of titers inoculated by mosquitoes. Five mice from each cohort of 10 animals were bled retroorbitally at 12, 24, 36, 48, 72, 96, and 120 hours post-infection, and the sera were titrated by plaque assay. Mice were monitored daily until signs of encephalitis, and then were observed 4 times daily to determine the time of death. The University of Texas Medical Branch Institutional Animal Care and Use Committee approved all experiments.

Statistics

Cohorts of log-transformed data were normally distributed, except for the group comprising RT-PCR assays from mouse tail homogenate pellets on which single mosquitoes probed (this group was not compared statistically). A one-way ANOVA using Tukey's test for multiple comparisons and an unpaired t-test were used to compare all normally distributed cohort data using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA).

RESULTS

In Vivo vs. *In Vitro* Transmission Titers

To determine if saliva collection accurately approximates the amount of VEEV transmitted during a mosquito blood meal, virus content from *in vitro* collected saliva versus virus deposited at sites of *in vivo* blood feeding were quantified. The mean amount of virus transmitted by a single mosquito feeding on the distal portion of a mouse tail, as detected by plaque assay of the supernatant of the tail homogenate, was 1.1 ± 1.0 $\log_{10}$ PFU (11 PFU) and using real time RT-PCR was 0.8 ± 0.9 $\log_{10}$ equivalent PFU (7 PFU). The mean amount of virus transmitted by a single mosquito to the mouse tail, as detected by both plaque assay and RT-PCR, was significantly lower ($p<0.001$) than the mean

amount of virus (3.6 $^{\pm}$ 1.5 $\log_{10}$ PFU, or 4,300 PFU) deposited into capillary tubes during 30-45 min of salivation (Figure 35). However, the time for engorgement on the mouse tail (<3 minutes) was much shorter than the 45 min allowed for *in vitro* salivation. Therefore, we matched times of saliva collection to the exact engorgement times of the mosquitoes. Significantly less ($p<0.05$) VEEV was still detected following *in vivo* transmission compared to <3 min of *in vitro* salivation (mean

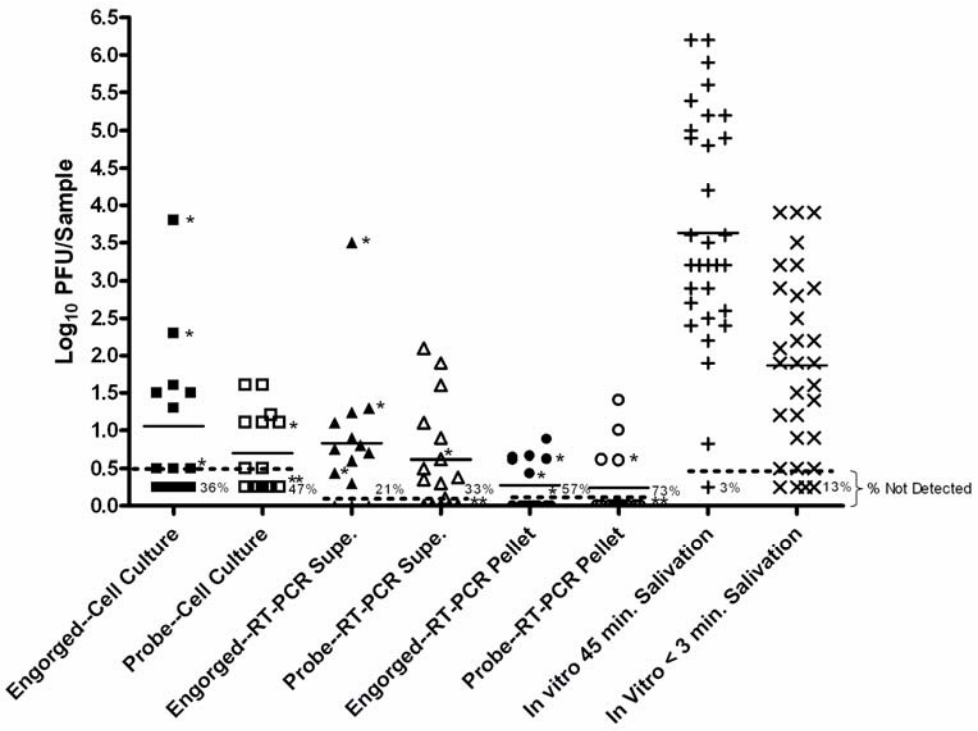

Figure 35. Titers of VEEV Transmitted *In Vitro* or *In Vivo* by *Ae. taeniorhynchus*. Cohorts are labeled as engorged (mosquito engorged to completion, closed symbols) or probed (mosquito probed but did not engorge, open symbols), followed by the assay used to determine the virus titer [cell culture assays (squares) or real-time RT-PCR] of the tail homogenate supernatant [(supe.) triangles] or pellet (circles). The last two cohorts represent VEEV titers in the saliva of mosquitoes allowed to salivate for 45 minutes (crosses) or for the same range of times (<3 min) required for mosquitoes to engorge completely on mouse tails, repeated in triplicate (x). Solid horizontal lines indicate means and horizontal dotted lines indicate the detection limits for the assays. The symbols below the dashed lines indicate samples from infected mosquitoes (CPE-positive bodies and legs/wings) that were below the limit of detection for the assay, and the numbers in parentheses indicate the percentages for these negative samples. *Denotes mice that died.

of 1.9 $\pm$ 1.2 $\log_{10}$ PFU, or 74 PFU).

The effect of the time of probing/blood feeding on the titer of virus salivated was addressed using timed saliva collections and mouse tail exposures. The amount of VEEV collected from mosquitoes that salivated *in vitro* for <3 min was significantly less than the amount collected from mosquitoes allowed to salivate for 45 min ($p<0.0001$). However, there was no significant difference in the amount of virus transmitted by mosquitoes allowed to completely engorge versus mosquitoes only allowed to probe without engorgement ($p>0.05$; 95% CI= -0.8-1.5 $\log_{10}$ PFU for the difference in the mean titers).

To address the possibility that some of the virus injected by feeding mosquitoes rapidly binds to or penetrates cells, and therefore is not measured by plaque assay, we also examined VEEV RNA content in mouse tails. No significant difference was detected between mean virus content in the mouse tail homogenate supernatants assayed by RT-PCR or plaque assay (Figure 35). Detection of relatively small amounts of viral RNA in the tail homogenate pellets indicated that most or all of the virus remained in the supernatant, and that infectious virus was not underestimated due to rapid penetration of cells or binding of virus to connective tissue (Figure 35).

Location of VEEV Deposition

To assess the intra- versus extravascular locations of VEEV deposition by mosquitoes, the distal portions of mouse tails were amputated immediately after engorgement, and mice were held for signs of infection. Forty percent (4/10) of control mice whose tails were not removed after mosquito feeding survived, compared to significantly more (79%, 23/29) that had their tails amputated ($p=0.04$, Fisher's exact test). No mice that survived developed neutralizing antibodies (data not shown) indicating that infection was invariably fatal and suggesting that a systemic VEEV infection did not occur in surviving animals. These data suggested that nearly all of the saliva and associated virus were deposited extravascularly and confined to the bite site by a lack of immediate vascular dissemination. Tail amputation nearly doubled survival rates by removing this virus before it had a chance to replicate and disseminate.

Virus Transmitted vs. Time of Engorgement

To assess the temporal pattern of virus deposition during blood feeding, the amount of VEEV transmitted was compared to the time required for mosquito engorgement. Figure 36 shows no significant correlation between the feeding time and the amount of VEEV in mouse tails, suggesting that most of the virus/saliva was deposited early during probing, with minimal virus deposition during engorgement.

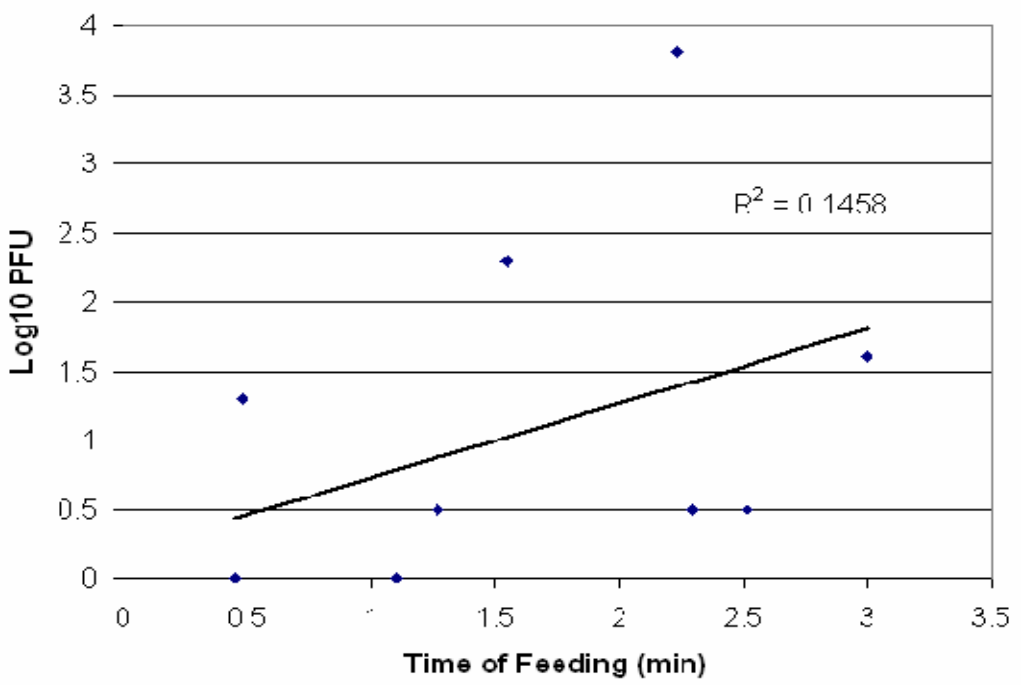

Figure 36. Comparison of the Amount of VEEV Transmitted into a Mouse Tail Versus the Time Required for Complete Engorgement. Samples below the limit of detection were not included.

Effect on Murine Pathogenesis of Needle vs. Mosquito Infection

To determine if mosquito saliva affects murine pathogenesis, mice were infected by either the bite of a single mosquito, or by intradermal needle inoculation. Two doses of VEEV were used to represent the range of titers injected during blood feeding (Figure 35). Needle infection with a high dose produced viremia with no significant difference from that generated by mosquito transmission (Figure 37). In contrast, viremia in mice

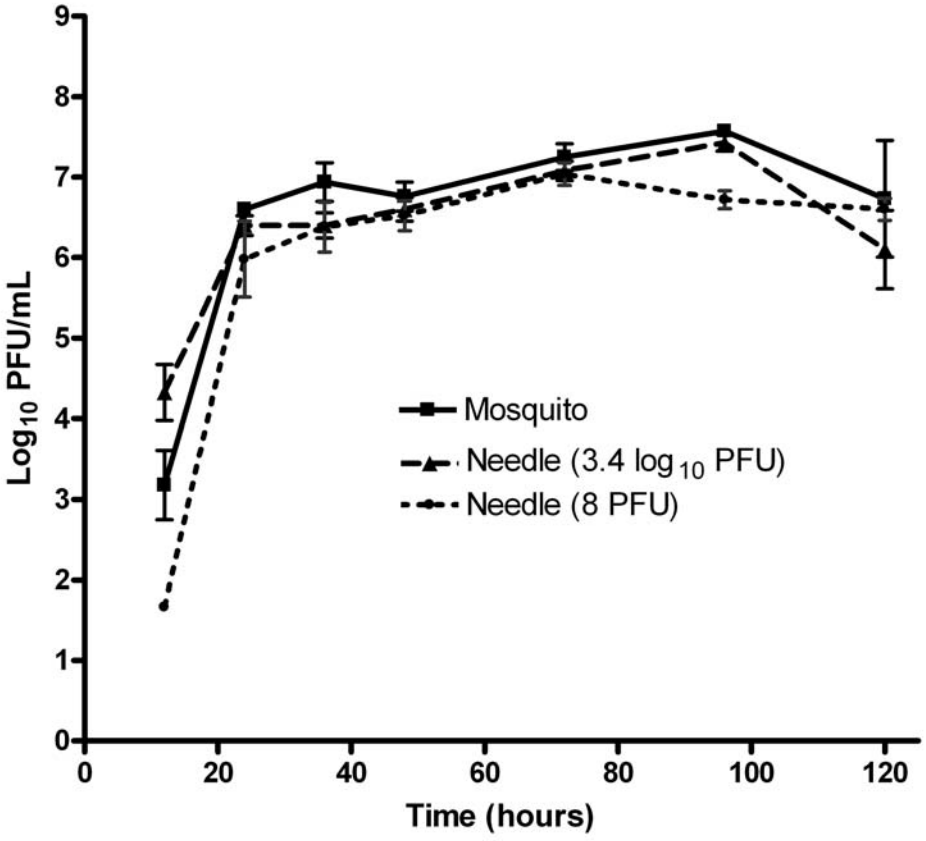

Figure 37. Viremia in NIH Swiss Mice Infected by a Single Mosquito Bite, or Intradermally Via Needle Inoculation With Two Different VEEV Doses Representing the Range of Doses Delivered During Blood Feeding (see Figure 35). Five animals per cohort were bled at each time point. Bars indicate standard deviations.

infected by a mosquito bite was significantly higher than that following a low dose needle inoculation for the 12 hr ($p<0.05$) and 96 hr ($p<0.001$) time points. A significant difference ($p<0.001$) was also observed at the 12 and 96 hr time points for mice infected with the high versus low dose needle inoculations. No significant difference in the mean survival times of mice infected by either mosquito (5.9 ± 0.6 days) or needle inoculation using 8 PFU (6.4 ± 0.7days) or 3.4 $\log_{10}$ PFU (6.3 ± 0.4 days) was detected (Figure 38).

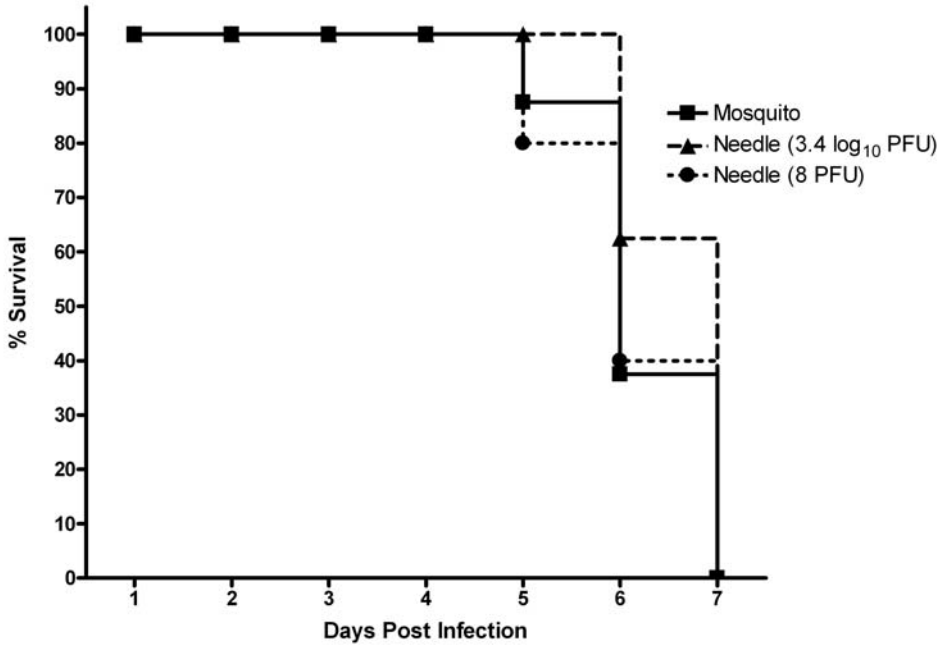

Figure 38. Survival of Cohorts of 10 NIH Swiss Mice Infected with VEEV by Either a Single Mosquito Bite, or Via Intradermal Needle Inoculation With Two Doses Representing the Range of Virus Titers Delivered During Mosquito Blood Feeding (see Figure 35).

DISCUSSION

Because the effect of mosquito transmission on infection by VEEV has not been addressed, we assessed the infectious dose delivered by a natural vector, *Ae.*

taeniorhynchus, compared to estimates from saliva collections. We also evaluated the effect of mosquito transmission on murine pathogenesis.

In Vivo vs. *In Vitro* Transmission

Our results indicate that *Ae. taeniorhynchus* mosquitoes transmit significantly less VEEV *in vivo* compared to the amount deposited *in vitro* into a capillary tube, even when the saliva collection times are matched to engorgement times. Most studies utilizing capillary tubes for saliva collection allow mosquitoes more time to deposit saliva than is required to engorge on a host. The results of our study caution against extended times of saliva collection in capillary tubes because mosquitoes allowed to salivate for 45 min expel significantly more VEEV than those that salivate for <3 min, the approximate maximum time required for natural engorgement.

Our study also assessed the location of saliva deposition by amputating the distal portion of the mouse tail after blood feeding. As reasoned by Turell et al. (208, 212), if an arbovirus were deposited intravascularly, it would quickly circulate beyond the bite site and animals with tail amputations would still become infected and die. Turell et al. reported that when the tails of suckling mice are exposed to a VEEV-infected *Ae. taeniorhynchus* and have their tail amputated ≤10 min later, 31-37% survive compared to 4% survival for mice whose tails are not amputated (30). Our results indicating that mortality is decreased by approximately one-half with tail amputation suggest that saliva and VEEV are deposited both intra- and extravascularly. These results are slightly different than those of Turell et al. (208, 212), who concluded that mosquitoes primarily inoculate virus extravascularly with only small amounts deposited intravascularly, or that intravascular transmission occurs only occasionally. An explanation for the mortality differences between our studies and those of Turell et al. is that they used suckling mice, while we used adult mice. Two of our mice that had only been probed by an infected mosquito also became infected and died. Surprisingly, no VEEV was detected in the tail homogenate of these mice by either cell culture or RT-PCR. Because the 50% mouse subcutaneous lethal dose (LD_{50}) for VEEV strain 3908 administered in the tail is 12 PFU

(DRS, unpublished), more than the LD_{50} for inoculation in the thigh (194), and an amount detectable using our methods, the virus was probably deposited primarily intravascularly in these 2 animals.

Unexpectedly, 40% of our mice with intact tails survived after an infected mosquito fed to completion. NIH Swiss mice are highly susceptible to VEEV, with mortality rates typically 100%. These results and those from our previous study (194), which reported that infected mosquitoes often deposit less than 12 PFU of VEEV into capillary tubes, suggest that systemically infected *Ae. taeniorhynchus* frequently transmit little or no VEEV. In contrast to the 40% survival rate of mice with intact tails, 100% of mice infected by mosquito bite at a site other than the tail (to evaluate the effect of mosquito transmission on viremia and mortality) died. This may be due to a difference in the site of virus deposition. The LD_{50} for VEEV strain 3908 administered in the tail is 12 PFU while the LD_{50} for VEEV administered subcutaneously in the hind limb is less than 1 PFU (DRS, unpublished). Alternatively, mosquitoes may deposit different amounts of virus at different anatomical sites due to the difficulty/ease of accessibility to blood vessels.

Time of Engorgement and Infectious Dose Transmitted

The amount of VEEV transmitted by *Ae. taeniorhynchus* did not correlate with the time to complete engorgement. However, I did not count how many times the mosquito probed before beginning to engorge. Assuming that most mosquito saliva is injected during the intradermal probing period that precedes canulation of a blood vessel, and that infection of the host correlates with the duration of salivation during probing, probing frequency could affect the transmission outcome and should be investigated.

Effects on VEE Pathogenesis of Needle vs. Mosquito Infection

Because mosquitoes transmit a wide range of arbovirus doses, I inoculated mice with 2 doses representing the range of VEEV titers transmitted. No significant difference in viremia was detected between mice infected by a mosquito versus a high needle dose. However, mice infected by a mosquito bite exhibited significantly higher mean viremia titers at the early (12 hr) and late (96 hr) time points compared to a low needle dose.

Because mice injected with the high dose also had significantly higher mean viremia titers at some time points compared to the low dose cohort (Figure 37), the difference in the mosquito bite versus low dose needle infection may only reflect that some mosquitoes transmitted higher doses than 8 PFU. The only way to confirm conclusively this possible slight effect of mosquito transmission on early and late VEEV viremia would be to duplicate the exact distribution of *in vivo* transmission titers using needle inoculations. Even then, the volumes inoculated by mosquitoes versus needles would differ as well as the exact intradermal sites of deposition. Another approach would be to co-inject mosquito saliva with virus (43, 188), but the same volume and site discrepancies would apply.

My results agree with reports of little or no enhancement of other alphaviral infections by mosquito transmission (157). In several studies describing the enhancement of arboviral infection by mosquito transmission, multiple mosquitoes were transmitting to a single vertebrate (43, 109, 142), or salivary gland extracts from many mosquitoes were inoculated along with virus (43, 188). Salivary gland extracts contain additional proteins and cellular mileu not present in the mosquito saliva alone, which could lead to artificial results. Additionally, the mosquito salivates a small percentage of it's total saliva content instead of emptying the gland, which is simulated by using entire salivary gland extracts. Because natural infection rates of mosquitoes are typically low, simultaneous transmission by more than one vector is probably rare. Additionally, the use of an inaccurate dose or inoculation site to simulate natural transmission may give misleading results. The artificial conditions used for some of these experiments demonstrating potentiation of arbovirus infection by mosquito transmission may therefore exaggerate the true effect.

Significance for Pathogenesis Studies

In conclusion, *Ae. taeniorhynchus* mosquitoes transmit significantly less VEEV *in vivo* compared to the amount deposited *in vitro* into capillary tubes. Mosquito transmission has little or no effect on the overall murine viremia profile and none on mortality. These results should be considered when designing arboviral pathogenesis

studies to simulate natural infection. For VEEV, a range of relatively small doses from about 10-1,000 PFU is recommended to simulate *Ae. taeniorhynchus*-borne infections, which is lower than doses used in past VEEV pathogenesis studies (54, 63, 86, 87, 117, 179). Additonally, some previous pathogenesis studies of VEEV used the foot pad of a mouse as the site of virus inoculation (63, 117), which is not the most likely anatomical location of mosquito feeding. The results of my study suggest that the anatomical site of mosquito transmission may have an effect on the susceptibility of the vertebrate to virus infection. Alternatively, mosquitoes may deposit different amounts of virus at different anatomical sites due to the difficulty/ease of locating and canulating blood vessels; therefore, the site of virus inoculation should be considered for future VEEV pathogensis studies. I suggest using the hind-limb of laboratory rodents for future pathogensis studies. Because VEEV saliva titers differ among mosquito species (194), comparable studies should be conducted with other epizootic and enzootic vectors.

CHAPTER 6: VENEZUELAN EQUINE ENCEPHALITIS VIRUS INFECTIOUS CLONE ERRORS THAT AFFECTS PROPER FUNCTION

T7 PROMOTER

The plasmid vectors used for Alphavirus infectious cDNA clone production often contain promoters derived from bacteriophages T3, T7, and/or SP6. These promoters allow *in vitro* transcription to be carried out from linear DNA when incubated with the appropriate DNA-dependent RNA polymerase and ribonucleotide precursors.

Figure 39. T7 Promoter Sequence

The infectious clone for VEEV strain 68U201, IE.AA, was not transcribing RNA efficiently. Following sequencing of the T7 promoter region it was discovered that the G nucleotide, which is located in the promoter initiation site (Figure 39), was missing. Although T7 polymerase has been observed to initiate with ATP (42), it prefers to initiate with GTP (85). The IE.AA clone was corrected by designing primers that would insert the missing G nucleotide by PCR amplification. The primers also contained restriction sites that would allow the successful incorporation of the corrected promoter into the existing infectious clone. The PCR product was digested by restriction enzymes XbaI and XmaI while the IE.AA infectious clone was digested with combinations of XmaI, AscI and AscI, XbaI. The correct fragments were then ligated to create the IE.AA infectious clone with the corrected promoter, which resulted in transcription that is more efficient.

3' CONSERVED SEQUENCE ELEMENT

The 3' end of the alphavirus genome consists of a polyadenylate tail [poly (A)] preceded by a highly conserved sequence element (CSE) 19 nucleotides in length (195). The 3' CSE and poly (A) tail are thought to be a part of the core promoter for minus-strand RNA synthesis during genome replication (72). Kuhn et al. found that mutations

within the 3' CSE inhibited virus production (104) and Hardy et al. found that deletion or substitution of the 13 nucleotides within the 3' end of the 3' CSE markedly inhibited minus-strand RNA synthesis.

A replicon system was created for VEEV strain 3908 (Chapter 2) to identify primary sites of virus replication by the expression of the reporter gene green fluorescent protein (GFP). The infectious clone used for the creation of the replicon particles was pM1.3908. By sequencing this clone, an additional G nucleotide was located in the 3' CSE immediately before the start of the poly (A) tail (Figure 40), which was found to inhibit the infection of cells with the replicon particles. However, virus replication was detected when cells were infected with virus derived from the full length infectious clone. This was most likely due to the ability of the virus to overcome this error by mutation during replication, which has been demonstrated in previous studies (51, 153). This error in the pM1.3908 helper for the replicon system and full length infectious clone was corrected by designing primers to delete the additional G nucleotide by PCR amplification. The primers also contained restriction sites that would allow the successful insertion of the corrected 3' CSE into the existing infectious clones. Both the PCR product and pM1.3908 full length infectious clone and helper plasmid was digested with the restriction enzymes MluI and SacII. The correct fragments were then ligated to create the infectious clones with the corrected 3' CSE, which resulted in the efficient infection of cells with the replicon particles.

ATTTTGTTTTTAATATTTCG-Poly (A)

Figure 40. Alphavirus 3' Conserved Sequence Element Based on VEE Trinidad Donkey Strain. The additional nucleotide found in pM1. 3908 is underlined.

VIRUS CONSTRUCTS CONTAINING GFP

The gene encoding GFP was inserted into VEEV strains 3908 and 68U201 (Chapter 2 and 3) as a marker of infected cells. Following sequencing of the constructs, several mutations were found in GFP, which included mutations of Phe115 to Leu, His231 to Leu, and Met233 to Leu based on the GFP sequence reported by Tsien et al.

(205). A major difference in fluorescent intensity was observed when cells were infected with equivalent concentrations of replicon particles with either the wild type or the mutated GFP (Figure 41). These mutations have not been previously reported in the literature to affect the fluorescence properties of GFP. The origin of the mutations in GFP was from the use of Taq DNA polymerase, which is known to have a higher error rate than the Pfu DNA polymerase. The use of the incorrect polymerase was due to my inexperience in proper cloning techniques. These mutations were corrected for the virus constructs used in Chapter 2 and 3 by digesting out the mutated GFP and inserting the wild-type GFP by ligation.

Other problems to consider when using GFP as a reporter gene includes that the insertion of GFP attenuates the virus (Chapter 3) and is eventually lost from the genome because it represents additional, unnecessary genetic material, as previously reported (146). Other studies report the loss of GFP in alphavirus constructs following 3 or 4 passages in cell culture. By observing plaques from the titration of mosquitoes infected with VEEV 3908/GFP, I found that virus populations within infected mosquitoes first began to lose GFP six days post infection. Despite these limitations, viral constructs expressing GFP are still useful tools for identifying infected cells, especially early in the course of infection.

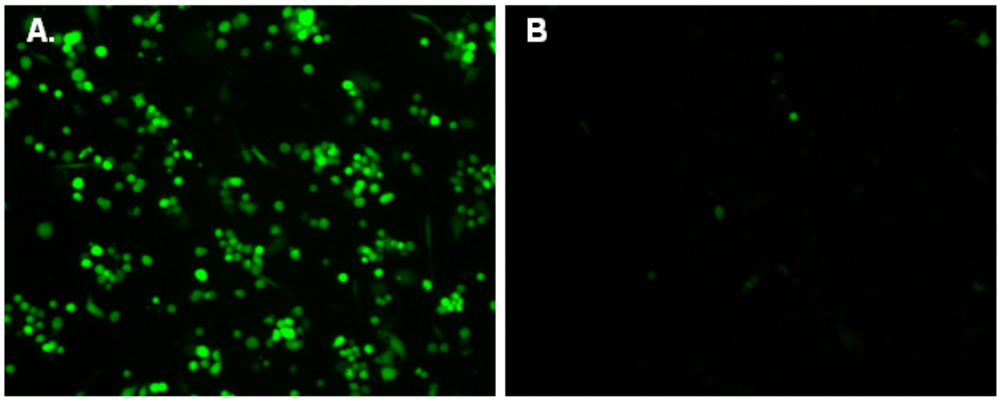

Figure 41. Vero Cells Infected with VEEV 3908 Replicon Particles Expressing Wild Type (A) or Mutated (B) GFP.

CHAPTER 7: CONCLUSIONS AND FUTURE DIRECTIONS

INTRODUCTION

Arthropod-borne (arbo) viruses are important causes of morbidity and mortality worldwide (58) and are of growing concern to human health (67). Despite the public health importance of arboviral diseases, the mechanisms of virus/host interactions and transmission of many vector-borne pathogens remains poorly understood. This dissertation focuses on an important reemerging arboviral pathogen in the Americas, Venezuelan equine encephalitis virus (VEEV), and it's interactions with the important epidemic VEEV mosquito vector, *Aedes taeniorhynchus*. The results of this dissertation improve understanding of the VEEV/vector interactions affecting vector competence [defined as the innate ability of a vector to acquire a pathogen and to successfully transmit it to another susceptible host (228)] and provide insight for modeling VEEV transmission in nature.

The overall objective of this dissertation was to study the pathogenesis and transmission of VEEV with regard to both the mosquito vector and vertebrate host. Detailed studies addressing the initial infection, dissemination, and transmission of VEEV in *Ae. taeniorhynchus* mosquitoes were lacking. Therefore, my first goal was to provide a better understanding of the virus/vector interactions necessary for the transmission of VEEV by this important epidemic mosquito vector.

Past studies in the Weaver laboratory that have focused on the emergence of epidemic VEEV have shown that mutations in the E2 envelope glycoprotein of enzootic progenitor strains convert the VEEV serotype from enzootic ID to epidemic IAB or IC, leading to enhanced equine viremia and virulence (5, 226). In addition, E2 mutations enhance the ability of epidemic strains to infect the proven epidemic mosquito vector, *Ae. taeniorhynchus* (16, 17). This vector is the only species identified thus far that is more susceptible to most epidemic versus enzootic VEEV strains. Adaptation of VEEV to utilize this vector may be an important determinant of epidemic transmission; therefore,

studies to further characterize VEEV/*Ae. taeniorhynchus* interactions were a focus of this dissertation.

The two representative VEEV strains that are a focus of this dissertation are the epidemic subtype IC strain 3908 and the enzootic subtype IE strain 68U201. Epidemic strain 3908 was isolated from a febrile human during one of the largest outbreaks on record, which occured in Zulia State, Venezuela in 1995, during which *Ae. taeniorhynchus* mosquitoes were implicated as an important vector (235). The enzootic VEEV strain 68U201 was isolated from a sentinel hamster in a typical enzootic VEE habitat in La Avellana, Guatemala, in 1968, where *Ae. taeniorhynchus* mosquitoes are abundant (32). Additionally, I chose to focus on epidemic strain 3908 and enzootic strain 68U201 due to their relatively low passage histories before undergoing cDNA clone production (17, 150), the existing studies of their ability to infect *Ae. taeniorhynchus* (16, 17, 102, 141, 226), and the availability of infectious cDNA clones within the laboratory.

CHARACTERIZATION OF THE MIDGUT INFECTION BARRIER FOR VEEV IN *AE. TAENIORHYNCHUS*

Previous studies have already demonstrated that *Ae. taeniorhynchus* mosquitoes are highly susceptible to oral infection with epidemic strain 3908, but are relatively refractory to enzootic strain 68U201. The first question I wanted to address is why *Ae. taeniorhynchus* mosquitoes are more susceptible to infection with epidemic strain 3908 compared to enzootic strain 68U201. I hypothesized that differential interactions of epidemic versus enzootic VEEV strains with midgut epithelial cells determine infection of *Ae. taeniorhynchus*. The experiments using purified, radiolabelled virus and replicon particles expressing GFP (Chapter 2) supported my hypothesis by demonstrating that significantly more epidemic subtype IC virus bound to and infected mosquito midguts compared to the enzootic subtype IE VEEV. These studies provided evidence supporting the hypothesis that *Ae. taeniorhynchus* mosquitoes are more susceptible to epidemic versus enzootic VEEV due to initial interactions of the virus with midgut epithelial cells. However, additional studies are needed to determine if the midgut infection and binding differences are explained by receptor interaction. Interestingly, a wide range of VEEV

strain 3908 particles bound to and infected the midgut. For example, as few as 1 and as many as 70 GFP-expressing cell(s) were detected in a single midgut. It would be interesting to determine if the number of cells initially infected in the midgut correlates with the dissemination rate from the midgut into the hemocoel. Clusters of midgut epithelial cells were often found to be expressing GFP, suggesting that selected cells are preferentially targeted by VEEV. I speculate that "bare cells," which were described previously to lack microvilli and to occur singly or in clusters throughout the posterior midgut (250), might represent a preferential target for VEEV infection. Further characterization of these cells to determine if and why they are preferentially invaded is needed. Additionally, cells morphologically resembling endocrine cells appeared to be infected on some occasions. Further characterization of these cells is needed to determine their identity.

Replicon particles were also injected intrathoracically to test the hypothesis that midgut infection is the primary barrier affecting the transmissibility of epidemic versus enzootic VEEV strains. The GFP was consistently detected in mosquitoes intrathoracically infected with both epidemic and enzootic strains, indicating that midgut infection is the primary barrier to enzootic VEEV infection. However, more GFP expressing cells were observed in mosquitoes infected intrathoracically with the epidemic strain compared to the enzootic strain. In agreement with these results, when fully infectious VEEV was injected intrathoracically, the enzootic strain replicated to slightly lower titers compared to the epidemic strain. Also, the salivary glands were not found to be infected when mosquitoes were orally infected with the enzootic strain, even though a small percentage of mosquitoes developed a disseminated infection (Chapter 3). Kramer et al. (102) found that *Ae. taeniorhynchus* mosquitoes intrathoracically infected with VEEV enzootic strain 68U201 do transmit virus at a low frequency (less than 40%). In contrast, my results indicated when mosquitoes are injected intrathoracically with epidemic VEEV strain 3908 they transmit at a much higher frequency (near 100%) compared to infection with enzootic VEEV. These results suggest the possible role of a salivary gland infection barrier in *Ae. taeniorhynchus* mosquitoes infected with enzootic

VEEV strain 68U201, possibly due to a lower level of replication in the hemocoel associated cells and tissues.

The results from Chapter 2 suggest that interactions of the virus with midgut epithelial cells allow epidemic subtype IC VEEV to infect *Ae. taeniorhynchus* efficiently, which is the primary barrier affecting the transmissibility of epidemic versus enzootic VEEV. These results provide important implications regarding the emergence of epidemic VEEV involving *Ae. taeniorhynchus* mosquitoes. Future studies with additional epidemic (subtype IAB) and enzootic (subtype ID) VEEV strains are needed to determine if comparable virus-receptor interactions mediate the emergence of all epidemic VEEV strains via changes in epidemic vector infectivity. Additionally, studies are needed to assess the role of nonstructural vs. structural protein genes in initial midgut cell infection and replication.

Comprehending the species-specific refractoriness of midgut epithelial cells to infection is an important topic in arbovirology, which provides insight for understanding mechanisms of vector competence. My results agree with those of others (80, 130) in support of the most likely hypothesis to explain this refractoriness--the presence of specific receptor sites on the midgut epithelial of susceptible mosquito speices are modified or absent on the midgut epithelia of refractory mosquitoes (71). This hypothesis should be investigated for other arboviruses and their vectors.

INFECTION AND DISSEMINATION OF VEEV IN *AE. TAENIORHYNCHUS*

In agreement with the results presented in chapter 2, studies following the infection and dissemination of VEEV strains 3908 and 68U201 in *Ae. taeniorhynchus* mosquitoes by immunohistochemical antigen detection also found that midgut epithelial cells were not infected efficiently by strain 68U201 (Chapter 2). However, dissemination did occur for a small percentage of mosquitoes infected with VEEV strain 68U201. Since midgut epithelial cells were not found to be infected efficiently with this strain, the route of virus escape from the midgut into the hemocoel remains to be determined. In fact, the route of arbovirus egress from the midgut lumen to the hemocoel remains a fundamental

mechanism to be determined for all arboviruses. The basal lamina of midgut epithelial cells has pore sizes considerably smaller than arboviruses. Recent studies by Romoser et al. (169, 170) suggest that tracheae and/or modified "spongy" basal lamina associated with midgut muscles may act as tissue conduits for virus escape from the midgut into the hemocoel. Their ultrastructural studies revealed that tracheae/tracheoles associated with muscles that surround the posterior midgut appear to penetrate the midgut epithelium in *Culex pipien* mosquitoes. Since VEEV readily infects tracheae associated with the midgut [Chapter 2 and (170)] it is very likely that dissemination from the midgut occurs via tracheae if they are found to pentrate the midgut epithelium in *Ae. taeniorhynchus* mosquitoes, which I speculate to be highly probable. I speculate that on very rare occasions the tracheae penetrate the midgut epithelium and are exposed to the luminal surface of the midgut, allowing for their direct infection. This scenario could explain the small rate of virus dissemination in mosquitoes infected with VEEV strain 68U201, where the midgut epithelial cells were not found to be infected, but dissemination did occur. However, my study did not find evidence of tracheae cells associated with the midgut to be infected with the replicon particles following oral infection. A larger sample size is needed to answer this question more conclusively. Another possible mechanism of virus escape into the hemocoel is via a "leaky" midgut. Although no evidence of a "leaky" midgut was found in this study, future work with larger sample sizes are needed to conclusively decipher the role of this mechanism of VEEV dissemination from the midgut. Additionally, electron microscopy studies of VEEV infection of *Ae. taeniorhynchus* mosquitoes to determine the role of these tissue conduits in virus egress into the hemocoel.

Another potential route of virus escape from the midgut to the hemocoel is through the epithelial cells of the intussuscepted foregut. Virus was detected in the epithelial cells of the anterior portion of the anterior midgut and the cardia on day 2 post-infection (Chapter 3). Virus may have infected this region as the bloodmeal passed through the anterior midgut on its way to the posterior midgut. Also, a portion of the bloodmeal may have been directed into the diverticulum. Over time the diverticulum

contents are re-directed to the posterior midgut through the anterior midgut where cells may become infected. It is possible that virus can spread in a cell-to-cell manner from the cardia to the adjacent intussuscepted foregut, where virus can hypothetically escape into the hemocoel without traversing a basal lamina (167). However, my results suggest that dissemination also occurred from the posterior midgut, which is probably the more common route. Further studies are needed to determine the frequency of intussuscepted foregut infection following VEEV infection of *Ae. taeniorhynchus* mosquitoes. I hypothesize that VEEV primarily disseminates to the hemocoel via the posterior midgut within tracheae (see above) and only occasionally escapes through the epithelial cells of the intussuscepted foregut.

For both VEEV strain 3908 and 68U201, the nervous tissue of mosqitoes was frequently infected (Chapter 3). Additionally, replicon particles expressing GFP often infected nervous tissue when injected intrathoracically (Chapter 2); therefore, nervous tissue appears to be a primary site of replication following virus dissemination into the hemocoel. Because my and previous studies (105, 223) demonstrate VEEV infection of mosquito nervous tissue, it would be interesting to determine if mosquito behavior is altered by viral infection.

Viral antigen was detected by immunohistochemistry in some unexpected locations within the mosquito (Chapter 3). For example, on day 1 post-infection antigen staining seemed to concentrate along the brush border and in the surrounding lumen of the midgut for mosquitoes infected with the high titer 3908 bloodmeal. Further studies are needed to confirm that viral antigen accumulates along the brush border following replication and if virus is shed into the lumen. A possible explanation for virus accumulation along the apical side of the midgut is an effect of bloodmeal digestion. Shortly after the bloodmeal, the midgut epithelial cells begin absorbing nutrients from the bloodmeal, which pass through the cells to the hemocoel from the basolateral membranes. The activity occurring on the basal side of the midgut (ie nutrient transport to the hemocoel) shortly after the bloodmeal may cause virus to accumulate along the apical side early in infection. This may also aid in the infection of other midgut epithelial

cells since virus would bud into the lumen. However, other studies found that alphaviruses bud primarily from the basolateral membranes of infected midgut epithelial cells (223, 239); therefore, the antigen detection along the brush border may be due to non-specific staining and requires further investigation. I also observed antigen staining in the diverticulum on day 1 post-infection. Additionally, viral antigen was detected in the ovaries, but transovarial transmission has never been reported for VEEV. Electron microscopy studies are needed to clarify these observations because non-specific staining is a common problem of immunohistochemical assays.

The results of this study provide a better understanding of the patterns of replication and dissemination of epizootic and enzootic VEEV in *Ae. taeniorhynchus* mosquitoes. Some common patterns of infection and dissemination occur with VEEV in *Ae. taeniorhynchus* mosquitoes that has been observed for other arboviruses and their vectors, such as consistent infection of tracheal cells (170, 190), early replication in the anterior region of the midgut (16, 40, 107, 167, 168, 241), and frequent infection of the nervous tissue (53). It appears that some arboviruses may infect and disseminate within their vectors via common mechanisms, which represents an important topic of future research leading to a better understanding of mechanisms affecting vector competence of arboviruses.

TRANSMISSION AND PATHOGENESIS OF VEEV

Another major gap in knowledge regarding VEEV pathogenesis in the vertebrate host is that the primary sites of replication following the bite of an infected mosquito have not yet been determined. Most pathogenesis studies of VEE have been conducted following a subcutaneous needle inoculation of the virus. Therefore, the second goal of this dissertation was to address the pathogenesis of VEEV following natural (i.e. mosquito bite) versus artificial (i.e. needle inoculation) infection of the vertebrate host. In order to address this question, significant efforts were made to determine the precise dose transmitted by infected mosquitoes.

Vector saliva enhances infection with many pathogens (135, 160, 192, 200-202) and mosquito saliva is reported to enhance infection by some (43, 109, 142, 187, 188), but not all (3, 157, 178) arboviruses. Previous studies clearly demonstrate that saliva from vectors such as ticks and sandflies enhance pathogen infection, but the results are variable for studies designed to determine if mosquito saliva enhances the infection of arboviruses. A major flaw of some previous studies of the effect of mosquito saliva on arbovirus infection was the lack of a thorough investigation of how much virus the mosquito transmits. Some studies showing arboviral enhancement by mosquito transmission allowed multiple mosquitoes to transmit to a single vertebrate (43, 109, 142, 187) while others supplemented the virus inoculum with salivary gland extracts from multiple mosquitoes (43, 188). I wanted to determine if transmission from a single mosquito alters VEEV pathogenesis. In contrast to the above studies, efforts were made to determine accurately the dose transmitted by infected mosquitoes. First, the method and virus titer used for mosquito infection were evaluated. My results (Chapter 4) show that the virus titer used for the infection of mosquitoes has little or no effect on the amount of virus found in saliva following extrinsic incubation. The method of oral infection (artificial bloodmeal versus viremic animal) also does not appear to affect the titer of VEEV in saliva. However, intrathoracic inoculation generates lower saliva titers than oral infection when using the same incubation period (10 days). An interesting finding of this study was that *Ae. taeniorhynchus* mosquitoes transmitted on average significantly more (1.2 $\log_{10}$ PFU) virus than *Ae. albopictus* mosquitoes. The higher saliva titers in this proven vector, compared to *Ae. albopictus* that are susceptible to infection but have not been implicated in natural transmission, further support the hypothesis from previous studies (17, 141), that adaptation for *Ae. taeniorhynchus* transmission contributes to VEEV emergence; however, further studies looking at saliva titers and transmission efficiency are needed. Mosquitoes transmitted a very wide range of virus in their saliva, which appears not to be correlated with the degree of virus within the mosquito body. Additionally, about 25% of saliva samples contained low levels of virus that were only detected by suckling mouse inoculation. *Ae. albopictus* transmitted a

median titer of 0.6 $\log_{10}$ PFU, a mean titer of 1.0 $\log_{10}$ PFU, and a range of 0.25-4.2 $\log_{10}$ PFU, while *Ae. taeniorhynchus* mosquitoes transmitted a median titer of 1.8 $\log_{10}$ PFU, a mean titer of 2.2 $\log_{10}$ PFU, and a range of 0.25-6.2 $\log_{10}$ PFU.

One limitation of the studies in chapter 4 was that mosquitoes were forced to salivate into capillary tubes filled with immersion oil. It was not known if saliva collection in capillary tubes (*in vitro* method) would accurately estimate the amount of virus mosquitoes transmit during feeding on a live vertebrate host (*in vivo* method); therefore, experiments in chapter 5 were designed to compare the two saliva collection methods. My results demonstrated that *Ae. taeniorhynchus* mosquitoes transmit significantly less VEEV *in vivo* (geometric mean of 11 PFU) into mouse tails when compared to the amount deposited *in vitro* into oil-filled capillary tubes (geometric mean of 74 PFU). Similar to the results in chapter 4, a large number of samples contained no detectable virus (36% for cell culture assays and 21% for real time RT-PCR). It is interesting that *Ae. taeniorhynchus* mosquitoes often transmitted low amounts of virus or no virus at all. During large outbreaks in nature, only 2 to 4% of the probable primary vectors were found to be infected with VEEV (197); therefore, only a small percentage of mosquitoes are needed to maintain VEEV epidemic circulation during a large scale outbreak.

My results provide important insights into the transmission dynamics of VEEV and arboviruses in general. The time that it took mosquitoes to feed to repletion did not correlate with the amount of virus transmitted, and mosquitoes deposited virus both intra- and extravascularly. Differing vertebrate mortality rates were observed depending on the mosquito feeding site (Chapter 5); therefore, the anatomical site of mosquito transmission may have an effect on the susceptibility of the vertebrate to virus infection. Alternatively, mosquitoes may deposit different amounts of virus at different anatomical sites due to the difficulty/ease of locating and canulating blood vessels.

Because my studies found that mosquitoes transmit a wide range of arbovirus doses, I inoculated mice intradermally with two doses representing the range of VEEV titers transmitted naturally. My results indicated little or no overall effect of mosquito

transmission on murine viremia or mortality when compared to needle inoculations (Chapter 5). My study is the first to use *in vivo* methods to determine the accurate dose transmitted by infected mosquitoes and to use this dose for pathogenesis studies simulating natural infection. Additionally, unlike most previous studies, I only allowed a single infected mosquito to transmit to a single vertebrate. Further studies with other VEEV vertebrate hosts are needed to determine if mosquito saliva has no effect on VEEV infection of other hosts. However, I speculate that no major effects on VEEV infection from mosquito saliva will be observed in any vertebrate hosts, and that mosquito saliva only functions to facilitate blood feeding.

The results of these studies found that *Ae. taeniorhynchus* mosquitoes transmit a range of VEEV doses (often low amounts of virus); therefore, future arbovirus pathogenesis studies simulating natural transmission should use a range of virus doses, which should be lower than what has been used in past pathogenesis studies. However, different mosquito species were found to transmit different amounts of virus; therefore the particular virus dose used for other arboviruses should be determined in regards to it's mosquito vector. For VEEV, no overall effect of mosquito transmission on murine viremia or mortality occurs compared to needle inoculations. Future pathogenesis studies of VEEV simulating natural infection should be more concerned with the virus dose used and not the mode of transmission (i.e. mosquito vs. needle).

SUMMARY

It is interesting to point out that results in this dissertation suggest that VEEV faces two bottleneck events imposed by the mosquito vector. The first bottleneck is indicated by the observation that, on average, only 14 midgut epithelial cells are initially infected by VEEV subtype IC. Other studies also report a low number of midgut epithelial cells initially infected by other arboviruses within the mosquito vector (45, 139, 190). Concerning the transmission of other viruses in nature, infection by massive numbers of virus particles is most likely rare. For example, transmission of many viruses by respiratory droplets frequently occurs by single particles (31, 37, 52); therefore, the

fact that transmission of VEEV also occurs by a low number of virus particles is not inconceivable.

The second bottleneck occurs when *Ae. taeniorhynchus* mosquitoes transmit an average of only 11 PFU of VEEV subtype IC to a vertebrate host. The first bottleneck suggest that the mosquito vector selects for a specific virus population that is able to bind and infect the midgut epithelial cells. The virus then disseminates to the salivary glands where a small amount of virus is transmitted to the vertebrate host. The evolutionary consequences of these bottleneck events for VEEV has yet to be determined, but viruses facing such events can suffer a loss of fitness (41). However, VEEV and other arboviruses remain relatively stable in nature (28, 57, 227, 231). In fact, arboviruses in general are more genetically stable than RNA viruses transmitted exclusively among vertebrates. This is most likely due to the constraints imposed on arboviruses, which must cycle between two genetically distinct hosts. Studies with Vesicular stomatitis virus found that the size of a genetic bottleneck required to maintain fitness of VSV depends on the intitial virus population fitness (134). When viral populations of VSV reaches neutral fitness levels, five-particle to five-particle transmissions remains relatively stable (133). These studies provide possible partial explanations for why VEEV remains relatively stable in nature while facing two apparent bottleneck events due to the mosquito vector. The evolutionary consequences of these bottleneck events on VEEV should be an important area of future research.

In summary, differential interactions of VEEV with midgut epithelial cells (possibly via receptors) determine the ability of epidemic IC versus enzootic IE VEEV strains to infect the important epidemic vector, *Ae. taeniorhynchus*. Additionally, midgut binding and infection is the primary barrier to enzootic IE VEEV infection. Following initial midgut infection, the epidemic subtype IC VEEV strain is pantropic in tissues of the mosquito vector *Ae. taeniorhynchus*, whereas the enzootic subtype IE strain does not infect the midgut efficiently and upon dissemination replicates mainly in nervous tissue. Initial transmission of epidemic subtype IC VEEV to a vertebrate host occurs on day 4 post-infection. The amount of epidemic VEEV subtype IC that *Ae. taeniorhynchus*

mosquitoes transmit ranges from 10-1,000 PFU. However, little or no overall effect of mosquito transmission on murine viremia or mortality occurs in comparison to needle inoculations. The results of this dissertation project enhance our understanding of the interactions between VEEV and the epidemic mosquito vector, *Ae. taeniorhynchus*. This study also increases our knowledge of the mechanisms of epidemic VEEV emergence and transmission. This information will aid our understanding of factors affecting vector competence, improve design of vaccine candidates that will not be transmitted by mosquitoes, and enhance modeling of VEEV transmission in nature.

APPENDIX: IMMUNOHISTOCHEMSITRY ANTIGEN STAINING SCORES IN THE TISSUES OF INFECTED *AE. TAENIORHYNCHUS* MOSQUITOES

Tissue		Days Post-infection													
		1	2	3	4	5	6	7	8	9	10	11	16	21	
	Posterior Midgut	(3/3) 1+--2+	(3/3) 1+--2+	(2/3) 1+--2+	(3/3) 1+--2+	(3/3) 1+--2+	(3/3) 1+--2+	(3/3) 2+	(3/3) 1+--3+	(3/3) 2+	(3/3) 1+	(3/3) 1+--3+	(3/3) 2+--3+	(2/2) 3+	7 $\log_{10}$ 3908
		(0/3)	(0/3)	(0/3)	(0/3)	(1/3) 2+	(1/3) 1+	(2/4) 1+	(3/3) 1+--2+	(2/3) 1+	(1/4) 1+	(3/3) 1+--2+	(1/3) 2+	(2/3) 1+--2+	5 $\log_{10}$ 3908
		(0/3)	(0/3)	(0/3)	(2/3) 1+	(1/3) 1+	(1*/3) 1+	(0/3)	(0/3)	(1*/3) 1+	(1*/3) 1+	(0/3)	N/A	N/A	7 $\log_{10}$ 68u201
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(1/3) 1+	(2/3) 1+--2+	(2/3) 2+--3+	(1/3) 1+	(1/3) 3+	7 $\log_{10}$ 3908/GFP oral/whole section
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(1/3) 1+	(1/3) 2+	(1/3) 1+	(1/3) 1+	(1*/3) 1+	(2/3) 1+--3+	(3/3) 2+	7 $\log_{10}$ 3908/GFP oral/dissection
		(0/3)	(2*/3) 1+	(3*/3) 2+	(3*/3) 2+	(3*/3) 2+	(3*/3) 2+--3+	(3*/3) 2+	(3*/3) 1+--2+	(3*/3) 2+	(3*/3) 2+	(3*/3) 2+	(3*/3) 1+	(3*/3) 1+	4 $\log_{10}$ 3908/GFP IT/whole section
		(0/3)	(2*/3) 1+--2+	(3*/3) 1+	(3*/3) 1+--3+	(3*/3) 3+	(3*/3) 2+	(3*/3) 1+	(3*/3) 2+--3+	(3*/3) 1+--2+	(3*/3) 1+	(3*/3) 1+--2+	(3*/3) 1+--2+	(3*/3) 2+--3+	4 $\log_{10}$ 3908/GFP IT/dissection
	Anterior Midgut	(1/3) 1+	(1/3) 1+	(0/3)	(0/3)	(0/3)	(2/3) 1+	(1/3) 2+	(1/3) 1+	(3/3) 1+	(0/3)	(3/3) 1+--3+	(3/3) 2+--3+	(2/2) 2+	7 $\log_{10}$ 3908
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(1/3) 1+	(0/3)	(1/3) 1+	(0/3)	(0/3)	(1/3) 1+	5 $\log_{10}$ 3908

	Days Post-infection													
	1	2	3	4	5	6	7	8	9	10	11	16	21	
	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/2)	(0/3)	(0/3)	N/A	N/A	7 $\log_{10}$ 68u201
	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(1/3) 1+	(2/3) 1+--2+	(0/3)	(1/3) 3+	7 $\log_{10}$ 3908/GFP oral/whole section
	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	7 $\log_{10}$ 3908/GFP oral/dissection
	(0/3)	(2*/3) 1+	(3*/3) 1+	(3*/3) 2+	(3*/3) 2+	(3*/3) 2+	(3*/3) 2+	(3*/3) 2+	(3*/3) 2+	(3*/3) 2+	(3*/3) 2+	(3*/3) 1+	(3*/3) 1+	4 $\log_{10}$ 3908/GFP IT/whole section
	(0/3)	(3*/3) 1+	(2*/3) 1+	(3*/3) 2+--3+	(3*/3) 3+	(3*/3) 2+	(3*/3) 1+--2+	(3*/3) 2+--3+	(3*/3) 1+--2+	(3*/3) 1+--2+	(3*/3) 1+--2+	(3*/3) 1+--2+	(3*/3) 2+	4 $\log_{10}$ 3908/GFP IT/dissection
Hindgut	(0/3)	(1/3) 1+	(0/3)	(1/3) 1+	(0/3)	(1/3) 1+	(1/3) 1+	(1/3) 1+	(2/3) 1+	(1/3) 1+	(1/3) 1+	(3/3) 1+--2+	(0/2)	7 $\log_{10}$ 3908
	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	5 $\log_{10}$ 3908
	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(1/3) 2+	(1/3) 2+	(0/3)	N/A	N/A	7 $\log_{10}$ 68u201
	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(1/3) 2+	(1/3) 1+	(1/3) 1+	7 $\log_{10}$ 3908/GFP oral/whole section
	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	7 $\log_{10}$ 3908/GFP oral/dissection
	(0/3)	(0/3)	(0/3)	(3/3) 1+--2+	(3/3) 2+	(3/3) 2+--3+	(3/3) 2+--3+	(3/3) 1+--2+	(3/3) 2+--3+	(3/3) 2+	(3/3) 2+	(3/3) 1+	(3/3) 1+	4 $\log_{10}$ 3908/GFP IT/whole section

	Days Post-infection													
	1	2	3	4	5	6	7	8	9	10	11	16	21	
	(0/3)	(1/3) 1+	(0/3)	(0/3)	(1/3) 1+	(0/3)	(0/3)	(2/3) 1+-- 2+	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	4 $\log_{10}$ 3908/GFP IT/dissection
Cardia	(1/3) 1+	(1/3) 1+	(0/3)	(2/3) 2+-- 3+	(2/3) 1+	(3/3) 1+	(2/3) 2+	(1/3) 2+	(1/3) 1+	(2/3) 1+	(0/3)	(3/3) 1+-- 2+	(1/2) 2+	7 $\log_{10}$ 3908
	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(1/3) 1+	(1/3) 2+	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	5 $\log_{10}$ 3908
	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(1*/3) 1+	(0/3)	N/A	N/A	7 $\log_{10}$ 68u201
	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(1/3) 1+	(0/3)	(0/3)	(1/3) 1+	(2/3) 1+	(2/3) 1+	(0/3)	(1/3) 1+	7 $\log_{10}$ 3908/GFP oral/whole section
	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	7 $\log_{10}$ 3908/GFP oral/dissection
	(0/3)	(0/3)	(0/3)	(2*/3) 1+	(3*/3) 1+	(3*/3) 1+	(3*/3) 1+-- 2+	(3*/3) 1+	(3*/3) 1+	(3*/3) 1+	(3*/3) 1+	(3*/3) 1+	(3*/3) 1+	4 $\log_{10}$ 3908/GFP IT/whole section
	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	4 $\log_{10}$ 3908/GFP IT/dissection
Intussucepted Foregut	(0/3)	(1/3) 1+	(0/3)	(1/3) 1+	(1/3) 1+	(0/3)	(1/3) 2+	(1/3) 2+	(2/3) 1+-- 2+	(1/3) 1+	(1/3) 2+	(3/3) 1+	(1/2) 1+	7 $\log_{10}$ 3908
	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(1/4) 1+	(1/3) 1+	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	5 $\log_{10}$ 3908
	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(1/3) 1+	(0/3)	(0/3)	(0/2)	(1/3) 1+	(0/3)	N/A	N/A	7 $\log_{10}$ 68u201

		Days Post-infection													
		1	2	3	4	5	6	7	8	9	10	11	16	21	
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(1/3) 1+	(0/3)	(1/3) 1+	7 log_{10} 3908/GFP oral/whole section
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	7 log_{10} 3908/GFP oral/dissection
		(0/3)	(0/3)	(1/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+-- 3+	(3/3) 1+-- 2+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	4 log_{10} 3908/GFP IT/whole section
		(0/3)	(0/3)	(0/3)	(1/3) 1+	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(1/3) 1+	(0/3)	(0/3)	4 log_{10} 3908/GFP IT/dissection
	Salivary Gland Lateral Lobe	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(1/3) 1+	(1/3) 1+	(1/3) 3+	(1/3) 2+	(1/3) 1+	(3/3) 1+-- 3+	(2/3) 2+-- 3+	7 log_{10} 3908
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(2/3) 1+	5 log_{10} 3908
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	N/A	N/A	7 log_{10} 68u201
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	7 log_{10} 3908/GFP oral/whole section
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	7 log_{10} 3908/GFP oral/dissection
		(0/3)	(0/3)	(0/3)	(2/3) 1+	(3/3) 1+	(3/3) 1+-- 2+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 2+	(3/3) 1+	(3/3) 1+	(3/3) 1+	4 log_{10} 3908/GFP IT/whole section

	Days Post-infection														
		1	2	3	4	5	6	7	8	9	10	11	16	21	
		(0/3)	(0/3)	(0/3)	(2/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 2+	(3/3) 1+	(3/3) 2+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	4 $\log_{10}$ 3908/GFP IT/dissection
	Salivary Gland Medial Lobe	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(1/3) 1+	(0/3)	(0/3)	(0/3)	(0/3)	7 $\log_{10}$ 3908
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	5 $\log_{10}$ 3908
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	N/A	N/A	7 $\log_{10}$ 68u201
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	7 $\log_{10}$ 3908/GFP oral/whole section
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	7 $\log_{10}$ 3908/GFP oral/dissection
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	4 $\log_{10}$ 3908/GFP IT/whole section
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	4 $\log_{10}$ 3908/GFP IT/dissection
	Dorsal Diverticulum	(1/3) 2+	(1/3) 1+	(0/3)	(0/3)	(0/3)	(0/3)	(1/3) 2+	(1/3) 2+	(1/3) 3+	(1/3) 3+	(1/3) 3+	(1/3) 3+	(1/3) 3+	7 $\log_{10}$ 3908
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	5 $\log_{10}$ 3908
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(1/3) 1+	(0/3)	(0/3)	N/A	N/A	7 $\log_{10}$ 68u201
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	7 $\log_{10}$ 3908/GFP oral/whole section

		Days Post-infection													
		1	2	3	4	5	6	7	8	9	10	11	16	21	
		(0/3)	(0/3)	(1/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+-- 3+	(3/3) 1+-- 2+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	4 $\log_{10}$ 3908/GFP IT/whole section
	Ventral Diverticulum	(1/3) 2+	(0/3)	(0/3)	(0/3)	(0/3)	(1/3) 2+	(1/3) 2+	(0/3)	(3/3) 1+-- 3+	(0/3)	(1/3) 3+	(1/3) 3+	(1/3) 3+	7 $\log_{10}$ 3908
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	5 $\log_{10}$ 3908
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(1/3) 1+	(0/3)	(0/3)	N/A	N/A	7 $\log_{10}$ 68u201
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	7 $\log_{10}$ 3908/GFP oral/whole section
		(0/3)	(0/3)	(1/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+-- 3+	(3/3) 1+-- 2+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	4 $\log_{10}$ 3908/GFP IT/whole section
	Oesophagus	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(1/3) 1+	(1/3) 1+	(1/3) 1+	(1/3) 1+	(1/3) 1+	(0/3)	(0/3)	7 $\log_{10}$ 3908
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	5 $\log_{10}$ 3908
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	N/A	N/A	7 $\log_{10}$ 68u201
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	7 $\log_{10}$ 3908/GFP oral/whole section
		(0/3)	(0/3)	(1/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+-- 3+	(3/3) 1+-- 2+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	4 $\log_{10}$ 3908/GFP IT/whole section

	Days Post-infection													
	1	2	3	4	5	6	7	8	9	10	11	16	21	
Abdominal Nerve Ganglia	(0/3)	(0/3)	(0/3)	(1/3) 1+	(2/3) 1+	(1/3) 1+	(1/3) 1+	(1/3) 2+	(1/3) 2+	(1/3) 3+	(1/3) 1+	(3/3) 2+--3+	(1/2) 2+	7 $\log_{10}$ 3908
	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(2/3) 1+	5 $\log_{10}$ 3908
	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(1/3) 2+	(0/3)	(0/3)	(1/3) 2+	(0/3)	(0/3)	N/A	N/A	7 $\log_{10}$ 68u201
	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(1/3) 2+	(0/3)	(1/3) 3+	7 $\log_{10}$ 3908/GFP oral/whole section
	(0/3)	(0/3)	(0/3)	(3/3) 1+--2+	(3/3) 3+	(3/3) 2+--3+	(3/3) 2+--3+	(3/3) 2+--3+	(3/3) 3+	(3/3) 3+	(3/3) 3+	(3/3) 2+	(3/3) 2+	4 $\log_{10}$ 3908/GFP IT/whole section
Thoracic Nerve Ganglia	(0/3)	(0/3)	(0/3)	(1/3) 1+	(0/3)	(1/3) 1+	(1/3) 1+	(1/3) 1+	(3/3) 1+	(1/3) 1+	(1/3) 1+	(3/3) 1+--2+	(3/3) 1+--2+	7 $\log_{10}$ 3908
	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	5 $\log_{10}$ 3908
	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(1/3) 1+	(0/3)	(0/3)	(0/2)	(1/3) 1+	(0/3)	N/A	N/A	7 $\log_{10}$ 68u201
	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(1/3) 1+	(1/3) 1+	(0/3)	(1/3) 1+	7 $\log_{10}$ 3908/GFP oral/whole section
	(0/3)	(0/3)	(1/3) 1+	(3/3) 1+	(3/3) 2+	(3/3) 2+--3+	(3/3) 2+	(3/3) 2+	(3/3) 2+	(3/3) 2+	(3/3) 2+	(3/3) 1+	(3/3) 1+	4 $\log_{10}$ 3908/GFP IT/whole section
Cephalic Ganglia	(0/3)	(0/3)	(0/3)	(2/3) 1+	(0/3)	(1/3) 1+	(1/3) 2+	(1/3) 2+	(3/3) 1+--2+	(1/3) 1+	(1/3) 1+	(3/3) 1+--2+	(3/3) 2+	7 $\log_{10}$ 3908

		Days Post-infection													
		1	2	3	4	5	6	7	8	9	10	11	16	21	
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(1/3) 1+	5 $\log_{10}$ 3908
		(0/3)	(0/3)	(0/3)	(1/3) 1+	(1/3) 1+	(1/3) 1+	(0/3)	(0/3)	(1/3) 1+	(1/3) 1+	(0/3)	N/A	N/A	7 $\log_{10}$ 68u201
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	7 $\log_{10}$ 3908/GFP oral/whole section
		(0/3)	(0/3)	(1/3) 1+	(3/3) 1+	(3/3) 2+	(3/3) 2+-- 3+	(3/3) 2+	(3/3) 2+	(3/3) 2+	(3/3) 2+	(3/3) 2+	(3/3) 1+	(3/3) 1+	4 $\log_{10}$ 3908/GFP IT/whole section
	Johnston's Organ	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(1/3) 1+	(1/3) 1+	(1/3) 1+	(0/3)	(3/3) 1+	(2/3) 1+	7 $\log_{10}$ 3908
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	5 $\log_{10}$ 3908
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(1/3) 1+	(0/3)	(0/3)	(1/3) 1+	(0/3)	(0/3)	N/A	N/A	7 $\log_{10}$ 68u201
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	7 $\log_{10}$ 3908/GFP oral/whole section
		(0/3)	(0/3)	(0/3)	(1/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	4 $\log_{10}$ 3908/GFP IT/whole section
	Ommatidia	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(1/3) 1+	(1/3) 1+	(1/3) 1+	(1/3) 1+	(1/3) 1+	(0/3)	(3/3) 1+	(2/3) 1+	7 $\log_{10}$ 3908
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	5 $\log_{10}$ 3908
		(0/3)	(0/3)	(0/3)	(0/3)	(1/3) 1+	(0/3)	(0/3)	(0/3)	(1/3) 1+	(0/3)	(0/3)	N/A	N/A	7 $\log_{10}$ 68u201

		Days Post-infection													
		1	2	3	4	5	6	7	8	9	10	11	16	21	
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	7 log_{10} 3908/GFP oral/whole section
		(0/3)	(0/3)	(0/3)	(2/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	4 log_{10} 3908/GFP IT/whole section
	Abdominal Fat Body	(0/3)	(0/3)	(0/3)	(1/3) 1+	(2/3) 1+	(2/3) 1+	(1/3) 1+	(1/3) 2+	(1/3) 1+	(1/3) 2+	(1/3) 1+	(3/3) 1+-- 3+	(2/3) 1+	7 log_{10} 3908
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(2/3) 2+	5 log_{10} 3908
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(1/3) 1+	(0/3)	N/A	N/A	7 log_{10} 68u201
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(1/3) 1+	(0/3)	(1/3) 1+	7 log_{10} 3908/GFP oral/whole section
		(1/3) 1+	(1/3) 1+	(2/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	4 log_{10} 3908/GFP IT/whole section
	Thoracic Fat Body	(0/3)	(0/3)	(0/3)	(0/3)	(2/3) 1+	(2/3) 1+	(2/3) 1+	(1/3) 1+	(2/3) 1+	(1/3) 1+	(0/3)	(3/3) 1+-- 3+	(3/3) 1+-- 2+	7 log_{10} 3908
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(2/3) 1+	(1/3) 1+	(0/3)	(0/3)	(0/3)	(0/3)	(2/3) 2+	5 log_{10} 3908
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(1/3) 1+	(0/3)	(0/3)	(0/3)	(1/3) 1+	(0/3)	N/A	N/A	7 log_{10} 68u201

		Days Post-infection													
		1	2	3	4	5	6	7	8	9	10	11	16	21	
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(1/3) 1+	(0/3)	(1/3) 1+	7 log_{10} 3908/GFP oral/whole section
		(0/3)	(1/3) 1+	(2/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	4 log_{10} 3908/GFP IT/whole section
	Cephalic Fat Body	(0/3)	(0/3)	(0/3)	(2/3) 1+	(2/3) 1+	(1/3) 1+	(1/3) 1+	(1/3) 1+	(1/3) 1+	(1/3) 1+	(1/3) 1+	(3/3) 1+	(3/3) 1+-- 2+	7 log_{10} 3908
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(1/3) 1+	5 log_{10} 3908
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(1/3) 1+	(1/3) 1+	(0/3)	N/A	N/A	7 log_{10} 68u201
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(1/3) 1+	(1/3) 1+	(0/3)	(0/3)	7 log_{10} 3908/GFP oral/whole section
		(0/3)	(0/3)	(1/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	4 log_{10} 3908/GFP IT/whole section
	Malpighian Tubules	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(2/3) 1+	(0/3)	7 log_{10} 3908
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	5 log_{10} 3908
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	N/A	N/A	7 log_{10} 68u201
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	7 log_{10} 3908/GFP oral/whole section

		Days Post-infection													
		1	2	3	4	5	6	7	8	9	10	11	16	21	
		(0/3)	(0/3)	(0/3)	(1/3) 1+	(0/3)	(0/3)	(0/3)	(0/3)	(1/3) 1+	(0/3)	(0/3)	(0/3)	(0/3)	4 $\log_{10}$ 3908/GFP IT/whole section
	Ovaries	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(3/3) 1+--3+	(3/3) 1+--2+	7 $\log_{10}$ 3908
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	5 $\log_{10}$ 3908
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	N/A	N/A	7 $\log_{10}$ 68u201
		(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	(0/3)	7 $\log_{10}$ 3908/GFP oral/whole section
		(0/3)	(0/3)	(0/3)	(1/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+	(3/3) 1+--2+	(3/3) 1+	(3/3) 1+--2+	(3/3) 1+	(3/3) 1+	4 $\log_{10}$ 3908/GFP IT/whole section

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Education

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Publications

Smith, D.R., C.A. Rossi, T.M. Kijek, E.A. Henchal, and G.V. Ludwig. 2001. Comparison of Dissociation-Enhanced Lanthanide Fluorescent Immunoassays to Enzyme-Linked Immunosorbent Assays for Detection of Staphylococcal Enterotoxin B, Yersinia pestis-Specific F1 Antigen, and Venezuelan Equine Encephalitis Virus. Clinical and Diagnostic Laboratory Immunology. **8(6)**: 1070-1075.

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